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Single cytomegalovirus strain associated with fetal loss and then congenital infection of a subsequent child born to the same mother

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Abstract

Background. Intrauterine transmission of cytomegalovirus (CMV) can occur even in CMV-seropositive mothers. Previous studies demonstrated re-infection with a newly acquired CMV strain during pregnancy had a major in such transmission. Although reactivation of latent CMV infections is another plausible cause, no direct evidence has been documented.

Objectives. We sought to identify the route(s) of CMV infection occurring in consecutive pregnancies and resulting in symptomatic congenital infections, and to analyze maternal risk factors for these infections.

Study design. A newborn identified with congenital CMV infection in our newborn screening program developed hearing loss and subsequent nystagmus. The mother had had an elective abortion due to a severe fetal CMV infection 32 months prior to delivery. We analyzed maternal serological changes and compared CMV genomic sequences in specimens obtained from her first healthy child, the aborted fetus, and the case reported here. We also analyzed immunological and genetic risk factors for CMV disease.

Results. Our major findings were as follows: 1) the aborted fetus and the present case were infected with the same strain as that infecting the first child. 2) The congenital infection that resulted in the abortion was due to a primary infection. 3) CMV DNA was undetectable in the mother's blood from 3 months after the abortion. These results strongly suggested that maternal viral reactivation caused the congenital infection in the present case. However, the mother had no risk factors for CMV diseases.

Conclusions. Viral reactivation in an apparently immunocompetent and normal mother can cause symptomatic congenital CMV infection.

Key words: congenital cytomegalovirus infection, neonatal screening, transmission, siblings, viral reactivation.

Background

Congenital cytomegalovirus (CMV) infection occurs in 0.2-2% of births and causes developmental abnormalities in a small percentage of newborns.[1,2] In addition, infants asymptomatic at birth may develop late-onset sequelae, such as sensorineural hearing loss (SNHL) and developmental delay.[1,2] Our recent retrospective analyses using dried umbilical cord specimens demonstrated that a proportion of cases of SNHL[3] and developmental delays[4] can be ascribed to congenital CMV infection.

Transplacental transmission of CMV may occur even in seropositive women after non-primary infection. The non-primary infection consist of two different routes, those are re-infection with a newly acquired CMV strain and reactivation of a latent CMV infection. By use of a serological assay that can distinguish between the two gH genotypes, Boppana et al.[5] clearly demonstrated that two thirds of congenital infections in seropositive women were caused by re-infection. However, reactivation as a route for congenital infection has not been previously demonstrated by direct evidence.

Besides primary infection, several maternal risk factors have been reported for congenital CMV infection; for example, reduced T cell-mediated immune responses against CMV[6,7] and a short interval between consecutive deliveries.[8] In addition, specific amino acid sequence alterations in the Toll-like receptor 2 (TLR2) gene have been associated with a higher incidence of CMV disease in immunocompromised individuals.[9]

In this report, we demonstrated that virus re-activation can cause symptomatic congenital CMV infections and there were no obvious maternal risk factors for the case reported.

Objectives

An infant identified in newborn CMV screening developed hearing loss and subsequent nystagmus. Surprisingly, the mother had a history of elective abortion due to a severe congenital CMV infection. The objectives of the present study are to identify the route(s) of infections that caused the symptomatic infections in the consecutive pregnancies, and to analyze maternal risk factors for congenital CMV infections.

Study Designs

Identification of the case. The collection and the use of materials for this study was approved by the Ethical Committee in our institute. And informed consent was obtained from each infant's parents. Newborn screening was carried out using urine specimens from newborns were collected onto filter papers inserted in diapers, and the presence of CMV DNA on the filter papers was directly examined by real-time PCR.[10,11]

Serological assays. The serological test for CMV was done with a commercial CMV ELISA kit (Enzygnost anti-CMV, Dade Behring), and the IgG antibody avidity was measured by incorporating a urea denaturation procedure as described by Blackburn et al.[12] The antibody avidity index (AI) was calculated as: AI = [mean absorbance at 450 nm (OD_{450}) of the urea-filled wells/mean OD_{450} of the control well] × 100 (%). An AI $<$ 35 % was considered to be suggestive of recent infection, and an AI $\geqq$ 35% was considered to be suggestive of past infection.

