Human Cytomegalovirus UL144 Is Associated with Viremia and Infant Development Sequelae in Congenital Infection

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Human cytomegalovirus (HCMV) strains may be genotyped based on polymorphisms that exist within the UL144 gene, which is one of 19 viral genes lost in attenuated laboratory strains. In the present study, UL144 genotypes in congenitally infected babies (congenital cytomegalovirus [cCMV]) were determined, and the relationship between the genotype, viral load, cytokine profile, and patient developmental outcome was investigated. All cCMV infections identified during 2006 and 2007 were included (n = 29). A portion of the infants were clinically assessed at birth and at 12 to 18 months postinfection for cCMV clinical sequelae (n = 18/29). The plasma viral load (PVL) was requested for 23/29 patients, and the UL144 genotypes were determined (n = 27/29). The cytokine profile in patient plasma or serum was assessed (n = 20/29). UL144 genotypes A, B, and C were detected within the cCMV population at 33.3%, 29.6%, and 25.9%, respectively. UL144 A and C were associated with a high PVL (P < 0.04). Furthermore, a significant association between the developmental outcome and UL144 A and C was observed (P < 0.04). Only patients infected with UL144 B and A/B were described as having a normal clinical outcome. In addition, a significant correlation between interleukin 10 (IL-10) levels and the PVL was observed (P < 0.04); however, there was no association between the genotype and the cytokine profile. The present study determined that the specific detection of UL144 genotypes A and C was indicative of serious cCMV infection and more likely to lead to long-term cCMV-associated clinical manifestations. The inclusion of HCMV UL144 genotyping along with the recommended PVL monitoring following cCMV diagnosis may aid prediction of the clinical outcome.

Congenital cytomegalovirus (cCMV) infection is the most common congenital infection in the developed world, affecting approximately 1% of live-born neonates. It is a frequent cause of mental retardation and the leading nongenetic cause of sensorineural hearing loss (SNHL) (7, 11). cCMV disease can result from either primary maternal infection or reactivation from latency during pregnancy, although generally, the most severe clinical syndromes follow primary infection. However, at present there is no way of definitively identifying at birth those infants who will develop sequelae and those who will not.

The clinical significance of human cytomegalovirus (HCMV) molecular epidemiology is unclear and controversial, as the 236-kb viral genome suggests that a large number of polymorphic strains may potentially exist (10). HCMV infects many different cell types, resulting in a diverse range of clinical manifestations and suggesting that the clinical outcome may be related to both genetic variation among HCMV strains and the host immune response(s). Previous studies have investigated genetic polymorphisms that exist within the envelope glycoprotein genes, as their encoded proteins are targets for neutralizing antibodies. However, the glycoprotein B (gB) gene, which encodes a putative target for HCMV vaccination, has shown no consistent relationship with disease outcome (3, 16, 25).

HCMV strains may be genotyped based on polymorphisms that exist within the UL144 gene, which is one of 19 viral genes lost in attenuated laboratory strains (4). The majority of these deleted genes are nonessential for viral replication; however, expression in vivo may contribute to disease pathogenesis (8). Three main HCMV genotypes, based on the ectodomain of the UL144 protein, have been described: UL144 A, UL144 B, and UL144 C (1, 2, 16, 24). Conflicting reports on the association between the UL144 genotypes and the viral load, clinical presentation, and clinical outcome have been published. Arav-Boger and colleagues concluded that infection with the UL144 A and C strains was associated with unfavorable clinical outcome in neonates (2). In addition, genotype UL144 C was linked to termination of pregnancy following detection of HCMV in the amniotic fluid (1). In direct contrast, UL144 genetic polymorphisms were associated with neither clinical presentation nor viral-load levels in the amniotic fluid in a French population (18, 19). Furthermore, Bale and colleagues found no relationship between the UL144 genotype and the congenital clinical outcome (3).

The present study addresses these inconsistent findings. The UL144 genotypes, detected in a well-defined, geographically distinct group of congenitally infected infants, were analyzed with respect to the viral loads, immunological cytokine profiles, and developmental outcomes of affected infants. Our findings show that the HCMV genotypes UL144 A and C are significantly associated with high plasma viral loads (PVLs) and long-term cCMV clinical sequelae.

