Mixed Infection and Strain Diversity in Congenital Cytomegalovirus Infection


Departments of Pediatrics, Epidemiology and International Health, Microbiology, and Neuroscience, University of Alabama, Birmingham; Department of Pediatrics, Carolinas Medical Center, Charlotte, North Carolina; Department of Pediatrics, University of Pittsburgh and the Children’s Hospital of Pittsburgh of UPMC, Pennsylvania; Department of Pediatrics, University of Texas Southwestern Medical Center, Dallas; Cincinnati Children’s Hospital Medical Center and University of Cincinnati, Ohio; Department of Pediatrics, Saint Peter’s University Hospital, New Brunswick, New Jersey; Drexel University College of Medicine, Philadelphia, Pennsylvania; and Department of Pediatrics, University of Mississippi Medical Center, Jackson

Background. Cytomegalovirus (CMV), the most common cause of congenital infection, exhibits extensive genetic variability. We sought to determine whether multiple CMV strains can be transmitted to the fetus and to describe the distribution of genotypes in the saliva, urine, and blood.

Methods. Study subjects consisted of a convenience sampling of 28 infants found to be CMV-positive on newborn screening as part of an ongoing study. Genotyping was performed on saliva specimens obtained during newborn screening and urine, saliva, and blood obtained at a later time point within the first 3 weeks of life.

Results. Six (21.4%) of the 28 saliva samples obtained within the first 2 days of life contained a CMV genotype. Multiple CMV genotypes were found in 39% (5/13) of urine, saliva, and blood samples obtained within the first 3 weeks of life from 13 of the 28 newborns. There was no predominance of a CMV genotype at a specific site; however, 4 infants demonstrated distinct CMV strains in different compartments.

Conclusions. Infection with multiple CMV strains occurs in infants with congenital CMV infection. The impact of intrauterine infection with multiple virus strains on the pathogenesis and long-term outcome remains to be elucidated.

Cytomegalovirus (CMV) is a frequent cause of congenital infection worldwide. Between 20,000 and 40,000 children are born each year in the United States with congenital CMV infection; ~15% of those will develop permanent sequelae, the most common being sensorineural hearing loss (SNHL) [1, 2]. The reason only some children develop SNHL or other sequelae after congenital CMV infection is not understood but could be related to both host and viral factors.

Human CMV (HCMV) is a large virus with >200 open reading frames. CMV isolates from infected individuals have been shown to be genetically diverse [3]. Several regions of the HCMV genome have been used to define distinct genotypes based on clustering of polymorphisms. Mixed infection with multiple CMV strains occurs in various patient populations including immunocompetent and immunocompromised subjects [4–7]. However, infection with multiple strains has rarely been described in newborns with congenital CMV. This study sought to determine if congenital infection can result from multiple CMV strains by genotyping glycoproteins gB, gN, and gH in blood, urine, and saliva specimens of newborns with congenital CMV infection. In addition, the distribution of CMV genotypes in saliva, urine, and blood was examined.

METHODS

Study Population

From May 2007 through January 2009, 201 infants were found to be CMV-positive on newborn screening at the
7 hospitals participating in the National Institute on Deafness and Other Communication Disorders (NIDCD) CMV and Hearing Multicenter Screening Study (CHIMES Study) [8]. Screening for congenital CMV infection was performed by rapid culture of saliva specimens (screening saliva samples). Infants that screened positive for congenital CMV infection were enrolled in the follow-up component of the study and urine, saliva, and dried blood spot (DBS) samples (follow-up samples) were obtained to confirm infection. Institutional review board approval was obtained at each study site. Written informed consent was obtained from a parent for their newborn’s enrollment in the study. A convenience sampling of 28 study subjects from all 7 study sites was selected based on the availability of adequate remnant saliva, urine and blood specimens for genotyping. The demographic characteristics of these 28 subjects did not differ significantly from the demographics of the entire group of 201 children found to be CMV-positive during the study period (data not shown). Laboratory personnel were blinded to demographic characteristics, clinical findings, and previous genotyping results of study subjects.

