Objective To determine the relationship between the virus burden in infancy and hearing loss in congenital CMV infection.

Study design A cohort of 76 infants with congenital cytomegalovirus (CMV) infection identified by means of newborn virologic screening was monitored for outcome. The amount of infectious CMV was analyzed in urine specimens obtained during early infancy. Peripheral blood (PB) samples obtained during early infancy were available from 75 children and CMV DNA was quantitated with a real-time quantitative polymerase chain reaction.

Results Infants with clinical abnormalities at birth (symptomatic congenital CMV infection) had higher amounts of CMV in urine (P = .005) and CMV DNA in PB (P = .001) than infants with no symptoms. Eight children with and 4 children without symptoms had hearing loss. Among children without symptoms, those with hearing loss had a significantly greater amount of CMV in urine (P = .03) and PB virus burden (P = .02) during infancy than those with normal hearing. Infants with <5 × 10^3 pfu/mL of urine CMV and infants with <1 × 10^4 copies/mL of viral DNA in PB were at a lower risk for hearing loss.

Conclusion In children with asymptomatic congenital CMV infection, hearing loss was associated with increased amounts of urine CMV and PB CMV DNA during early infancy. (J Pediatr 2005;146:817-23)
METHODS

Study Population and Specimens

Between August 1994 and October 1998, 96 children with congenital CMV infection were identified by means of the presence of CMV in saliva specimens obtained during the first week of life at the University of Alabama at Birmingham Hospitals.28,29 of the 93 children who were congenitally infected and enrolled in follow-up, urine specimens collected during the first month of life were available from 83 children (65 without symptoms, 18 with symptoms). Seventy-six children (58 without symptoms, 18 with symptoms) underwent at least 2 follow-up hearing evaluations, with at least 1 hearing test at 1 year of age or older, and this group constituted the study population. Of the 76 study children, a PB sample was unavailable from 1 infant with asymptomatic infection. The demographic characteristics were not different between the study children and children enrolled in the follow-up from whom urine specimens, hearing outcome data, or both were unavailable. The 10 children with unavailable urine specimens had asymptomatic congenital CMV infection and had normal hearing. The 7 children with asymptomatic CMV infection who underwent only 1 hearing evaluation during infancy had normal hearing. The urine and PB specimens were collected at the time of the initial study visit during the first month of life. Urine samples were analyzed for the amount of CMV excretion immediately after sample collection to obtain DNA preparations from 200 mL of whole blood with commercial centrifuge columns and stored at −20°C (Qiagen, Valencia, Calif).

Infants were classified as having symptomatic infection when they shed CMV during the first week of life and exhibited any of the clinical findings suggestive of congenital infection at birth described previously.26 The study protocol was approved by the University of Alabama at Birmingham institutional review board for human use, and informed consent was obtained from the parents or guardians of the children enrolled in the study.

Follow-up of Children

Children enrolled in the study were monitored according to a standard protocol described previously.4 Audiologic evaluations consisted of assessment with auditory brainstem evoked response audiometry (ABR), immittance measures of middle ear function, and/or pure-tone and speech audiometry appropriate for the child’s developmental level. The study children were routinely tested with ABR between 3 and 8 weeks of age and re-tested at 6 to 12 months of age. Behavioral audiometric evaluations with visual reinforcement procedures were performed beginning at 9 months of age, with follow-up assessments every 6 months until valid pure tone thresholds could be obtained for each ear, which usually occurred between 2.5 and 3 years of age. Thereafter, children were seen annually unless test results or parental concerns indicated a need for additional testing. A child was considered to have SNHL when air conduction thresholds at 1 or more frequencies were greater than 20 dB in conjunction with normal tympanograms, normal otoscopic findings, and/or normal bone conduction thresholds.4,7 Progressive hearing loss was defined as sensorineural decrease in hearing ≥10 dB at any 1 frequency or ABR threshold, documented on 2 separate evaluations. Delayed or late-onset hearing loss was defined as 1 or more hearing evaluations with a normal threshold documented for each ear before the onset of SNHL. For the purposes of this study, children with conductive hearing loss in the absence of SNHL were not considered to have hearing loss.