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tation [abstract number O-12] at the 2008 Congenital Cytomegalovirus Conference, Centers for Disease Control and Prevention, Atlanta, GA, 5 November 2008.)

MATERIALS AND METHODS

Specimen and patient details. The National Virus Reference Laboratory of Ireland detected 29 individuals with cCMV by detection of early antigen fluorescent foci (DEAFF) analysis (12) of urine during 2006 and 2007. cCMV infection was confirmed by detection of the virus within the first 3 weeks of life. Ethical approval for all patients was granted from the source hospitals. CMV-positive patients identified during the same period were included in the study for comparison (n = 57), specifically, postnatally infected infants (postnatally acquired CMV [pCMV]; n = 19/57). Adult CMV infection was detected in patients with HIV infection, posttransplantation patients, and patients undergoing dialysis and in chronic respiratory tract infections.

Plasma viral load. The patient PVL was measured using the Cobas Amplicor HCMV Monitor Test (Roche Diagnostics, United Kingdom) according to the manufacturer's protocol. The assay's quantitation range was between 600 and 1,000,000 copies/ml. Samples with amplification for CMV DNA but with viral loads of <600 copies/ml were deemed positive, but the viral load was not recorded. Following a positive DEAFF test result, 23/29 cCMV patients requested PVL testing.

HCMV genotyping. Urine specimens were extracted on the Roche MagNA pure using the total nucleic acid kit (Roche Diagnostics, United Kingdom). The UL144 gene (737 bp) was amplified from the extracted urine as described by Lurain et al. (16). The amplicons were purified with a QiAQuick PCR purification kit (Qiagen, United Kingdom) and sequenced on an ABI Prism 3.10 using the Big Dye sequencing kit (Applied Biosystems) according to the manufacturer's instructions. Sequence trace files were analyzed by Lasergene SeqMan Pro software version 7.2.1 (DNAStar). Sequences were aligned in Clustal W (http://www.ebi.ac.uk/Tools/clustalw/index.html), and maximum-likelihood trees were constructed in PAUP* version 4.0 Beta 10 (Sinauer Associates, Inc., United Kingdom). The model of evolution was selected using Modeltest version 3.7 (21). Bootstrap resampling was carried out for 1,000 replicates of the data set. Genotypes were determined from the phylogenetic tree and Basic Local Alignment Search Tool analysis (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Luminex assay: cytokine and chemokine detection. Multianalyte cytokine and chemokine profiling was performed using the Luminex-100 system and the X平台 platform (Luminex Corp., TX). Plasma or serum specimens received within 2 weeks of the initial DEAFF-positive specimen (n = 20/29 cCMV patients) were analyzed for the presence of the following cytokines and chemokines by using a human cytokine Lincoplex kit according to the manufacturer's instructions (Millipore, Miami): interleukin 1α (IL-1α), IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), and macrophage inflammatory protein 1α (MIP-1α). No serum samples from healthy neonates were used as controls for ethical reasons. Neonatal cytokine and chemokine control levels were determined using HCMV-negative healthy adult serum specimens and from published neonatal and pediatric cytokine “control” values (14, 17, 22, 23). In addition, cCMV cytokine and chemokine levels were compared to those of other, noncongenital HCMV infections identified during the same period.

Clinical manifestation of disease and developmental outcome. Clinical information was obtained retrospectively by chart review for 18/29 cCMV patients. Infants were clinically assessed at birth for evidence of the following possible manifestations of cCMV infection: intrauterine growth retardation (IUGR), abnormalities, were used to categorize HCMV infection as

RESULTS

HCMV genotype. The UL144 gene was successfully amplified from 27/29 urine specimens from the cCMV-infected infants, and the sequences were genotyped following the construction of a maximum-likelihood phylogenetic tree (Fig. 1 and Table 1). All HCMV UL144 genotypes were detected (Table 2), and within each genotypic group, approximately 2 to 6 nucleotide differences were observed between strains. UL144 A consisted of two subtypes, A1 and A2, and there were approximately 10 nucleotide changes between the subtypes within the region sequenced. All genotypes were found to be circulating throughout the 2-year period, and there was no significant increase in a specific genotype at any time, indicating that no particular strain(s) dominated during the study period.

