Genetics and Molecular Research

Genetic mechanism associated with congenital cytomegalovirus infection and analysis of effects of the infection on pregnancy outcome

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ABSTRACT. We aimed to compare the diagnostic value of various detection methods for cytomegalovirus (CMV) infection, to investigate the genetic mechanism associated with CMV infection in pregnant women, and to analyze the risk of sequelae development in fetuses with CMV infection. A total of 300 participants who had the same immunosuppressive regimen and received preemptive therapy for CMV infection were prospectively enrolled in this study; they included 289 vaccine trial participants. The gB-absorbed CMV IgG assay was performed for each vaccine trial participant. The healthy women were divided into 2 groups, and amniotic fluids were collected from them at 15-18 weeks of gestation to test for CMV

seropositivity before conception by using IgM specific antibodies, CMV-DNA, and IgG analysis. In 104 cases, cord blood sera and urine specimens were also collected from the infants and examined. The sensitivity and specificity of immediate-early messenger RNA and pp67 (late) messenger RNA detection by the nucleic acid sequence-based amplification technique was comparable to those of virus isolation and PCR. Furthermore, an association between single nucleotide polymorphisms in the TLR-2 gene and congenital CMV infection was observed and confirmed. Moreover, CMV infection during early pregnancy has been shown to have a much more severe effect on the pregnancy outcome compared to infection during later stages of pregnancy.

Key words: Cytomegalovirus; Pregnancy; Gene diagnosis

INTRODUCTION

Cytomegalovirus (CMV) is the most common cause of intrauterine infection, occurring in 0.3-2.1% of live-born infants (Stagno & Whitley, 1985). Although only 10-16% of the infected fetuses are diagnosed with symptomatic congenital CMV infection (CCMVI) at birth, the disease causes severe symptoms including intrauterine growth restriction (IUGR), low birth weight, central nervous system disorder, multiple organ involvement with petechiae, hepatomegaly, splenomegaly, jaundice, pneumonia and encephalitis, leading to a high perinatal mortality rate and major neurological sequelae in approximately 91% of the surviving infants (Reynolds et al., 1974; Ross et al., 2005; Bate et al., 2010; Gorzer et al., 2010; Ikuta et al., 2012). In addition, 11-16% of infants with asymptomatic CCMVI will develop long-term sequelae, namely progressive sensorineural hearing difficulty and mental retardation.

The potential risk factors influencing the clinical outcomes of CCMVI include both viral and host factors such as viral loads in the blood, CMV-IgG avidity index in pregnant women, as well as cytotoxic T lymphocyte responses and innate immunity in fetuses (Dahle et al., 1974; Gupta et al., 1996; Bate et al., 2010; Gorzer et al., 2010; Yamamoto et al., 2010; Ikuta et al., 2012). Ideally, the levels of systemic viral load in patients with symptomatic CCMVI should be monitored quantitatively to assess the clinical status, since qualitative measurements such as viral culture may only reflect viral infection rather than active disease (Ahlfors et al., 1983; Boppana et al., 2001). Monitoring of viral load by nucleic acid- or antigenemia-based assays has been shown to be particularly valuable in bone marrow and stem cell transplant patients. These assessments may not only predict the outcome of CMV infection, but also provide a solid basis for decisions on prophylactic or preemptive antiviral therapies.

This study was aimed at investigating the latest prevalence and risk of multiple CMV infection in pregnant women, as well as the types of maternal CMV infection associated with congenital CMV infection, to explore the underlying molecular mechanism of action and to build an analytic model to predict the pregnancy outcome in CMV infection. Specifically, the factors affecting the pregnancy outcome were analyzed by a variety of molecular techniques including fluorescence real-time quantitative PCR, sequencing, microarray, and mass spectrometry. The candidate factors include CMV load, CMV gB genotype, the immune-related genetic susceptibility in pregnant women, and infection by other pathogens such as *Toxoplasma gondii* (TOX), rubella

virus (RV), herpes simplex virus (HSV), parvovirus, Epstein-Barr virus (EBV), and hepatitis B virus. Future studies will need to be performed on a much larger number of cases to build a mathematical model for the prediction of the pregnancy outcome in CCMVI. Our results provide a scientific basis for the prevention of serious clinical outcomes in CCMVI.