Quantitative Titration of Urine CMV

The amount of CMV in urine was determined by plating serial 5-fold dilutions of the urine specimens in 24-well plates seeded with human fibroblasts, as described previously, and the TCID50 was calculated with the Reed and Muench method.26 The urine samples were assayed immediately after collection, and the results were reported as plaque forming units per milliliter of urine (pfu/mL).

Real-time Polymerase Chain Reaction

The investigators who performed the real-time polymerase chain reaction (PCR) assays were blinded to the results of the audiologic follow-up. The CMV primers were selected from the highly conserved AD-1 region of the major envelope glycoprotein B.31–33 The forward primer was 5′-AGG TCT TCA AGG AAC TCA GCA AGA and the reverse primer was 5′-ACC CCG TCA GCC ATT CTC TCG GC was labeled at the 5′ end with fluorescent dye 6-carboxyfluorescein as the reporter dye and the 3′ end with the quencher dye 6-carboxytetramethylrhodamine.

Real-time PCR Conditions

The PCR was performed with an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, Calif). The reaction mixture contained CMV primers at 400 nM concentration and the probe at 150 nM concentration. TaqMan universal master mix (2X), containing AmpliTaq Gold DNA polymerase, deoxynucleoside triphosphates with dUTP, AmpErase UNG, Passive Reference 1, and optimized buffer was obtained from Perkin-Elmer Applied Biosystems. Each 25 μL mixture contained 20 μL of the master mix and 5 μL of the test sample. The PCR cycle parameters were 2 minutes of incubation at 50°C and 10 min at 95°C, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute.
Quantitative PCR

To establish the standard curve, a plasmid (pTZG) containing the target sequence of the CMV glycoprotein B was constructed. The purified plasmid was quantitated spectrophotometrically, and the copy number of molecules was calculated. Quantitation of CMV DNA in test samples was achieved by using serial 10-fold dilutions of the previously quantified plasmid standards. Plasmid standards and test samples were run in triplicate, and the average values were used to determine the CMV viral load. CMV virus burden in whole blood was expressed as CMV genomic equivalents per milliliter of blood (ge/mL). To control for the sample preparation and amplification, primers for amplification of a housekeeping gene, G3PDH, was included in each PCR assay. The PCR conditions were optimized by amplifying the target sequence from the plasmid pTZG, and the sensitivity of the assay has been determined to be approximately 50 genomic equivalents per 1 mL of blood.

Data Analysis

The demographic characteristics, newborn findings, outcome data, and the results of urine CMV titration and the PB real-time PCR were collected on case report forms and entered into SAS V8 data sets (SAS Institute, Cary, NC). Relative risk (RR) and 95% CI were calculated to assess the risk of hearing loss among the study children. The relationship between the virus burden and hearing loss in children with asymptomatic and symptomatic congenital CMV infection was examined with non-parametric methods, and statistical significance was determined with the Wilcoxon rank sum test. The statistical significance of outcome data among the 3 groups of children with differing amounts of viruria and PB viral load was determined with the $\chi^2$ test for trend analysis.

RESULTS

Characteristics of Children

The demographic characteristics of the children enrolled in the study according to their hearing status are shown in Table I. Most of the study children were African American and born to single young mothers (<20 years) who received prenatal care at the public health clinics. A third of the children with SNHL (4/12) were premature (<37 weeks gestation), whereas only 9% of the children with normal hearing (6/64) were preterm (RR = 3.3; 95% CI, 1.2-9.0). However, the number of infants born at <32 weeks gestation was not different between the group of children with SNHL and the group of children with normal hearing (Table I). Ten of the children with symptoms were part of an earlier study of predictors for hearing loss in children with symptomatic...
congenital CMV infection.\textsuperscript{17} There were no significant differences between the groups of children with and without symptoms in various demographic characteristics, including race, sex, marital status of the mother, source of the prenatal care, and maternal age. One infant with symptomatic congenital CMV infection received ganciclovir for 6 weeks during early infancy as part of a phase III clinical trial, and this child had SNHL that was detected during the first month of life.

