Detection of Cytomegalovirus (CMV) DNA by Polymerase Chain Reaction Is Associated with Hearing Loss in Newborns with Symptomatic Congenital CMV Infection Involving the Central Nervous System

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Objective. The study sought to determine the relationship between cytomegalovirus (CMV) viremia during early infancy and clinical and laboratory outcome events, particularly hearing loss in infants with symptomatic congenital CMV infection involving the central nervous system (CNS).

Study design. A total of 147 infant patients were enrolled prospectively in 2 clinical trials evaluating ganciclovir for the treatment of symptomatic congenital CMV infection involving the CNS. Aliquots of serum collected at enrollment in either of the 2 trials were available from 50 of the infants, and the degree of viremia was determined by real-time quantitative polymerase chain reaction.

Results. Of the 50 infants from whom serum samples were available, 37 had detectable CMV DNA in the serum sample collected at enrollment and were classified as viremic. Viremic infants were more likely to have (1) hearing loss both at enrollment ($P = .045$) and at the 6-month follow-up testing ($P = .035$) and (2) other indicators of active CMV disease, including elevated levels of alanine aminotransferase, petechial rash, and organomegaly.

Conclusion. In children with symptomatic congenital CMV infection involving the CNS, viremia during early infancy is associated with hearing loss and systemic CMV disease.

Congenital cytomegalovirus (CMV) infection is the leading nongenetic cause of neurosensory hearing loss in developed countries, including the United States [1–6]. Although central nervous system (CNS) manifestations at birth in children with clinically apparent (or symptomatic) congenital CMV infection predict cognitive and motor deficits, they do not predict hearing loss [2, 4].

The National Institute of Allergy and Infectious Diseases (NIAID) Collaborative Antiviral Study Group (CASG) conducted a series of studies to assess the effect that intravenous-ganciclovir treatment of symptomatic congenital CMV disease involving the CNS has on hearing, particularly the stabilization or improvement of hearing. Prospectively collected serum samples from these studies were probed by polymerase chain reaction (PCR) to determine the relationship between baseline viremia, as determined by detection of CMV DNA, and both hearing outcome and laboratory outcome. The primary purpose of this study, then, was to determine whether the detection of CMV DNA by PCR was associated with clinical- and laboratory-outcome events, particularly hearing loss.

PATIENTS AND METHODS

Study Population

During 1985–1999 the CASG enrolled 147 infants in 2 clinical trials (phase II and phase III) that evaluated ganciclovir treatment of neonates with symptomatic congenital CMV infections [7, 8]; the first trial assessed the pharmacokinetics and pharmacodynamics of intravenous-ganciclovir therapy, as well as a measure of this drug's efficacy with regard to viruria [9, 10], and the second trial compared ganciclovir treatment to no treatment, to determine the safety and efficacy of ganciclovir therapy, as recently reported [8]. The entry criteria for both studies were identical and have been detailed elsewhere [7, 8]. In brief, all infants had both a urine culture positive for CMV and evidence of symptomatic disease involving the CNS (e.g., microcephaly, intracranial calcifications, abnormal cerebrospinal fluid (CSF) indices, chorioretinitis, and/or hearing deficits). Additional inclusion criteria included age ⩽1 month at enrollment, gestational age ⩾32 weeks, and birth weight ⩾1200 g. Infants were excluded if death was imminent, they had received other antiviral agents or immune globulin, the creatinine level was >1.5 mg/dL at the time of screening, and/or either HIV infection or hydranencephaly was present.

During the course of the study period, each infant had serial clinical, laboratory, and audiologic (brain-stem evoked response [BSER] audiometric) evaluations. Aliquots of serum, urine, and CSF were archived and frozen for potential future analyses, as feasible.

Serum samples were taken from 50 of the 147 infants when they were enrolled in the 2 studies; these samples were defined as baseline samples. The infants from whom these samples were obtained were equally divided between the treatment and no-treatment groups, and those enrolled in the phase III trial represented the vast majority.

Clinical, Audiologic, and Laboratory Data

The infants were serially followed, as defined in the previously reported clinical trials, for extent of clinical involvement (including progression, stabilization, or improvement in disease), laboratory evaluation, and audiologic assessment by BSER audiometry.