MATERIAL AND METHODS

Participants and specimens

A total of 300 healthy pregnant women were selected, that included 289 vaccine trial participants. The participants were divided into 2 groups-the first trimester group and later infection group in this study. The gB-absorbed CMV IgG assay was performed for each vaccine trial participant as described previously (Gouarin et al., 2002; Watzinger et al., 2004; Cheeran et al., 2009). Amniotic fluid (AF) was collected from these women at 15-18 weeks of gestation to test for CMV-seropositive before conception by using IgM specific antibodies, CMV-DNA, and IgG analysis. In 104 cases, cord blood sera and urine specimens were collected from the infants and examined by different HCMV assays.

Detection of CMV DNA

Sample preparation

Amniotic fluid samples (200-550 µL) were centrifuged at 12,000 *g* for 15 min at room temperature. A total of 10 mL urine sample was centrifuged at 850 *g* for 20 min, and the virus was pelleted by centrifugation at 36,000 rpm for 1 h. The pellet was suspended in 200 µL lysis buffer containing 55 mM KCl, 10 mM Tris-HC1 (pH 8.0), 2.5 mM $MgC1_2$, 0.6% Nonidet P40, 0.6% Tween 20, and 100 µg/mL proteinase K. The mixture was incubated at 55°C for 2 h to lyse the cells and then at 94°C for 5 min to inactivate the proteinase K. The lysed samples were stored at -25°C until use. Serum and cord blood serum samples (150 µL) were mixed with 500 µL lysis buffer and processed as described above.

Polymerase chain reaction (PCR)

A 406-bp DNA segment was amplified from the genome of CMV by PCR. The amplification reaction mixture consisted of 20 mM (NH4) 2804, 76 mM Tris-HC1 (pH 8.9), 0.2% (w/v) Tween, 1.0 mM $MgCl_2$, 200 µM deoxynucleoside triphosphates, 50 pmol each primer, 10 µL DNA sample, and 2.0 units Taq polymerase (Advanced Biotechnologies ltd; Surrey, UK) in a final reaction volume of 100 µL. The samples were overlaid with 100 µL mineral oil (Amresco ltd; Ohio, USA) and heated at 92°C for 10 min for template denaturation, followed by 40 cycles, each consisting of 93°C for 1 min, 56°C for 2 min, and 73°C for 3 min, and a final extension step at 73°C for 7 min. The DNA sequence of the forward and reverse primer was 5'Biotin-CCA AGC GGC CTC TGA TAA CCA AGC C-3' (nucleotide position 722-725) and 5'-CAG CAC CAT CCT CCT CTT CCT CTG G-3' (nucleotide position 1161-1151), respectively. Biotinylated PCR products were analyzed by gel electrophoresis on a 1.3% agarose gel. The resultant RNA:DNA hybrids were captured through biotin onto the surface of streptavidin-coated microwells. Immobilized hybrids were reacted with an antiRNA-DNA

specific antibody conjugated to alkaline phosphatase and detected using a colorimetric substrate. Absorbance values of each sample was determined under 403 nm and compared with that of positive and negative controls supplied by DIGENE diagnostics Inc. (Silver Spring, MD, USA). Positive cutoff values were obtained from the mean Abs value of the amplified negative PCR control.

gB-absorbed CMV IgG and IgM assay

IgG capture ELISA was used to detect gB-absorbed CMV IgG and IgM. Recombinant Staphylococcal protein A (R&D systems Inc, Minneapolis, USA) was added to the solid phase for capture saliva IgG (R&D systems Inc, Minneapolis, USA), followed by binding of biotinylated tachyzoite saline antigen (R&D systems Inc, Minneapolis, USA), which was quantitated by using avidin peroxidase (R&D systems Inc, Minneapolis, USA). The reaction was revealed using two chromogenic peroxidase substrates (R&D systems Inc, Minneapolis, USA), such as OPD and TMB, with appropriate controls in each plate.