Results of Follow-up

The children enrolled in the study were observed for a mean duration of 34.1 ± 17.9 months, and the median number of hearing evaluations was 6 (range, 2-17). Twelve of the 76 children enrolled in the study (16%) had SNHL (Table I). Significantly more children with symptomatic infection (8/18, 44%) had SNHL, compared with 4 of the 58 children with an asymptomatic infection (7%; RR = 4.3; 95% CI, 2.1-8.6). The children enrolled in the study underwent at least 2 hearing evaluations, at least 1 test when they were 12 months of age or older (Table II). Delayed onset hearing loss, progressive hearing loss, or both was observed in two thirds of the children with hearing deficit (8/12).

CMV Disease in Newborns and Viral Load

The mean urine CMV level in the group of infants with symptomatic congenital CMV infection (2.4 × 10^5 ± 9.5 × 10^5 pfu/mL) was greater than that of infants with asymptomatic infection (3.9 × 10^4 ± 9.7 × 10^4 pfu/mL), and this difference was statistically significant (P = .009). Similarly, infants with symptomatic infection had a significantly higher mean amount of CMV DNA in PB than infants with asymptomatic infection (4.0 × 10^5 ± 3.6 × 10^5 copies/mL and 8.2 × 10^4 ± 4.1 × 10^5 copies/mL, respectively; P = .001).

Table II. Follow-up parameters in children with congenital cytomegalovirus infection according to the presence of clinical findings at birth and the results of hearing assessments on follow-up

<table>
<thead>
<tr>
<th>Finding</th>
<th>Asymptomatic infants (n = 85)</th>
<th>Symptomatic infants (n = 18)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Hearing loss (n = 4)</td>
<td>Normal hearing (n = 54)</td>
</tr>
<tr>
<td>Mean duration of follow-up (months, ± SD)</td>
<td>39.3 ± 23.9</td>
<td>33.5 ± 17.6</td>
</tr>
<tr>
<td>Median number of hearing evaluations (range)</td>
<td>7 (2-14)</td>
<td>6 (2-13)</td>
</tr>
<tr>
<td>Number of hearing evaluations</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>&gt;2</td>
<td>3</td>
<td>46</td>
</tr>
<tr>
<td>Mean amount of CMV in urine (pfu/mL ± SD)</td>
<td>1.6 × 10^5 ± 2.1 × 10^5*</td>
<td>2.9 × 10^4 ± 7.8 × 10^4</td>
</tr>
<tr>
<td>Mean PB blood virus burden (ge/mL ± SD)</td>
<td>8.7 × 10^5 ± 1.6 × 10^6*</td>
<td>1.1 × 10^4 ± 1.5 × 10^4</td>
</tr>
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*P = .03. †P = .02.

Viruria and Outcome

The group of 12 children with SNHL had a significantly higher mean level of urine CMV during early infancy than the 64 children with normal hearing (mean values, 3.8 × 10^5 ± 7.6 × 10^5 pfu/mL versus 3.0 × 10^4 ± 7.5 × 10^4 pfu/mL; P = .003). As can be seen in Figure 1A, the group of 4 children with asymptomatic infection and SNHL had significantly higher mean CMV urinary excretion than the children with asymptomatic infection and normal hearing (mean values, 1.6 × 10^5 ± 2.1 × 10^5 pfu/mL and 2.9 × 10^4 ± 7.8 × 10^4 pfu/mL, respectively; P = .03). Among children with symptomatic infection, no significant difference was observed in CMV viruria between the group of children with SNHL and the group of children with normal hearing (mean values, 4.9 × 10^5 ± 9.2 × 10^5 pfu/mL and 3.8 × 10^4 ± 5.9 × 10^4 pfu/mL, respectively; P = .24).