Description of audiologic assessments employed. Audiologic analyses were performed on the best evaluable ear ("functional" assessment) of each infant and on the total number of evaluable ears ("biologic" assessment), as described elsewhere [8]. The best-ear analysis correlates with functional hearing loss in daily living (e.g., a person with mild hearing loss in 1 ear and severe hearing loss in the other ear will function as a mildly hearing impaired person); total-ear analysis further assesses the biologic outcomes. In the analyses below, odd numbers of total ears by treatment category are reported because, at a given follow-up

visit, an infant may have had only 1 evaluable ear (e.g., otitis media [i.e., a nonevaluable ear] on 1 side and a normal-hearing ear [i.e., an evaluable ear] on the other side) [8]. In these cases, if the second ear was both evaluable and normal, the infant was included in the best-ear analysis and would contribute 1 ear to the total-ear analysis; on the other hand, if the evaluable ear was abnormal, the infant was not included in the best-ear analysis, because the nonevaluable ear could have potentially been either normal or less severely affected. However, these cases would still add 1 ear each to the total-ear analysis.

BSER definitions. The BSER thresholds were defined as follows: normal hearing, 0–20 dB; mild hearing loss, 21–45 dB; moderate hearing loss, 46–70 dB; and severe hearing loss, ⩾71 dB, as reported elsewhere [4, 5]. The BSER threshold was defined as the lowest intensity level at which wave V could be detected and replicated. Hearing classifications were determined by audiologists at each participating site and, in the phase III study, were confirmed by a single CASG Central Unit audiologist who was blinded with regard to the randomization group [8]. For the purposes of the study, these categories were then collapsed into normal versus abnormal.

Samples

Baseline serum samples were obtained from all infants. After the baseline study laboratory evaluations, remaining serum samples were aliquoted and frozen at −20°C. All aliquots were maintained in freezers with emergency-power backup, to insure stability, and all stored samples were used on only 1 occasion. Before evaluation by real-time PCR, Institutional Review Board approval to assess the stored samples was obtained. Serum samples were then processed by use of a commercially available DNA extraction kit (QIAamp DNA blood kit; QIAGEN). The staff performing the assays were blinded with regard to the randomization.

Real-Time PCR

The CMV primers were selected from the highly conserved AD-1 region of the major envelope glycoprotein B (gB) [11]. The forward primer was 5′-AGG TCT TCA AGG AAC TCA GCA AGA, and the reverse primer was 5′-CGG CAA TCG GTT TGT TGT AAA. The internal probe 5′-ACC CCG TCA GCC ATT CTC TCG GC was labeled at the 5′ end with fluorescent dye 6-carboxyfluorescein (i.e., FAM), as the reporter dye, and the 3′ end was labeled with quencher dye 6-carboxytetramethylrhodamine (i.e., TAMRA).

Real-time. The PCR assay was performed by use of the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Each reaction mixture contained a 400-nmol/L concentration of CMV primers and a 150-nmol/L concentration of probe. TaqMan universal master mix, containing AmpliTaq Gold DNA polymerase, deoxynucleoside triphosphates with

deoxyuridine triphosphate, AmpErase uracil-N-glycosylase, Passive Reference 1, and optimized buffers, was obtained from Perkin-Elmer Applied Biosystems. Each 25-μL mixture contained 20 μL of master mix and 5 μL of the test-sample DNA preparation. The PCR-cycle parameters were incubation at 50°C for 2 min and at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min [11].