UL144 genotypes A, B, and C were detected within the cCMV patient population at 33.3%, 29.6%, and 25.9%, respectively (Table 1). Three recombinant strains were also detected, 2 UL144 A/B sequences (7.4%) and 1 UL144 A/C sequence (3.7%). The cCMV genotypes were compared with those identified from other HCMV infections, specifically, those detected in pCMV infection and adult immunodeficient patients. No particular strain was associated with any HCMV-positive group. For cCMV and pCMV infections, UL144 A was the most prevalent strain and was detected at 33.3% and 42.1%, respectively. UL144 B was the second most common genotype present in cCMV (29.6%) and pCMV (31.6%)-infected patients. In adult HCMV infection, the opposite was observed. UL144 B was the most prevalent (39.5%), followed closely by UL144 A (31.5%). UL144 C was the least common genotype detected in all patient groups analyzed: cCMV (25.9%), pCMV (21.1%), and adult HCMV (15.8%) infection. Recombinant strains were rare, but both A/B and A/C were detected; however, no UL144 B/C recombinants were identified.

Plasma viral load in cCMV infection. Assessment of the PVL was recommended following confirmation of cCMV infection, and follow-up blood specimens for 23 of the 29 congenitally infected infants were sent for analysis (Table 1). HCMV viremia was confirmed in 20/23 patients tested, and the viral loads ranged from 600 to >1,000,000 copies/ml (median, 2,770 copies/ml; mean, 57,963 copies/ml). The patients for whom a PVL was determined were analyzed according to their associated UL144 genotypes (n = 18/20). The median PVLs for infections caused by genotypes UL144 A (n = 7; PVL range, 600 to >1,000,000 copies/ml) and UL144 C (n = 4; PVL range, 708 to 975,175 copies/ml) were 19,800 and 8,635 copies/ml, respectively. UL144 A and C were significantly associated with high PVLs (P < 0.04; Mann-Whitney U test) compared with the PVLs achieved following UL144 B infection (n = 5; PVL range, 600 to 2,870 copies/ml) (Fig. 2). Too few recombinant strains were identified to be included in this analysis (UL144 A/C, n = 1; UL144 A/B, n = 1); however, the PVL for the one A/C strain was 25,600 copies/ml, which is within the high PVL range of this study (Table 1).

cCMV clinical manifestations and developmental outcome. Clinical manifestations of cCMV infection at birth, such as jaundice, microcephaly, hepatosplenomegaly, and intracranial abnormalities, were used to categorize HCMV infection as...
symptomatic. No specific HCMV genotype was significantly linked with symptomatic infection, and all UL144 genotypes were detected in symptomatic neonates. However, as there is no neonatal HCMV screening program in Ireland, the majority of newborns included in the study were symptomatic at the time of testing, and only 3 out of the 18 patients for whom the full clinical data were available were asymptomatic. It should be noted, though, that all 3 asymptomatic cases were caused by infection with a UL144 B HCMV strain (Table 1).

For the above reasons, our analysis focused on the prediction of cCMV outcome at 12 to 18 months postinfection as opposed to the manifestations of disease at initial diagnosis. Long-term clinical conditions, including SNHL (bilateral or unilateral), mental retardation, and developmental delay at 12 to 18 months postinfection, were used to determine if the infected neonate had continuing cCMV sequelae. Follow-up clinical data were available for the surviving cCMV neonates (16/18). Patient developmental delay was categorized based on communicative development (receptive and expressive), motor development, and global general development. Following assessment, a total of 11 children had some form of developmental delay, 6 of whom developed SNHL. Five patients had normal development at 12 to 18 months postinfection. Finally, 2 cCMV-related deaths were recorded (Table 3).