The relationship between the amount of CMV in urine and SNHL was further examined with the χ² test for linear trend analysis (Figure 2). The study population was arbitrarily divided in 3 groups according to the amount of CMV in urine: children with a concentration <3.5 × 10^3 pfu/mL, children with a concentration between 3.5 × 10^3 and 2.5 × 10^4 pfu/mL, and children with a concentration of >2.5 × 10^4 pfu/mL. The subjects were distributed equally among the 3 groups. As can be seen in Figure 2, only 1 of 26 children with urine CMV <3.5 × 10^3 pfu/mL (4%) had SNHL, whereas 3 of 26 children with urine CMV between 3.5 × 10^3 and 2.5 × 10^4 pfu/mL (12%) had SNHL, and 8 of 24 children with urine CMV >2.5 × 10^4 pfu/mL (33%) had SNHL (P <.01).

PB Virus Burden and Outcome

The amount of CMV DNA in PB samples from 5 infants with asymptomatic infection was less than the level of detection for the real-time PCR assay, and none of these
5 children had hearing loss. The group of 12 infants (4 with asymptomatic infection and 8 with symptomatic infection) who had SNHL had significantly higher mean CMV DNA values than the infants with normal hearing (mean values, $3.7 \times 10^3 \pm 9.7 \times 10^3$ ge/mL and $5.1 \times 10^4 \pm 2.4 \times 10^5$ ge/mL, respectively; $P < .0001$). Among children with asymptomatic infection, the 4 children with SNHL had higher mean PB CMV DNA amounts ($8.7 \times 10^5 \pm 1.6 \times 10^6$ ge/mL) than children with normal hearing ($1.1 \times 10^4 \pm 1.5 \times 10^4$ ge/mL; $P = .02$; Figure 1B). In infants with symptomatic infection, the level of PB virus burden during early infancy was not different between the group of children with and the children without hearing loss (Figure 1B).

Similar to the results of urinary virus shedding, an association between the PB virus burden during infancy and SNHL was observed when the children enrolled in the study were divided arbitrarily in 3 groups of an equal number of children: children with a viral load < $3.5 \times 10^3$ ge/mL, children with a viral load between $3.5 \times 10^3$ and $2.5 \times 10^4$ ge/mL, and children with a viral load > $2.5 \times 10^4$ ge/mL. The results were analyzed with the $\chi^2$ test for linear trend analysis. As shown in Figure 2, none of the 25 children with a viral load < $3.5 \times 10^3$ ge/mL had SNHL, whereas 2 of the 25 children in the group with virus burden between $3.5 \times 10^3$ ge/mL and $2.5 \times 10^4$ ge/mL (8%) and 10 of the 25 children with viral load > $2.5 \times 10^4$ ge/mL (40%) had SNHL ($P = .0001$).

**DISCUSSION**

In infants with asymptomatic congenital CMV infection, a high virus burden during the first month of life is associated with SNHL. This association was apparent whether virus burden was assessed by using the quantity of infectious CMV in urine or the amount of PB CMV DNA. The results of this prospective study indicate that there is a threshold level of virus burden below which the risk of hearing loss is very low. None of the 41 children with a PB viral load < $1.0 \times 10^4$ ge/mL and only 1 of 26 infants with urine CMV levels < $5.0 \times 10^3$ pfu/mL had SNHL. These
findings imply that it may be possible to identify children with asymptomatic congenital CMV infection at increased risk for SNHL by measuring virus burden during early infancy. If confirmed in future studies of congenital CMV infection with larger sample sizes, early identification of children who are at risk for SNHL will greatly improve the counseling provided to the parents of infected neonates. Because most children with congenital CMV infection develop normally without any sequelae, the ability to identify children at risk for SNHL early in life can lead to a better use of resources by targeting these children for closer monitoring and intervention. Finally, early identification of at-risk children will be crucial for the evaluation of future antiviral therapies to prevent or reduce the incidence of CMV-related hearing loss.