CMV-specific T cell responses. Heparinized blood specimens were obtained from 25 healthy volunteers and from the mother of the present case. The median age was 33.1 (range 22-46) and the mother was 32 years old. Of the 25 healthy controls, 16 (64 %) were anti-CMV IgG positive and 9 (36 %) were seronegative.

a) CMV-specific $CD4^+$ and $CD8^+$ T cell frequencies

Frequencies of CMV-specific T cells were determined by flow cytometry (FCA) using commercial assays (BD FastImmune CD4 and CD8 Intracellular Cytokine Detection Kits, Becton-Dickinson), according to the manufacturer's instructions. Frequencies of $CD4^+$, $CD8^+$, $CD69^+$, and IFN-γ^+ T cells were analyzed in a FACS Calibur instrument (Becton Dickinson), and determined using Cell Quest software (version 3.2). Ten thousand cells were counted for each sample in duplicate.

b) CMV-specific lymphoproliferative response

The lymphoproliferative response (LPR) was evaluated by [methyl-^{3}H]-thymidine incorporation as previously described.[6] The cell division index (CDI) was calculated as: CDI = [net counts per min

of the wells incubated with CMV antigen]/[net counts per min of the wells incubated without CMV antigen]. A CDI > 1.5 was considered to be a positive response, which means a greater proliferation than that of any of the 9 seronegative individuals tested (ranges 0.32 to 1.35).

Molecular assays. DNA samples were collected from each case of congenital infection and maternal peripheral blood using QIAamp DNA Mini Kit (Qiagen). Real-time PCR for CMV UL83 was performed as described previously.[3] For genotyping, DNA fragments of the glycoprotein O (gO, UL74), gN (UL73), and UL144 genes were amplified and sequenced as previously described.[13,14]

Maternal TLR2 gene exon 1 was amplified by PCR in order to analyze, not only the polymorphism at amino acid position 753, but also the full-length sequence. Primer sequences and thermal cycling conditions are listed in supplementary data 1.

Results

Clinical findings of two consecutive congenital CMV infections. The obstetrical history of the mother is gravida III, para II (Figure 1A); her first pregnancy and delivery were without complications and the child, "Baby A", has been healthy. The second pregnancy was terminated at 21 weeks after *in utero* diagnosis of severe CMV infection (abortion of "Baby B"). Twenty-two months after the abortion, the mother became pregnant again. Although there were no apparent complications during the third pregnancy, the child, "Baby C", was identified with congenital CMV infection in our newborn CMV screening program. Further details of the second pregnancy/Baby B and the third pregnancy/Baby C are as follows.

Second pregnancy/Baby B: The mother had no clinical symptoms during pregnancy. However, intrauterine growth retardation (IUGR), fetal ascites, and hepatosplenomegaly were found in ultrasound sonography at 19 weeks. Maternal serum was positive for anti-CMV IgM and IgG. Needle sampling of amniotic fluid and fetal ascites was, therefore, done at 21 weeks 2 days. CMV DNA was detected both of them, indicating fetal CMV infection. Exacerbation of IUGR and fetal ascites were observed, and an elective abortion was performed based on the couple's decision at 21 weeks 4 days.

Third pregnancy/Baby C: The birth weight of 2880g was appropriate for the gestational age of 38 weeks. Our screening program detected CMV in a urine specimen collected at 2 days after birth. CMV loads in urine and plasma specimens obtained at day 14 for confirmation were 1.4×10^6 copies/ml and 8.9×10^3 copies/ml, respectively. Serum was positive for anti-CMV IgM, and CMV was isolated in fibroblast cells from the urine specimen. No clinical or laboratory abnormalities commonly associated with congenital CMV infection, including microcephaly, hepatosplenomegaly, thrombocytopenia, and brain CT abnormalities, were present. However, the automated auditory brainstem response (ABR) evaluation showed hearing loss, profound in the left ear and moderate in the right ear on the basis of the reported classification.[15] The infant also developed mild infantile

nystagmus. At 18 months, he has little difficulty in daily conversation and no abnormalities were detected by Enjyoji test[16], which is the standard Japanese method for evaluating development in young children. His total developmental quotient (DQ) score was > 100, which is in the normal range.