SNP detection

Blood samples (5 µL) were obtained from each participant for genomic DNA extraction using the E.Z.N.A. Blood Midi Kit (Omega Bio-Tek, Norcorss, GA, USA). TLR2, TLR4, TLR9 SNPs were selected and genotyped using the Sequenom iPLEX platform (Sequenom Inc., San Diego, CA, USA), and laboratory persons who conducted the genotyping assays were blinded to patients' information. Quality controls were implemented in each assay for genotyping, and SNPs with call rate > 98% were included for further analysis. Differences in the prevalence of genotyped polymorphisms between the CMV infection group and the healthy control group were estimated by odds ratios (ORs), 95% confidence intervals (95% CIs), and P values. ORs and 95% CIs were calculated for each genotype in comparison to the reference genotype (homozygous genotype for the prevalent allele). P values were derived from the Chi-square test. P values < 0.05 were considered to indicate statistical significance. To adjust the P values for multiple comparisons, Bonferroni multiple correction of P values was applied.

NASBA assay for HCMV immediate-early messenger RNA (IEmRNA) and pp67 (late) messenger RNA

HCMV IEmRNA and pp67 mRNA were determined by the nucleic acid sequence-based amplification (NASBA) technique, following the manufacturer (Organon, Teknika, USA) instructions. In brief, nucleic acids from 100 µL whole blood were isolated by the method described by Lazzarotto T et al. (2000). System control (SC) RNA was added to samples before nucleic acid isolation as a positive control for RNA isolation, amplification, and detection. The SC RNA could be distinguished from wild-type (wt) RNA by the insertion of a 134-nt fragment (Enders et al., 2001; Gouarin et al., 2001). SC and wt IEmRNAs were amplified with a primer that contained a T7 promoter and a reverse primer. Amplification products were detected by electrochemiluminescence with a common capture probe coupled to magnetic beads and by 2 specific (wt- and SC-specific) ruthenium-labeled oligonucleotide detection probes (Enders et al., 2001; Gouarin et al., 2001). NASBA sensitivity for IEmRNA was 70 copies/10 µL whole blood.

Virologic analysis of antigenemia and DNAemia

Virologic analysis was performed by prospective quantitation of antigenemia and DNAemia. Antigenemia was quantitated by counting the number of peripheral blood leukocytes positive for pp65 (Lazzarotto et al., 2000). Viral DNA was quantitated in 10 µL whole blood by PCR in the presence of 100 copies of an internal control and in parallel with a series of external standards (Enders et al., 2001). Single-step PCR consistently amplified samples containing >10 genome equivalents (GEs), whereas samples containing 1-10 GEs were detected by nested PCR and were assigned an arbitrary value of 5 GEs.

Antigenemia Assay of CMV

The CMV antigenemia assays were performed using ethylenediaminetetraacetic acid-treated whole blood samples, according to previously reported methods (Ho et al., 1998). The blood samples were fractionated by dextran sedimentation and erythrocyte lysis. A cytospin slide was prepared after centrifugation of the blood samples, fixed with formaldehyde, sequentially immunostained with a monoclonal antibody (C10/C11) against the pp65 antigen (Biotest, Dreieich, Germany), and reacted with fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody (Biotest, Dreieich, Germany). The results were expressed as the number of CMV pp65-positive cells per slide. Positive CMV antigenemia was defined as at least 1 pp65-positive cell per 2 x 10^5 PMNs.

Statistical Analyses

The prevalence of congenital CMV among children with permanent childhood hearing impairment (PCHI) was calculated. Statistical analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). The X^2-test was used to determine the differences in baseline characteristics (Stagno et al., 1985; Mitchell et al., 2002). Linear regression modeling was used to analyze the developmental outcome based on the CDI-NL. Adjustment was made for age at examination and severity of hearing loss. P values < 0.05 were considered to indicate statistical significance.