The pathogenic features of congenital CMV infection that result in SNHL have not been defined. Systemic virus burden has been shown to predict the likelihood of CMV disease in individuals who are immunocompromised, such as patients with acquired immunodeficiency syndrome and allograft recipients.18-21 In women with primary CMV infection during pregnancy, ≥10^3 ge/mL of CMV DNA in amniotic fluid between 21 and 25 weeks gestation was predictive of intratérine transmission.23,24 Higher amniotic fluid viral loads were also associated with symptomatic congenital infection. Children with symptomatic congenital CMV infection born to mothers with primary maternal CMV infection had significantly higher viral loads than children with asymptomatic infection.25 We reported recently that disseminated infection at birth in infants with symptomatic congenital CMV infection as evidenced by the presence of petechiae, hepatitis, and thrombocytopenia was associated with an increased likelihood of SNHL.26 By using the χ² test for trend analysis, an association between the amount of CMV in urine and SNHL was also observed in that study.17 Studies of a limited number of temporal bones from infants with congenital CMV infection and experiments in the guinea pig model of congenital CMV infection demonstrated the presence of CMV antigens in the cells of the inner ear.34-36 These observations and the results of this study suggest that increased virus burden and continued viral replication in the affected organ systems leading to the loss of non-regenerating inner ear hair cells, spiral ganglion cells, or both could play an important role in the pathogenesis of CMV-related SNHL.

The relationship between a high virus burden in infancy and the likelihood of SNHL suggests a role for antiviral therapy in decreasing the incidence and severity of CMV-related hearing loss. However, a number of unresolved issues remain about the role of antiviral therapy in children with congenital CMV infection; these include the optimal timing and duration of antiviral therapy and the target population that would receive the most benefit from the therapy. In a phase II study of ganciclovir treatment of symptomatic congenital CMV infection, urinary CMV excretion decreased with antiviral therapy; however, viruria returned to near pretreatment levels after the cessation of therapy.27 A randomized controlled trial has examined the effect of ganciclovir therapy for 6 weeks during early infancy on hearing outcome in children with symptomatic congenital CMV infection involving the central nervous system.38 Of the 100 children enrolled in the study, the results of baseline and 6-month follow-up hearing evaluations were only available in 42 patients. Twenty-one of 25 infants treated with ganciclovir (84%) had improved hearing or maintained normal hearing between baseline and 6 months, compared with 10 of 17 children randomized to no treatment (59%; P = .06). In addition, none of the 25 children treated with ganciclovir had worsening in hearing between baseline and 6 months, versus 7 of the 17 children who were control subjects (41%; P < .01). When the hearing outcome at 1 year or older was compared between the groups, 68% of the children who were control subjects (13/19) had worsening of hearing, compared with 21% of children in the ganciclovir-treated group (5/24; P <.01). However, significant adverse effects were seen in about two thirds of infants receiving ganciclovir.37,38 Therefore, further studies are needed before a recommendation for the use of antiviral therapy in the clinical treatment of children with congenital CMV infection can be made.

In conclusion, children with asymptomatic congenital CMV infection with higher amounts of infectious CMV in urine and CMV DNA in PB during early infancy are more likely to have SNHL. The demonstration that the risk of hearing loss increases when subjects are grouped according to increasing virus burden breakpoints suggests the role of virus burden and viral replication in the pathogenesis of congenital CMV infection. The exact role of virus burden in the pathogenesis of SNHL (in particular, delayed onset hearing loss, progressive hearing loss, or both) will need to be defined in future prospective studies that include a larger number of children with congenital CMV infection with sufficient follow-up.

REFERENCES