Congenital infection due to reactivation. We analyzed the genetic background of the CMV strains that caused the two congenital infections. The nucleotide sequences of the polymorphic CMV genes, gO, gN, and UL144 were completely identical among the two strains, indicating that the same CMV strain was associated with both congenital infections. Maternal CMV IgM and IgG AI at the time of the abortion were 3.2 mg/dl and 37.9%, respectively, whereas 3 months later, CMV IgM was 5.1 mg/dl, and the AI increased to 67.5%. A dramatic increase in AI score over a 3-month period is consistent with the occurrence of a primary CMV infection during the second pregnancy. CMV DNA was undetectable (<200 copies/μl) in maternal serum specimens collected 3 months after the abortion of Baby B, 3 months after the conception of Baby C, and 19 months after the delivery of Baby C. This suggests that there was no continuous CMV viremia between the second and third pregnancies (Figure 1B). All results strongly suggest that a maternal primary infection caused the abortion of Baby B, and that maternal reactivation of the latent CMV infection resulted in a congenital infection in Baby C.

Risk factors for congenital CMV infection in the mother.

CMV-specific T cell responses of the mother: CMV-specific T cell responses of the mother were compared with those of healthy individuals. Frequencies of CMV-specific $CD4^+$ and $CD8^+$ T cells in the mother at 19 months after delivery of the Baby C were 5.31 % and 3.10 %, respectively. These values are similar to the frequencies of CMV-specific, IFN-γ producing $CD4^+$ and $CD8^+$ T cells in CMV-seropositive healthy individuals ($CD4^+$ median of 4.64 %, range 1.64–11.0 %; $CD8^+$

median of 3.39 %, range 1.05–10.57 %). As measured by the cell division index, the median lymphoproliferative response among CMV-seropositive individuals was 1.75 (range = 0.95 to 4.47). Ten of sixteen (62.5%) CMV-positive healthy individuals had detectable CMV-specific lymphoproliferative responses. The mother had a positive lymphoproliferative response, with a cell division index of 2.30 (Figure 2-B). These results indicated that the mother had a normal capacity to generate CMV-specific T cell responses (Figure 2-A).

Maternal TLR2. The TLR2 gene of the mother had no mutations and no R753Q polymorphism, indicating normal innate immunity against CMV.

Discussion

Intrauterine CMV transmission can result in severe clinical consequences, thus it is important to identify the route of the mother-to-fetus transmission. Here, we first provide molecular evidence of reactivation as the route of transmission resulting in symptomatic congenital CMV infection. In our study, we found that the same CMV strain infected two babies (B and C), demonstrating complete correspondence between their three already-known hypervariable viral genes.[17] The number of nucleotide sequences for CMV-gO, gN, and UL144 currently enrolled in Genbank are about 200, 300, and 500, respectively. Although we cannot accurately calculate the possibility, the incidental correspondence of all three nucleotide sequences between two different strains of CMV is nearly impossible. In addition, the fact that no CMV DNA was detected in maternal serum between the two pregnancies suggests the absence of persistent viremia and suggestive for establishment of latent infection at the time of conception of Baby C. Thus, reactivation was the causal route for transmission to Baby C.

Epidemiologic studies have already shown that maternal primary infection has a much higher risk for congenital infection than does recurrent infection.[18] Moreover, manifestations of congenital infection due to recurrent maternal infection are generally less severe.[19-21] This indicates that maternal CMV-specific immune function is protective against congenital CMV infection. Therefore, it is assumed that the acquired immune function can be more effective against reactivation than against reinfection and that viral reactivation is the least possible infectious route for symptomatic congenital infection. From this context, our case is surprising, therefore, we attempted to evaluate maternal risk factors for congenital CMV infection.

Several risk factors have been reported for congenital CMV infection besides maternal primary infection. For example, CMV-specific lymphoproliferative responses in mothers who transmitted virus to the fetus were weaker than those who did not.[6,7] Fowler et al.[8] demonstrated that a short interval between maternal primary infection and conception could be a risk factor for congenital

infection. Although some have argued that such a prediction was overstated.[22] Recent studies have indicated that TRL2 recognizes CMV and triggers innate immunity and inflammation against it[9,23] and that a TLR2 single-nucleotide polymorphism is associated with a lower level of inflammation against CMV[24] and a higher rate of CMV diseases in liver transplant patients.[25] However, the mother of the case had no such risk factors.

In conclusion, not only reinfection but also reactivation of CMV in apparently normal seropositive mothers can cause symptomatic congenital infection. Further studies will be required to clarify how frequently reactivation-mediated congenital infection occurs and what triggers the reactivation during pregnancy.

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Declared Ethical approval. This study was approved by the Ethical Committee of Asahikawa Medical Collage (No. 372).