All UL144 genotypes were associated with cCMV-related manifestations and developmental delay; however, only patients infected with genotypes UL144 B and A/B (n = 5) could be described as having a normal and unaffected clinical outcome (Table 2). In addition, a significant association between cCMV clinical sequelae and UL144 A and C (n = 7) was observed (P < 0.04; chi-square analysis). Furthermore, the two cCMV-related deaths were found to be caused by a UL144 A (PVL, 23,100 copies/ml) and a UL144 C (no CMV DNA detected in the blood) strain. Unfortunately, the sample population size precluded analysis of individual cCMV outcomes with genotype.

cCMV-induced cytokine response. The levels of 13 cytokines and chemokines were determined in sera or plasma of the cCMV patients (n = 20), the pCMV infants (n = 9), and a proportion of adult HCMV-infected individuals (n = 27). IL-8 and IL-10 were significantly increased following cCMV infection (Table 1) (P < 0.02 and P < 0.0001, respectively; Mann-Whitney U analysis) and in all HCMV-positive individuals (Fig. 3). In addition, a significant positive correlation between IL-10 levels and PVLs was observed (P < 0.03; r = 0.527; n = 17; Spearman correlation). IL-10 levels, PVLs, and clinical data were available for 10 cCMV patients, and high PVLs and IL-10 levels were associated with cCMV long-term clinical manifestations (Fig. 4). No significant differences in the levels of all other cytokines and chemokines assayed were detected (data not shown).

**DISCUSSION**

The present study investigated the relationship between the UL144 genotype and the cCMV clinical outcome at 12 to 18 months postinfection and determined that the specific detection of UL144 genotypes A and C was indicative of serious cCMV infection and more likely to lead to long-term cCMV-

![Maximum-likelihood phylogenetic tree based on UL144 gene sequences from Irish congenital human cytomegalovirus infections identified during 2006 and 2007. UL144 gene sequences from cCMV and pCMV infections were genotyped using a phylogenetic tree (black). Adult and reference HCMV sequences are shown in gray. The GenBank accession numbers for the reference sequences (gray and boxed) are as follows: NW004 (AF179196), NW007 (AF179197), NW009 (AF179199), NW012 (AF179200), NW013 (AF179201), NW016 (AF179204), NW020 (AF179206), NW024 (AF179209), PT001 (AF084976), PT002 (AF084977), PT015 (AF084990), PT016 (AF084991), PT017 (AF084992), PT019 (AF084994), PT023 (AF084998), PT025 (AF085000), PT028 (AF085005), and PT030 (AF085004).](http://jcm.asm.org/)
associated clinical manifestations. The HCMV UL144 gene encodes a member of the TNF-α receptor superfamily and is found in HCMV clinical isolates only, suggesting it has a role in virulence and pathogenesis. The expressed UL144 protein binds the B- and T-lymphocyte attenuator (BTLA) protein (9) and also activates the chemokine CCL22 (20). Molecular epidemiology based on the UL144 gene has been carried out in Italian (1, 2), French (18, 19), American (3, 16), and Japanese (25) populations. In our geographically distinct Irish cCMV population, UL144 A was the most prevalent, followed closely by UL144 B. As with other studies, UL144 C was the least detected, and few recombinant strains were identified. All UL144 genotypes were detected in the cCMV patient group, and this is consistent with two previous studies that concluded all of the UL144 genotypes were transmissible from mother to child (1, 19). The prevalence of UL144 genotypes in the present study was more similar to those found in congenital infection in France (18) than to the investigations carried out in the United States, per-

<table>
<thead>
<tr>
<th>Population</th>
<th>No. (%) in UL144 genotype</th>
<th>Recombinant</th>
<th>Total</th>
<th>Untyped</th>
</tr>
</thead>
<tbody>
<tr>
<td>cCMV</td>
<td>9d (33.3)</td>
<td>8 (29.6)</td>
<td>7 (25.9)</td>
<td>2 (7.4)</td>
</tr>
<tr>
<td>pCMV</td>
<td>8e (42.1)</td>
<td>6 (31.6)</td>
<td>4 (21.1)</td>
<td>1 (5.3)</td>
</tr>
<tr>
<td>Adult</td>
<td>12 (31.5)</td>
<td>15 (39.5)</td>
<td>6 (15.8)</td>
<td>4 (10.5)</td>
</tr>
</tbody>
</table>

a The relative HCMV UL144 genotype prevalences in cCMV infection were compared with those detected in pCMV infection and adult HCMV-infected patients.

b HCMV genotypes were based on sequences of the UL144 gene. Genotypes were assigned as either UL144 A (A1/A2), UL144 B, UL144 C, or UL144 recombinant strain (A/B, A/C, or B/C).

c A genotype could not be assigned for 2 cCMV patients, as the UL144 sequence would not amplify. NA, not applicable.

d A1, 6; A2, 3.

e A1, 5; A2, 3.
haps indicating that the relative prevalence of genotypes differs between Europe and the United States (2, 16).