Quantitative PCR. To establish the standard curve, pTZG, a plasmid containing the target sequence of the CMV gB, was constructed. The purified plasmid was quantitated spectrophotometrically, and the copy number of molecules was calculated. Quantitation of CMV DNA in the test samples was achieved by means of serial 10-fold dilutions of the previously quantitated plasmid standards. The plasmid standards and test samples were quantitated in triplicate, and the average values were used to determine the CMV viral load. The CMV virus burden in serum was expressed as CMV genomic equivalents per milliliter (ge/mL). To control for preparation and amplification of the sample, primers for amplification of a housekeeping gene, *G3PDH,* were included in each PCR assay. The PCR conditions were optimized by amplification of the target sequence from the plasmid pTZG, with the sensitivity of the assay being ~50 ge/mL of sample. Real-time PCR provided both a quantitative assessment of viral load and a qualitative assessment—specifically, whether CMV DNA positivity (hereafter referred to as "viremia") was present. For the purposes of these analyses, infants with ≥200 ge/mL were considered to be viremic (this cutoff is the equivalent of 1 virion/5 μL of test sample). In addition, the quantitative viral load was stratified into 3 subgroups for analysis: <200, 200–5400, and ≥5400 ge/mL; these groups were chosen to represent, respectively, the <25, 25–75, and >75 percentiles of the distribution of viral load.

Data Analyses

The results of the real-time PCR were entered into the SAS V9 data sets (SAS Institute) and were combined with the clinical-trial data containing the monitoring and outcome results from the phase II and III studies, including the groups' demographic characteristics, the results of clinical and laboratory monitoring, and the outcome results. Differences between the viremic and nonviremic groups, with respect to (1) hearing status both at baseline and at follow-up (at both 6 months and ≥12 months) and (2) other relevant clinical and laboratory parameters (organomegaly, petechial rash, thrombocytopenia, neutropenia, microcephaly, and intrauterine growth retardation [IUGR] and levels of alanine transaminase [ALT], creatinine, and bilirubin) were investigated by χ^2 analyses. A logistic regression model was applied to examine factors independently related to hearing loss at baseline and at the 6-month follow-up.

RESULTS

Demographic characteristics and detection of viremia. Of the 50 infants from whom baseline serum samples were taken, 37 had real-time–PCR results that were positive for CMV (≥200 ge/mL) and therefore were considered to be viremic at baseline. The distribution of infants, stratified on the basis of treatment status and other baseline demographic data, is shown in table 1. In these 50 infants, the median CMV load at baseline was 810 ge/mL (range, 0–720,000 ge/mL); 13 infants had PCR results that were negative for CMV (<200 ge/mL), and 37 infants had PCR results that were positive for CMV (24 infants had CMV levels of 200–5400 ge/mL, and 13 infants had CMV levels >5400 ge/mL).

Correlation between viremia and hearing outcome. In the best-ear analysis, infants who were viremic at baseline were statistically more likely to have hearing loss both at baseline ($P = .045$) and at the 6-month follow-up ($P = .035$), and the results of assessment approached significance at the ≥12-month follow-up ($P = .054$); in the total-ear analysis as well, these infants were statistically more likely to have hearing loss at all 3 time intervals (baseline, $P = .001$; 6-month follow-up, $P = .003$; ≥12-month follow-up, $P = .02$) (table 2).

Grouping according to virus load, on the basis of the breakpoints (<200, 200–5400, and ≥5400 ge/mL) described above, demonstrated that, in viremic infants, an increase in virus load was no more predictive of hearing loss than was qualitative identification of viremia (<200 vs. ≥200 ge/mL) (table 3).

Table 1. Demographic characteristics and treatment-group assignments for infants with symptomatic congenital cytomegalovirus (CMV) infection, stratified on the basis of results of real-time polymerase chain reaction performed on baseline serum samples (viremia = ≥200 genomic equivalents/mL).

| Characteristic | Baseline CMV viremia | | P |
	Present (n = 37)	Absent (n = 13)	
Age, days			.08
Median	12	17	
Range	1–34	8–33	
Sex			.75
Female	18 (49)	5 (35)	
Male	19 (51)	8 (62)	
Race			.15
White	24 (65)	6 (46)	
Black	7 (19)	6 (46)	
Other	6 (16)	1 (8)	
Treatment status			.33
Ganciclovir	18 (49)	9 (69)	
No ganciclovir	19 (51)	4 (31)	

NOTE. Data are no. (%) of infants, unless indicated otherwise.