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Figure legends

Figure 1. (A) Family tree including two consecutive siblings with symptomatic congenital CMV infection. (B) CMV-specific IgG, IgM and avidity index (AI) of the mother at the indicated time points are shown.

Figure 2. (A) Frequencies of CMV-specific IFN-γ producing $CD4^+$ and $CD8^+$ T cells determined by intracellular cytokine staining and (B) the CMV-specific lymphoproliferative response of the mother. Healthy controls and the mother are indicated with open circles and a closed triangle, respectively. The mean of the responses of the CMV-seropositive controls in each assay is shown as a short horizontal bar.

Supplementary data Legends

Supplementary data 1. Primer sequences and thermal cycling conditions for the amplification of four DNA fragments spanning exon 1 of the TLR2 gene are shown.

Title: Single cytomegalovirus strain associated with fetal loss and subsequent congenital infection of the next child born to the same mother

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Running title: Reactivation as a cause of symptomatic congenital CMV infection.

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We would like to dedicate this study to Prof. Fujieda who passed away in March, 2010.

word counts:

abstract 250 words, manuscript 2361 words

Figure 1

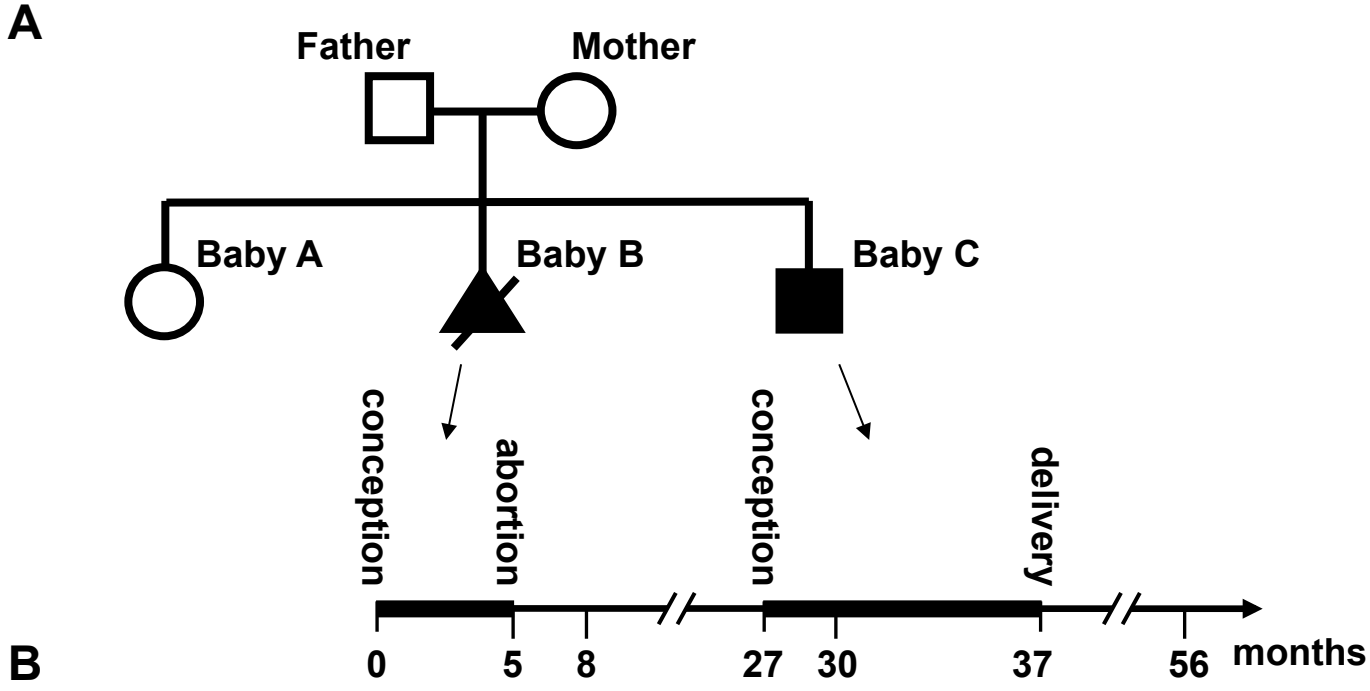
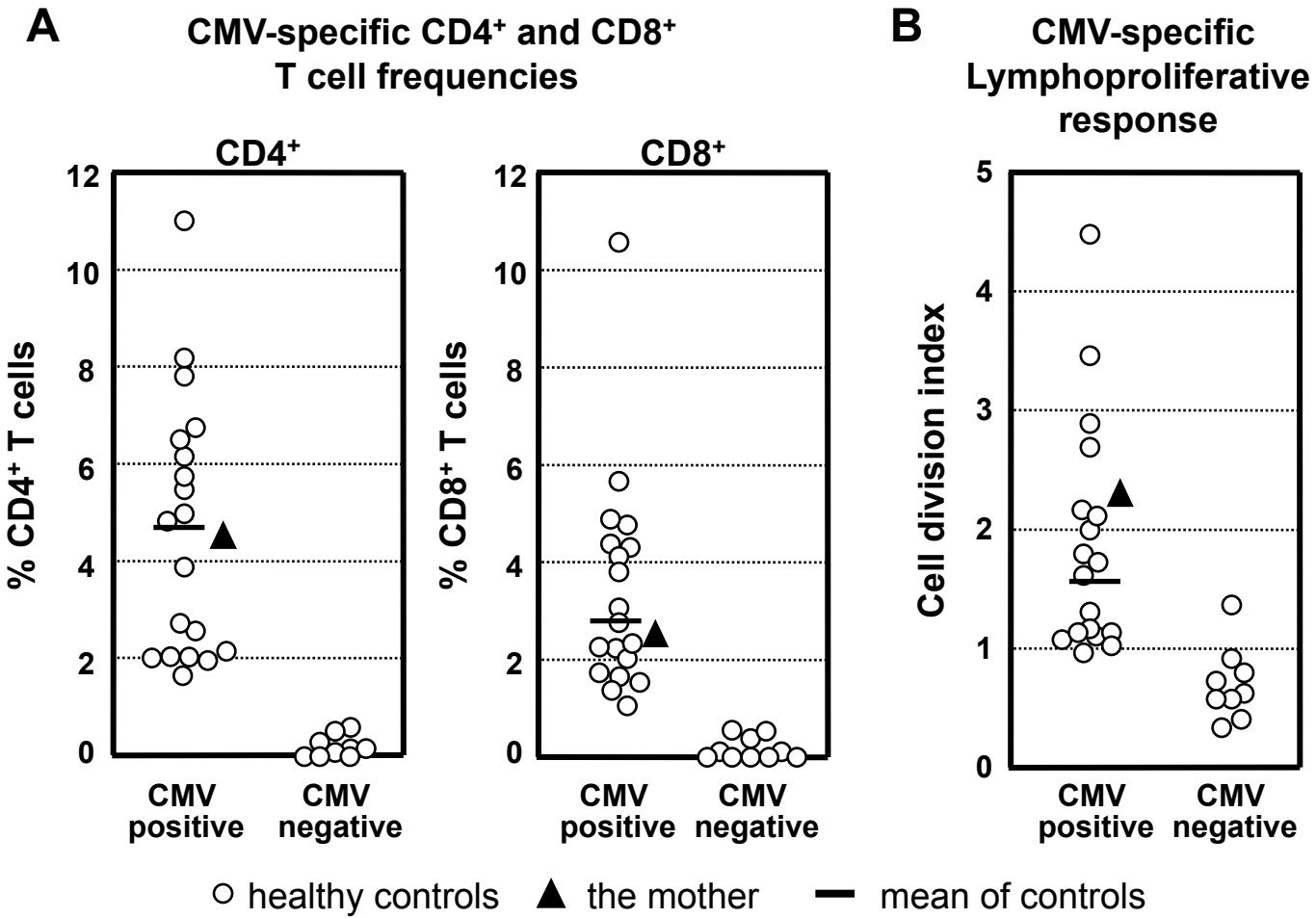

A
Father
Mother
Baby A
Baby B
Baby C
conception
abortion
conception
delivery
B
0
5
8
27
30
37
56
months
Anti-CMV IgG 20.0 22.5 14.0 12.7
Anti-CMV IgM 3.2 5.1 1.2 1.1
Avidity index (%) 37.9 67.5 54.6
CMV DNA - - -

Figure 2


A
CMV-specific CD4+ and CD8+ T cell frequencies
CD4+
CD8+
% CD4+ T cells
% CD8+ T cells
12
10
8
6
4
2
0
CMV positive
CMV negative
B
CMV-specific Lymphoproliferative response
Cell division index
5
4
3
2
1
0
healthy controls
the mother
mean of controls