Characterization of CMV Genotypes
DNA was extracted from urine and saliva samples using commercial spin columns (Qiagen, Inc). DNA extraction from DBS samples was performed from 2 3-mm punches as described elsewhere [8]. For gN (UL73) genotyping, samples underwent polymerase chain reaction (PCR) to amplify the gN region using primers and conditions previously reported [7]. To reduce the possibility of PCR artifact, positive and negative controls were included with each PCR run. PCR products were directly cloned into the TOPO TA cloning vector pCR 2.1 (Invitrogen Inc) and up to ten individual colonies were screened for the presence of the gN insert [7]. The nucleotide sequences were compared with the published sequences of the 7 described gN genotypes (GeneBank accession numbers AF309971, AF309976, AF309980, AF309773, AF309987, AF309997, and AF310004). Genotyping of gB (UL55) and gH (UL75) was performed as described using the TaqMan platform [6, 9]. Specimens in which no gB genotype could be determined by this method underwent PCR to amplify the 961–1738 bp gB region with the following primers: gBlong-Fw (5’ cac agg tgt ggt ctt ct) and gBlong-rev (5’ gtc gtt agt agc agc gtc ct). The PCR conditions were optimized for HotStart Taq polymerase (5’) and included initial denaturation at 94°C for 4 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 40 seconds, extension at 68°C for 1 minute, and final extension at 68°C for 5 minutes. Nucleotide sequences of purified PCR products were compared with published sequences in the NCBI database (GeneBank accession numbers M60929, M60932, M60933, M60926, and GU180092). Different laboratory personnel performed genotyping on screening and follow-up samples (for blinding purposes).

RESULTS
Population Demographics
Study infants were primarily identified from the newborn nursery (27/28, 96%). Just over half were black, 5 (18%) were non-Hispanic white, 4 (14%) were Hispanic white, 1 (4%) was multiracial, and 1 (4%) was Asian. The gender of the study subjects was equally distributed (50% female).

CMV Genotyping of Samples
Screening saliva samples obtained at a median of 1 day (range, 0–2 days) of age from the 28 study subjects were analyzed for gN, gB, and gH genotypes. All 7 gN genotypes (1, 2, 3a, 3b, 4a, 4b, 4c), all 5 gB genotypes (1–5) and both gH genotypes (1, 2) were represented in the saliva samples (Figure 1). gN genotyping was performed on 26/28 screening saliva samples because the gN gene could not be amplified from 2 specimens. gN type 3a was the most commonly observed strain (32%) followed by type 4c (23%). Genotyping of gB and gH was performed on all 28 screening saliva samples. gB type 1 was the most common variant (42%) followed by type 2 (23%). In 2 subjects, gB genotypes could not be determined using the type-specific real-time PCR. However, nucleotide sequence analysis matched these to a previously described gB genotype 5 [10]. gH types 1 and 2 were distributed in 39% and 61% of specimens, respectively.

6 of the 28 (21.4%) newborn saliva samples had more than one CMV strain. 4 infants had 2 distinct CMV genotypes and 2 infants were shedding 3 genotypes in saliva specimens obtained within 2 days of birth. To further investigate CMV strain diversity, genotyping was carried out on urine, saliva, and DBS samples obtained at enrollment into the follow-up component of the study. To avoid the possibility of identifying CMV strains acquired postnatally, only samples from the group of 13 infants that were enrolled in the follow-up study
and had samples obtained within the first 3 weeks of life were examined. The average age at sample collection was 15.3 days (± 4.3 days). Genotyping of follow-up urine, saliva, and DBS samples showed that multiple CMV genotypes were present in 5 (39%) of 13 infants (Table 1). Four infants shed 2 different CMV strains, whereas 1 infant (Subject h) shed 3 different gN and gB genotypes. When both screening and follow-up samples were considered, approximately a third of study infants (9/28, 32%) were infected with multiple CMV strains. Interestingly, in 2 infants, (subjects b and j) a new genotype was detected in the saliva specimen taken at follow-up that was not present in the screening saliva specimen. In 1 infant (subject b), gB genotype 1 was detected in the screening saliva specimen; however, gB genotypes 2 and 3 were detected in the follow-up saliva specimen. Another infant (subject j) had gN genotype 4a in screening saliva, whereas the follow-up saliva contained type 1.