Table 2. Hearing evaluation determined by brain-stem evoked response (BSER) in infants with symptomatic congenital cytomegalovirus (CMV) infection, at baseline and at 6-month and ⩾12-month follow-up, stratified on the basis of results of real-time polymerase chain reaction used on baseline serum samples (viremia = ⩾200 genomic equivalents/ mL).

BSER	Baseline CMV viremia		P
	Present	Absent	
At baseline			
Best ear	(n = 32)	(n = 10)	.045
Normal	14 (44)	8 (80)	
Abnormal	18 (56)	2 (20)	
Total ear	(n = 64)	(n = 20)	.001
Normal	19 (30)	14 (70)	
Abnormal	45 (70)	6 (30)	
At 6 months follow-up			
Best ear	(n = 26)	(n = 7)	.035
Normal	15 (58)	7 (100)	
Abnormal	11 (42)	0 (0)	
Total ear	(n = 50)	(n = 14)	.003
Normal	20 (40)	12 (86)	
Abnormal	30 (60)	2 (14)	
At ⩾12 months follow-up			
Best ear	(n=24)	(n=5)	.054
Normal	8 (33)	4 (80)	
Abnormal	16 (67)	1 (20)	
Total ear	(n = 48)	(n = 11)	.02
Normal	13 (27)	7 (64)	
Abnormal	35 (73)	4 (36)	

NOTE. Data are no. (%) of infants, unless indicated otherwise.

Correlation between viremia and other laboratory and clinical parameters. Infants who were viremic at baseline were also more likely, at some point during the course of the study, to have petechial rash ($P = .01$), splenomegaly ($P = .02$), and elevated levels of ALT ($P = .006$) (table 4); the data suggest that this may also be true for thrombocytopenia, although the differences approached but did not attain statistical significance ($P = .10$).

In contrast to the data with regard to hearing, the amount of virus present in viremic infants at presentation (i.e., the baseline viral load) correlated with laboratory abnormalities at every point during the course of the study. Specifically, an increase in virus load correlated significantly, in a univariate analysis, with frequency of petechial rash, splenomegaly, and/ or thrombocytopenia and with elevated levels of ALT (table 5).

There was no apparent relationship between the baseline level of viremia and elevation in the level of either creatinine or bilirubin, nor did the incidence of neutropenia differ between viremic and nonviremic infants during the course of the study. There also was no correlation between the level of baseline of viremia and either microcephaly or IUGR.

Logistic regression modeling. The baseline factors of sex, splenomegaly, thrombocytopenia, petechial rash, CMV viremia at baseline, and elevated levels of ALT were included in a logistic regression model with backward elimination. The baseline factors of viremia ($P = .02$) and petechial rash ($P = .03$) were found to be independently associated with hearing loss.

Detection of viremia and effect of therapy. The observation that infants who were viremic at baseline were at higher risk for hearing loss at the 6-month follow-up prompted an analysis of hearing outcome with regard to ganciclovir therapy for that subgroup. Of the 26 infants who were viremic at baseline and who had a BSER evaluation at the 6-month follow-up, 10 (67%) of 15 ganciclovir recipients had normal hearing (best-ear analysis) at the 6-month follow-up, compared with 3 (27%) of 11 placebo recipients ($P = .05$).

DISCUSSION

Congenital CMV infection is the most common congenital infection in developed countries, occurring in ~1% of all live-born infants [3, 12, 13]; ~10% of these congenitally infected

Table 3. Hearing evaluation determined by brain-stem evoked response (BSER) in infants with symptomatic congenital cytomegalovirus (CMV) infection, at baseline and at 6-month and ⩾12-month follow-up, stratified on the basis of virus load expressed in terms of genomic equivalents per milliliter (ge/mL).

Virus load at baseline, ge/mL	BSER		P
	Normal	Abnormal	
	At baseline		
	(n = 22)	(n = 20)	.13
<200	8 (80)	2 (20)	
200–5400	10 (45)	12 (55)	
>5400	4 (40)	6 (60)	
	At 6 months follow-up		
	(n = 22)	(n = 11)	.11
<200	7 (100)	0 (0)	
200–5400	9 (56)	7 (44)	
>5400	6 (60)	4 (40)	
	At ⩾12 months follow-up		
	(n = 12)	(n = 17)	.16
<200	4 (80)	1 (20)	
200–5400	5 (33)	10 (67)	
>5400	3 (33)	6 (67)	

NOTE. Data are no. (%) of infants, unless indicated otherwise.