RESULTS

Clinical characteristics of infant subjects

A total of 39 infants were enrolled in this study including 20 infants in the CMV infection group and 19 infants in the control group. The dried umbilical cord specimen from each infant was analyzed for CMV-DNA detection. In the CMV infection group, 11 infants were asymptomatic and 9 had one or more of the following manifestations during initial examination: abnormal ABR in 8 cases, hepatosplenomegaly in 4, hepatitis in 3, jaundice in 1, thrombocytopenia with petechiae in 2, chorioretinitis in 2, and intracranial calcifications in 5. The clinical characteristics of these infant subjects were summarized in Table 1.

Prenatal diagnosis of CMV infection is a very delicate task and should never be based on the result of a single test. We have recently evaluated commercially available molecular assays

other than PCR analysis for the detection of CMV in AF samples (Revello et al., 1995). In this study, 69 AF samples were analyzed by virus isolation and PCR, and subsequently tested for CMV IE mRNA and pp67 mRNA by the NASBA technique. As shown in Table 2, similar sensitivity was observed with all four methods (Revello et al., 1995; Lazzarotto et al., 2000; Enders et al., 2001; Gouarin et al., 2001), whereas the specificity of all assays was 100% with the exception of one false-positive result detected by the NASBA assay of IE mRNA, yielding a specificity of 96.8%. These results suggested that NASBA assays for both IE mRNA and pp67 mRNA can be used as first choice detection methods or methods to confirm results obtained by PCR in those laboratories where virus isolation is not feasible.

Table 1. Clinical characteristics of enrolled infants.

	Control (N = 19)	CMVI		
		Total (N = 20)	Normal ABR (N = 11)	Abnormal ABR (N = 9)
Gestational age (weeks)	37 (36 - 41)	37 (31 - 41)	37 (35 - 41)	38 (31 - 39)
Birth weight (g)	2791 (2218 - 3688)	2916 (1378 - 3840)	3170 (2362 - 3840)	2319 (1378 - 3160)
Male/female	8/11	8/12	4/7	4/5
Postconceptional age at the time of brain CT (weeks)	37 (36 - 42)	39 (34 - 52)	41 (38 - 45)	39 (34 - 52)
Postconceptional age at the time of ABR evaluation (weeks)	38 (36 - 42)	41 (34 - 48)	41 (39 - 45)	39 (34 - 48)

ABR = auditory brainstem response.

Table 2. Diagnostic value (with respect to virus detection at birth) of different assays for prenatal diagnosis of congenital infection in 104 fetuses of 102 mothers with primary HCMV infection during pregnancy.

Sample	HCMV assay	Test result	Infected	Uninfected	Sensitivity	Specificity	PPV	NPV
Fetal blood	Antigenemia	Negative	18	45	61.2%	100%	100%	72.9%
		Positive	23	0				
	Viremia	Negative	25	39	35.9%	100%	100%	65.1%
		Positive	15	0				
	DNAemia	Negative	7	36	82.7%	100%	100%	84.3%
		Positive	33	0				
	IEmRNA	Negative	4	20	81.9%	100%	100%	86.1%
		Positive	15	0				
	pp67 mRNA	Negative	3	13	64.9%	100%	100%	72.6%
		Positive	9	0				
	IgM antibody	Negative	17	41	51.7%	100%	100%	68.7%
		Positive	24	0				
Amniotic fluid	Virus isolation	Negative	11	51	81.8%	100%	100%	82.8%
		Positive	43	0				
	DNA	Negative	4	45	91.5%	100%	100%	91.2%
		Positive	47	0				
	IEmRNA	Negative	5	44	91.2%	96.8%	95.3%	92.7%
		Positive	28	2				
	pp67 mRNA	Negative	2	43	92.1%	100%	100%	91.8%
		Positive	29	0				

PPV = positive predictive value; NPV = negative predictive value; IEmRNA = immediate-early messenger RNA; pp67 mRNA = pp67 (late) messenger RNA. (Revello and Gerna, unpublished data). N = Negative, P = Positive.