CMV Genotyping by Compartment

The presence of different viruses in different compartments was determined by analyzing saliva, urine, and blood specimens from the group of 13 infants obtained at enrollment into the follow-up study (Table 1). As shown in Table 1, no genotype predominated in saliva, urine, or blood compartments. However, 5 infants (subjects b, d, e, h, and j) had different CMV genomic variants in different compartments. Of the 6 infants whose screening saliva samples contained multiple CMV genotypes, follow-up saliva, urine, and blood samples were available from 4 (subjects d, h, k, and l). Multiple CMV genotypes were found in the follow-up samples of 2 of the 4 (subjects d and h) (Table 1).

DISCUSSION

This report demonstrates that there is great diversity among the strains that cause congenital CMV infections and congenitally infected neonates often harbor multiple CMV genotypic variants. Among the 28 saliva samples from infants obtained at birth and saliva, urine, and blood samples from 13 of the 28 subjects obtained at follow-up, all gN, gB and gH genotypes were found to cause congenital infection. Furthermore, mixed infection with >1 virus strain was detected in approximately one-third of the study infants. In addition, the presence of distinct virus strains in specimens from different compartments from the same child was demonstrated.

In studies of both immunocompetent and immunocompromised hosts, multiple CMV genotypes have been detected in older children and adults. Although studies have documented that these viruses are acquired over time through reinfections [4, 11–14], it is also possible that multiple viruses can be acquired at the time of primary CMV infection. It is generally believed that vertical transmission to the fetus occurs after maternal viremia. Whether this transmission occurs as a single event during pregnancy or through multiple placental transmission events over the course of the pregnancy is unknown. Studies of the guinea pig model of congenital CMV infection have shown that maternal viremia leads to placental infection, and, in some instances, a secondary viremia can occur and result in secondary seeding of the placenta. However, dissemination to the fetus is not always immediate and the placenta serves as a reservoir for the virus [15–17]. Studies of human placentas have also shown multiple CMV genotypes at the maternal-fetal interface [18]. Thus, virus transmission to the fetus resulting in

### Table 1. Distribution of CMV Genotypes in the Saliva, Urine, and Dried Blood Spot (DBS) of 13 Children With Congenital Infection

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**NOTE.** DBS, dried blood spot.

<sup>a</sup> Saliva samples taken within the first few days of life. Urine, saliva, and DBS samples were taken at a later time point within the first 3 weeks of life.

<sup>b</sup> CMV DNA was detected in only 4/13 DBS samples.

<sup>c</sup> Ellipses denotes sample unavailable for testing.
congenital infection with multiple CMV strains could occur as a single infection with codisseminating strains, or by multiple transmission events of individual viruses, or both.

Studies examining the association between virus diversity at a single polymorphic gene and outcome in children with congenital CMV infection reported conflicting results [19–22]. However, studies in the murine model and in immunocompromised patients suggest that coinfection with multiple strains of CMV could lead to enhanced pathogenicity [5, 23, 24]. In a study that examined the diversity of 3 polymorphic CMV genes in infants with congenital CMV infection, >1 virus strain was detected in 8/10 specimens obtained from stillborn infants but only a single genotype from 22 living newborns [25]. However, the interpretation of these findings is difficult because tissue-cultured viruses from living newborns were compared with paraffin-embedded tissue specimens from stillborn infants. Since propagation of virus in tissue culture often selects for a single virus strain [26–29], the inability to detect multiple virus strains in infants with congenital CMV infection in previous studies [22, 25] could be due to methodological issues.