Table 4. Clinical features and laboratory evaluations of infants with symptomatic congenital cytomegalovirus (CMV) infection, stratified on the basis of results of real-time polymerase chain reaction used on baseline serum samples (viremia = ≥200 genomic equivalents/mL).

| Characteristic | Baseline viremia | | P |
	Present	Absent	
Alanine transaminase	(n = 37)	(n = 12)	.006
Normal	11 (30)	9 (75)	
Abnormal[a]	26 (70)	3 (25)	
Petechial rash	(n = 36)	(n = 11)	.01
Absent	17 (47)	10 (91)	
Present	19 (53)	1 (9)	
Hepatomegaly	(n = 36)	(n = 12)	.11
Absent	9 (25)	6 (50)	
Present	27 (75)	6 (50)	
Splenomegaly	(n = 36)	(n = 12)	.02
Absent	10 (28)	8 (67)	
Present	26 (72)	4 (33)	
Thrombocytopenia[b]	(n = 37)	(n = 11)	.10
Absent	24 (65)	10 (91)	
Present	13 (35)	1 (9)	

NOTE. Data are no. (%) infants, unless indicated otherwise.
[a] >1.1 × normal at any point during the study period.
[b] Platelet count <75,000 at any point during the study period.

infants will be symptomatic at birth, and in ~75% of this symptomatic group the infection will involve the CNS [3]. Sensorineural impairment is a common complication of this infection, regardless of whether the latter is symptomatic; of those infants who are symptomatic at birth, ~65% will develop hearing loss, compared with ~5%–10% of those who are asymptomatic [1, 3]. To maximize the therapeutic benefit while minimizing drug exposure, defining the methods to predict hearing loss is of the utmost importance. The present study represents one approach to this problem, albeit in children infected with symptomatic congenital CMV disease involving the CNS.

For newborn infants with symptomatic congenital CMV infection, viremia at baseline correlates with both best-ear and total-ear hearing loss, both at baseline and at the 6-month follow-up; furthermore, viremia correlates significantly with total-ear hearing loss at the longer-term, ≥12-month follow-up. Viremia correlates with other indicators of active systemic disease, including petechial rash, splenomegaly, elevated levels of ALT, and perhaps thrombocytopenia. Other recent studies have indicated that a higher systemic virus burden (as evidenced by viral load) is associated with hearing loss in infants with congenital CMV infection [2, 11]. These findings become exceedingly relevant in the attempt to identify those infants

who are most likely to benefit from antiviral therapy; this is especially true because treatment with ganciclovir is associated with acute toxicity and also has the potential for long-term toxicity [8].

The study populations included only infants who were at significant risk for devastating outcomes, including hearing loss, that are due to congenital CMV infection. These populations were selected because of the known toxicities of ganciclovir, including its potential, in nonhuman models, for development of mutagenicity, teratogenicity, and carcinogenicity [7, 8, 14]. At the outset of these earlier studies, as well when the present article was accepted for publication, the potential risk:benefit ratio precluded ganciclovir use in those infants who are asymptomatically infected with CMV. Because the majority (~80%–85%) of infants asymptomatically infected with congenital CMV do not develop hearing loss, an accurate indicator of higher risk should help to identify those infants most likely to benefit from available treatment resources (counseling, close monitoring, early intervention, and controlled-trial evaluation of antiviral therapy). Given the inherent problems of the currently available antiviral therapy and the difficulty and risk involved in maintaining intravenous access for 6 weeks of treatment, it

Table 5. Clinical features and laboratory evaluations of infants with symptomatic congenital cytomegalovirus infection, stratified on the basis of virus load expressed in terms of genomic equivalents per milliliter (ge/mL).