Statistical analyses of genotype

As shown in Table 3, genotype and allelic frequencies of all SNPs of *TLR2, TLR4, TLR9* genes were in Hardy-Weinberg equilibrium among each group (data not shown) [18-20]. In this study, differences in the prevalence of genotyped polymorphisms between the CMV infection group

and the healthy control group were estimated by odds ratios (ORs), 95% confidence intervals (95% CIs), and P values. ORs and 95% CIs were calculated for each genotype in comparison to the reference genotype (homozygous genotype for the prevalent allele). P values were derived using the Chi-square test. P values < 0.05 were considered to indicate statistical significance. To adjust the P values for multiple comparisons, Bonferroni multiple correction of P values was applied.

Table 3. Association between single nucleotide polymorphisms in the TLR-2, 4, and 9 genes with CCMVI.

Gene	Genetic model	Genotype	Healthy control group (A) or CCMVI group (B)			
			A (%)	B (%)	OR (95% CI)	P value
TLR-2	Codominant	AA	19.7	34.5	1	NS
		AG	62.8	47.1	0.43	
		GG	17.4	18.4	0.60	
	Dominant	AA	19.7	34.5	1	0.030
		AG+GG	80.2	65.5	0.47	
	Recessive	AA+AG	82.6	81.6	1	NS
		GG	17.4	18.4	1.07	
TLR-4	Codominant	GG	57.1	51.7	1	NS
		GC	35.7	40.2	1.24	
		CC	7.1	8	1.24	
	Dominant	GG	57.1	51.7	1	NS
		GC+CC	42.9	48.3	1.24	
	Recessive	GG + GC	92.9	92	1	NS
		CC	7.1	8	1.14	
TLR-9	Codominant	AA	29.1	25.3	1	NS
		AG	43	54	1.44	
	Dominant	AA	29.1	25.3	1	NS
		AA+AG	70.9	74.7	1.21	

CCMVI = congenital cytomegalovirus infection; OR = odds ratio; CI = confidence interval; NS = not significant. P values were derived using the Chi-square test. P values < 0.05 were considered to indicate statistical significance.

Detection of CMV infection by CMV culture

The gB-absorbed CMV IgG assay was performed on each of the 289 vaccine trial participants. Subjects with positive results in the gB-absorbed CMV IgG assay were compared with those with negative results (Table 4). Serologic evidence of CMV infection was found in 26/289 (8.99%) study participants who had gB-absorbed CMV IgG levels >5.0 AU/mL. The gB-absorbed CMV IgG levels of their first positive serum ranged from 15.7 to 251.0 AU/mL with a mean of 77.0 AU/mL and a median of 43.6 AU/mL. Out of the 1142 tested serum samples, 1091 were negative (gB-absorbed CMV IgG level <5.0 AU/mL, range 0.0-3.7 AU/mL). All subjects with positive gB-absorbed CMV IgG results were also found to be positive upon repeat testing at their next study visit. The gB-absorbed CMV IgG levels of the first follow-up serum collected post-infection from the 23 positive subjects ranged from 14.0 to 255.0 AU/mL with a mean of 95.5 AU/mL and a median of 75.6 AU/mL. The median interval from the last negative to first positive gB-absorbed CMV IgG for these subjects was 14 weeks.

Examples of serial gB-absorbed CMV IgG results of 2 vaccine trial participants before and after CMV infection during the trial are shown in Figure 1 (Gaytant et al., 2002; Gouarin et al., 2002; Compton et al., 2003; Tanaka et al., 2006; Revello et al., 2008). Subjects A and B were both found to be infected with CMV at 9 months after the initial immunization. Their gB-absorbed CMV IgG levels prior to infection remained as low as that during the prevaccine visit, ranging from 0.0 to 1.7 AU/mL. In contrast, their serial gB-absorbed CMV IgG results post-infection increased dramatically and ranged from 43.4 to 181.0 AU/mL.

Table 4. gB-absorbed CMV IgG assay of vaccine trial participants.