Our results determined that neonates infected with HCMV UL144 A and C strains had significantly higher PVLs than those infected with UL144 B. Furthermore, the majority of patients with UL144 A and C infections developed cCMV-related clinical manifestations. Several publications have reported a relationship between the HCMV PVL and disease severity, specifically, hearing loss and systemic HCMV disease (5, 6, 15); however, this has not been a consistent observation, suggesting that other factors may be involved in the outcome of infection. Our results further corroborate that HCMV PVL is a predictor of the outcome of cCMV infection. Previous epidemiological studies by Arav-Boger and colleagues determined that UL144 A and C may be specifically associated with the most serious outcomes of cCMV and identified the UL144 C strain in symptomatic cCMV cases only (1, 2). In direct contrast, Picone and colleagues previously concluded that the UL144 genotype is not of prognostic value in cCMV infection (19). Both of these studies, however, focused solely on the relationship between UL144 and symptomatic infection. This is the first study focusing on the association between the UL144 genotype and cCMV-associated clinical manifestations of disease at 12 to 18 months postinfection.

The cellular immune responses to HCMV infection have been extensively studied and contribute greatly to disease outcome (13). Recently, we demonstrated the ability of congenitally infected neonates to mount proinflammatory Th1 responses to HCMV infection (14). Analysis of HCMV-infected newborns showed increased levels of circulating IL-8 and IL-10. Elevated cytokine and chemokine levels following HCMV infection were independent of the genotype, although rising IL-10 levels were linked with the PVL. However, while the circulating cytokine immunological profile is relevant to the pathogenesis of HCMV, it offered no further insight into predicting the outcome of infection and the action of UL144.

This paper represents the first attempt to detail cCMV molecular epidemiology in Ireland, and further studies, including expanding the study population, are needed to establish baseline prevalence rates of the disease. Our results further implicate the UL144 genotype as a determinant of cCMV infection. Recent UL144 transfection assays determined that 2 different UL144 genotypes induced similar

### Table 3. HCMV UL144 genotypes and cCMV long-term clinical sequelae

<table>
<thead>
<tr>
<th>UL144 genotype</th>
<th>No. of cCMV patients with clinical manifestation</th>
<th>Communicative delay</th>
<th>Motor delay</th>
<th>Global delay</th>
<th>Death following cCMV</th>
<th>Total</th>
<th>No. of concerns</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td>1</td>
<td>1</td>
<td>2</td>
<td></td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>A/B</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>1</td>
<td>1</td>
<td>2</td>
<td></td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td>1</td>
<td>2</td>
<td>1</td>
<td></td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>A/C</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td>3</td>
<td>5</td>
</tr>
<tr>
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<td></td>
<td>1</td>
<td>1</td>
<td>2</td>
<td></td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

Total 3 2 6 2 13 5

*The UL144 genotypes of cCMV-infected neonates were compared with cCMV long-term clinical manifestations and developmental concerns at 12 to 18 months postinfection. A significant association between cCMV clinical sequelae and UL144 genotypes A and C (n = 7) was observed (P < 0.04; chi-square analysis).

b cCMV infection that resulted in SNHL (n = 6).

c Denotes statistical significance with a P value of <0.05.
responses in cells (20). The correlation of UL144 genotypes A and C with cCMV disease severity may reflect the ability of the protein to affect its intracellular signaling pathway differently. It is clear, however, that not all the cCMV infections investigated that were caused by UL144 A and C strains resulted in a severe outcome, implying that other viral and host factors are also involved in disease progression. However, as the sample population was small, the significance of these results must be treated with caution, and further studies using larger patient cohorts are needed to corroborate our findings. The inclusion of HCMV UL144 genotyping, along with the recommended PVL monitoring following cCMV diagnosis, may aid prediction of the clinical outcome and help identify children who may benefit from antiviral therapy.

REFERENCES