Virus load at baseline, ge/mL	Characteristic		P
	Alanine transaminase		
	Normal (n = 20)	Abnormal (n = 29)	.003
<200	9 (75)	3 (25)	
200–5400	10 (42)	14 (58)	
>5400	1 (8)	12 (92)	
	Petechial rash		
	Absent (n = 27)	Present (n = 20)	.01
<200	10 (91)	1 (9)	
200–5400	13 (57)	10 (43)	
>5400	4 (31)	9 (69)	
	Splenomegaly		
	Absent (n = 18)	Present (n = 31)	.03
<200	8 (67)	4 (33)	
200–5400	8 (35)	16 (65)	
>5400	2 (15)	11 (85)	
	Thrombocytopenia		
	Absent (n = 24)	Present (n = 5)	.05
<200	10 (91)	1 (9)	
200–5400	18 (75)	6 (25)	
>5400	6 (46)	7 (54)	

NOTE. Data are no. (%) of infants, unless indicated otherwise.

would be helpful to identify those infants in whom the benefits of the therapy outweigh the risks.

The interpretation of the results of the present study has limitations. First, we have been able to evaluate samples from—and, therefore, to report associations for—only a small number of infants; nonetheless, this study presents one of the largest data sets of such severely infected children that has been acquired prospectively. Second, we have selected a population that represents only part of the spectrum of children with congenital CMV infection; thus, our results should not be extrapolated to all congenitally infected children. Third, we have assessed natural-history data from a randomized, controlled trial of antiviral therapy in which the treatment and its effects are themselves tested. With our data, we cannot fully control for the effect that ganciclovir therapy has on the observed association between baseline viremia and (1) hearing loss at the 6- and ≥12-month follow-ups and (2) clinical and laboratory abnormalities observed during the course of the study; however, the association between baseline viremia and hearing loss at baseline is prior to—and, therefore, unconfounded by—antiviral therapy. Fourth, the samples tested were serum samples, which should yield lower quantitative results than would be seen with whole-blood samples. The serum samples were frozen for varying periods before being processed, but all of them were maintained at a rigidly controlled temperature; the samples were used only once. Fifth, in contrast with other investigators [2, 11], we did not establish that viral load has a quantitative effect on hearing outcome but did establish that it has such an effect on other factors indicative of systemic disease; these findings may be related to the disease status of the infants (all had symptomatic disease involving the CNS), the nature of the samples analyzed (other investigators have analyzed urine or whole blood), and/or the relatively small population studied. Regardless, the methodology of the present study did associate viremia with hearing loss, not only at baseline but also at follow-up evaluation. As new treatment initiatives that assess outcome in less severely afflicted infants are developed, the contribution that virus load makes to outcome measures must be assessed. As mentioned above, we did not study asymptomatically infected infants (although one author of the present study recently has evaluated such a population and has reached conclusions similar to those reported here [11]).

A statement regarding the value that antiviral therapy has for congenital CMV infections is indicated, particularly in the context of the present study. Data from 1 randomized, controlled clinical trial and 3 open-label, nonrandomized studies indicate that ganciclovir has the potential to play a role in therapy for congenital CMV infections [7, 8, 15, 16]. Currently available data support only the treatment of infants with symptomatic infection involving the CNS and, even for orally administered drugs such as valganciclovir, should not be extrapolated to asymptomatically infected infants. The pediatric–infectious-diseases and neonatology communities await the development of drugs with improved therapeutic indices. Such drugs may include maribavir (GW-1263; currently under development by ViroPharma) [17] and derivatives of cidofovir that appear to have less renal toxicity (Chimerix) [18].

At the present time, treatment decisions cannot be based on the presence of viremia at baseline. Future prospective clinical trials should be designed to validate the data from the present study and to further identify the populations in greatest need of antiviral therapy. To that end, the NIAID CASG is currently enrolling infants in a large multicenter trial of orally administered valganciclovir as antiviral therapy for symptomatic congenital CMV infection, a trial in which quantitative real-time PCR is used on samples from all enrolled infants. Further prospective studies of both the natural history of CMV and promising antiviral therapies—especially those with toxicities less than that of the ganciclovir derivatives—will be necessary to advance our understanding of the role that viremia, viral load, or systemic virus burden plays in the pathophysiology of hearing loss in congenital CMV infection.

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