	CMV infection		Range of gB-absorbed CMV IgG levels (AU/mL)	Mean of gB-absorbed CMV IgG levels (AU/mL)	Median of gB-absorbed CMV IgG levels (AU/mL)
	Positive (n%)	Negative (n%)			
Participants (N = 289)	26 (8.99%)	263 (91.01%)	15.7 - 251.0*	77.0*	43.6*
Serum samples (N = 1142)	51 (4.46%)	1091 (95.54%)	0.0 - 3.7#	<5.0#	-
First follow-up for positive subjects	23 (100%)	0	14.0 - 255.0	95.5	75.6

*The gB-absorbed CMV IgG levels were detected in positive serum samples; #the gB-absorbed CMV IgG levels were detected in negative serum samples.

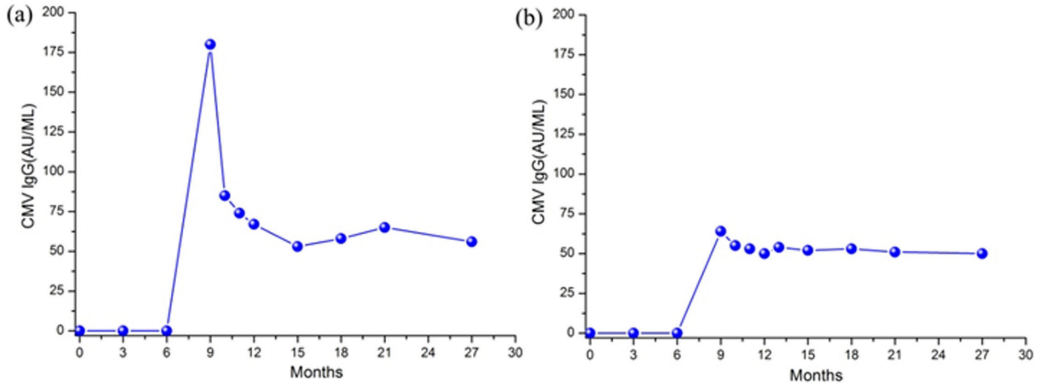

Figure 1. Serial gB-absorbed CMV IgG results for two vaccine trial participants before and after CMV infection

Pregnancy outcome after CMV infection

The overall rate of CNS sequelae in the first trimester group (12/36, 33%) was only slightly different from that in later infection group (6/40, 15%; P = 0.068, RR= 2.2). Four (12%) of the first trimester cases had more than two CNS sequelae, compared with none of the later infection group (P = 0.038) (Lukacsi et al., 2001; Kishore et al., 2011). Frequencies of the major CNS sequelae are shown in Figure 2.

All but five patients (one from the first trimester group and four from the later infection group) had follow-up evaluations for the determination of pregnancy outcome. The major abnormality was sensorineural hearing loss. The incidence of infants with sensorineural hearing loss in the first trimester group (9/36, 27%) was significantly higher than that in the later infection group (1/40, 2.5%; P = 0.01, RR = 9.6). Further, neuropsychologic examinations were completed on 24 infants in the first trimester group and 26 in the later infection group. The incidence of mentally retarded infants (IQ < 70) in the first trimester and later infection group was 17% and 7.7%, respectively, revealing no significant difference between the two groups. Moreover, seizure disorder, cerebral palsy, and chorioretinitis were confirmed in 3, 2, and 1 infants, respectively, from the first trimester group, whereas 1 case of seizures and 1 of chorioretinitis was observed in the later group.