In the current study, original saliva, urine, and DBS samples from congenitally infected infants were analyzed to avoid tissue culture selection of viruses, and real-time PCR and cloning of PCR products were used to genotype virus strains. Previous studies examining genotype distribution have used either virus from tissue cultures or samples from urine only. In addition, genotyping was performed by gene amplification and sequencing of the product [22, 25]. Thus, it is likely that only dominant or selected genotypes were detected. Although the implications of finding that congenital infection can be caused by multiple CMV strains, with respect to sequelae such as SNHL, are not yet known, the results from the ongoing NIDCD CHIMES study [8] that includes prospective follow-up of a large number of congenitally infected infants should provide a better understanding of the role of mixed infection on outcome.

In 13 study infants, urine, saliva, and DBS samples were available within the first 3 weeks of life and were examined to determine the CMV strain diversity in different compartments. Although there was not a predominant genotype, unique strains in different compartments were found in 5 of these infants. Compartmentalization of CMV strains has been reported in immunocompromised populations [30, 31]. CMV has the ability to grow in certain cell types, and variable cell tropism is conferred by particular viral genes that are present in clinical CMV strains [32, 33]. This observation has raised the possibility that CMV strain variation might explain differences in the biological behavior of different virus strains. The finding that infants with congenital CMV infection can harbor multiple CMV genotypes, and that unique genotypes are found in different compartments, underscores the need for examining the relationship between strain variation and biological characteristics of viruses.

In 2 study infants, genotypes that were detected in saliva at follow-up (at 2–3 weeks of life) were different from those detected in the saliva samples obtained at birth. This finding could have multiple explanations. Although it is possible that these infants acquired new strains during the time between screening and follow-up, this is unlikely because the follow-up samples were obtained between 2 and 3 weeks of age. Alternatively, both strains may have been present at both time points but not detected in the screening samples because relatively low numbers of the minor virus populations were present. In a recent study, investigators examined plasma and bronchoalveolar samples from 9 immunocompromised patients using a highly sensitive deep sequencing method. All 9 patients had mixed infections with 1 or 2 dominant genotypes and several low-abundance genotypes. In addition, the prevalence of the individual genotypes was shown to change over time, with strains that were initially minor becoming dominant [6]. The appearance of a “new” genotype not detected in the initial sample in our study infants may reflect a similar change in the prevalence of the individual strains.

A limitation of this study is that only a small proportion of the infected children identified in the NIDCD CHIMES study were included, which may have led to selection bias. However, this potential selection bias was unlikely to have affected the findings since the 28 study subjects have similar demographic characteristics as all CMV positive infants during the described time period. An additional limitation of the study is the use of real time PCR to detect gB and gH genotypes. The sensitivity of this assay is dependent on the relative amounts of viral DNA; thus, minor viral populations with low abundance of type-specific viral DNA could have been missed. This reduced sensitivity of the real-time PCR may have resulted in an overall underestimation of the true viral diversity within a sample and subject. Our conclusion that infection with multiple virus strains can occur in infants with congenital CMV infection remains valid, however. CMV is a large virus with >140 genes. In this study, only 3 loci (gB, gH, and gN, all known targets of neutralizing antibody) were examined to determine genetic diversity. Since many more polymorphic CMV genes have been identified, it is likely that the true virus strain diversity in the study population was underestimated. Although the relative frequency of mixed infection might change if a larger proportion of infected infants were included in the study and if more CMV loci were examined for diversity, the finding that some congenitally infected infants harbor multiple virus strains remains an interesting observation.

In summary, the present report demonstrates that there is great diversity in the CMV strains that cause congenital infection and that infection with multiple CMV strains occurs in congenital CMV infection. However, the relationship of specific genotypes and the implications of infection with multiple viral strains for the pathogenesis and long-term outcome in children with congenital CMV infection are not yet known.
References