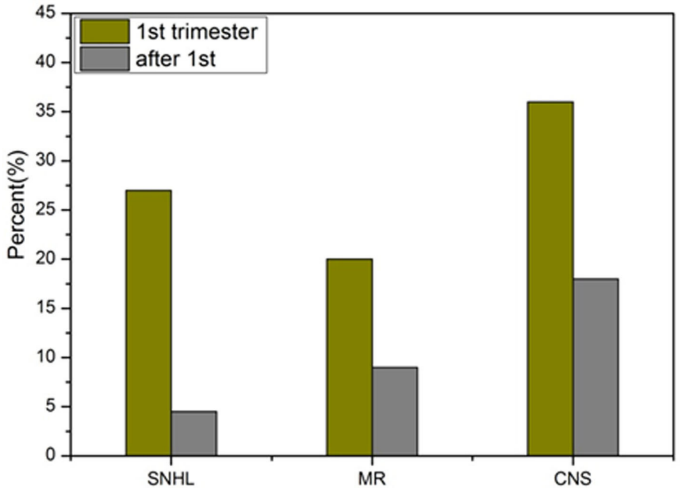

Figure 2. Percentage of patients with central nervous system sequelae in CCMVI according to gestational age at the time of maternal infection. SNHL = sensorineural hearing loss; MR = mental retardation (IQ < 70); CNS = central nervous system, indicating patients with any CNS sequelae. *P = 0.01; RR = 9.6. **P = 0.07; RR = 2.2.

DISCUSSION

While CMV can be transmitted from the mother to the fetus, congenital CMV infection can even occur following recurrent maternal infection. Therefore, the birth of one congenitally infected infant does not preclude the possibility of *in-utero* infection of a subsequent baby.

Moreover, a recent study has shown that 8 out of 47 infants with symptomatic CCMVI were born to mothers with a confirmed non-primary or recurrent infection (Lukacsi et al., 2001), suggesting that symptomatic CCMVI after recurrent maternal infection occurs more frequently than previously thought. However, the risk of major fetal injury associated with recurrent CMV infection is believed to be considerably lower than that of primary infection, probably due to the protective effect of pre-existing CMV antibodies (Gouarin et al., 2002; Tanaka et al., 2006; Revello et al., 2008). Live babies delivered in all the reported cases exhibited typical clinical features of congenital CMV infection, such as jaundice, hepatosplenomegaly, and thrombocytopenia. To the best of our knowledge, this is the first reported case of severe congenital CMV infection causing intrauterine fetal death after recurrent maternal infection, in which abnormal findings such as fetal hydrops were clearly identified by antenatal ultrasonography (Kishore et al., 2011).

Many infants born to mothers with primary or recurrent infection are asymptomatic and can develop normally despite viremia *in utero* and postnatally, and shedding of virus in urine and saliva for years after birth. Infants acquiring CMV postnatally exhibit similar patterns of viremia and viral excretion without symptoms. Even when CMV is acquired through transfusion in very low birth weight infants (weight lower than 1250 g) of seronegative mothers, the infants may become ill but do not develop the symptoms acquired *in utero* after primary maternal infection (Reynolds et al., 1974; Stagno & Whitley, 1985; Ross et al., 2005).

It is important to note the blind spot of the current clinical trial. It may be speculated that if CMV infections occur in recipients of CMV gB vaccine, they might be modified by the presence of vaccine-induced immunity, and therefore might not be detectable by the same approaches that

are effective in nonimmunized subjects with infection (Revello et al., 1995). To address this, in the future, we will confirm CMV infection in all participants in this trial by standard CMV IgG and immunoblot assays of serum, and PCR analyses of whole blood, urine, and saliva samples.

CMV infection has been known to significantly contribute to sensorineural hearing loss (SNHL) in many infants. Although most infants with CCMVI do not develop hearing loss, it is difficult to predict which infants with CCMVI will develop hearing loss and, among those who do develop loss, whether or not the loss will continue to deteriorate. Therefore, it is crucial to clarify the genetic mechanism associated with CMV infection and to build an analytical model for the prediction of pregnancy outcome in women with CMV infection (Compton et al., 2003). In this study, an association between SNPs in the TLR-2 gene and CMV infection was identified. Furthermore, it was shown that the incidence of sensorineural hearing loss in infants in the first trimester group was significantly higher than that in the later infection group, suggesting that CMV infection during early pregnancy has a much more severe effect on the pregnancy outcome.

Conflicts of interest

The authors declare no conflict of interest.

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