Human cytomegalovirus infection during pregnancy and detection of specific T cells by intracellular cytokine staining

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Objective: The flow cytometric assay was evaluated as a tool for real-time monitoring of human cytomegalovirus (HCMV)-specific cellular immunity in pregnant women.

Methods: We screened for HCMV infection in pregnant women in Sapporo, Japan, during the year 2000, by serologic assays, virus isolation from urine, and PCR to detect DNA in cervical swabs. The frequencies of HCMV-specific CD4+ T cells in pregnant women with serum anti-HCMV IgG antibody were detected by intracellular cytokine (ICC), interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) staining.

Results: The levels of intracellular cytokines in pregnant women with serum anti-HCMV IgG antibody were significantly higher than those in women without anti-HCMV IgG antibody (P = 0.011 for IFN-γ and P = 0.023 for TNF-α) but lower than those in non-pregnant women with serum anti-HCMV IgG antibody. Frequencies of HCMV-specific CD4+ T cells were higher in infants with symptomatic congenital infection than in infants with asymptomatic perinatal infection.

Conclusions: This ICC assay may reflect immunologic activity against HCMV infection in pregnant women with immunosuppressive conditions.


INTRODUCTION

Human cytomegalovirus (HCMV) causes fetal infection in 0.2-3.0% of all newborns,1 and has been recognized to cause fetal abnormalities of microcephaly, hydrocephalus, intracranial calcification, chorioretinitis, liver dysfunction, and thrombocytopenia, known as cytomegalic inclusion disease (CID). Approximately 90% of cases with fetal HCMV infection are characterized as asymptomatic at birth. Among symptomatic cases, 5% of newborns have nonspecific symptoms, and only the remaining 5% present with specific symptoms of CID.2

The mortality rate of symptomatic fetal HCMV infection is as high as 30%, and 90-95% of survivors are left with neurologic sequelae. Even among those asymptomatic at birth, 5-17% of infants with fetal HCMV infection may present with neurodevelopmental disorders, such as sensorineural hearing loss, spastic diplegia or quadriplegia, and mental retardation within the first 4 years of life.3 Although the natural history of intrauterine HCMV infection is not known completely, a proportion of fetuses are damaged before delivery, and it is sometimes difficult to obtain an accurate clinical diagnosis.

Primary infection during pregnancy is more likely to be transmitted to the fetus (average 40%)4 with more severe sequelae than reactivated latent infection. However, it was recently reported that reactivated latent infection was also transmitted to approximately 1% of fetuses, and that infants with symptomatic HCMV infection were born.5 Pregnant women were screened for HCMV infection mainly by virus isolation from urine, and serologic assays to detect anti-HCMV IgG and IgM antibodies. However, it has been pointed out that these conventional diagnostic assays are not always available for the diagnosis of primary or reactivated HCMV infection in pregnant women.

The number of virus-specific T cells is recognized as an important factor in adaptive immunity. Recently, a new assay for the detection of virus-specific T cells by intracellular cytokine (ICC) staining, using flow cytometry, was developed.6 This approach to counting the cells avoids the errors inherent in limiting dilution estimates of responder cell frequencies that result from apoptosis during longer-term incubation, and shows high sensitivity.

In the present study, we screened for HCMV infection in pregnant women in Sapporo, Japan, by serologic assays, virus isolation from urine, and PCR assay to detect viral DNA in cervical swabs. HCMV-specific CD4+ T cells were detected in pregnant women with serum anti-HCMV antibodies using flow cytometry.
MATERIALS AND METHODS

Screening of pregnant women for HCMV infection

Materials

Urinary specimens for virus isolation were obtained from 642 pregnant women visiting three obstetric clinics in Sapporo, Japan, during January to August 2000. Cervical swabs were obtained from 105 of 642 pregnant women for PCR assay to detect viral DNA. Another 537 women refused to have cervical swabs taken for the present study. Additionally, serum samples were obtained for serologic assay from another 173 pregnant women for the confirmation of prevalence of HCMV. The time of sample collection was the second trimester of pregnancy in all women who consented to sample collection.

Serologic tests

Anti-HCMV IgG and IgM were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Medac Diagnostika, Hamburg, Germany). (The cutoff was 0.183 for IgG and 0.181 for IgM.)

Isolation of HCMV from urine

HCMV from urinary specimens was isolated according to the standard tissue culture technique using MRC-5 cells. The presence of HCMV was confirmed by observation of its characteristic cytopathic effect (CPE) and by immunofluorescent staining with monoclonal antibodies against HCMV immediate early (IE) and early (E) antigens.

Detection of HCMV DNA in cervical swabs by PCR

Cervical fluid wiped off with swabs was suspended in 1 mL of phosphate-buffered saline (PBS) and stored frozen at -20°C until use. The sample was thawed and centrifuged at 1500 rev/min for 10 min, and DNA was extracted from 200 mL of the supernatant, using a QIAamp DNA Mini kit (QIAGEN GmbH, Hilden, Germany).

According to the method of Chou and Dennison, we amplified a part of the HCMV glycoprotein B (gB) gene using PCR assay. The reaction mixture in a volume of 50 μL contained 1.5 mM MgCl2, 10 mM Tris (pH 8.3), 12.5 U of Taq DNA polymerase (Promega, Madison, WI, USA), a dNTP mixture (1 mM each), and 0.1 mmol/L of each primer of gB1319 (5'-TGGAACTGGAACGTTTGGC-3') and gB1604 (5'-GAAACGCGCGGCAATCGG-3'). Physiologic saline served as, respectively, negative control and cell lysate of a laboratory strain AD169 (American Type Culture Collection). Infected MRC-5 cells served as positive control. After 35 cycles of 1 min of denaturation at 95°C, 2 min of annealing at 55°C, and 2 min of extension at 72°C, with GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT, USA), the reaction was allowed to proceed for an additional 5 min at 72°C. The PCR products were identified by 3% agarose gel electrophoresis.

Detection of cytokine-positive CD4+ T cells in specific response to HCMV antigen

Materials

Fourteen samples of whole blood were obtained from six pregnant women with serum anti-HCMV IgG antibody (serum anti-HCMV IgM antibody was detected from one woman, HCMV was isolated from urinary samples from two women, and HCMV DNA was detected by PCR in cervical swabs from two women), from six normal healthy pregnant and non-pregnant women without serum anti-HCMV IgG antibody, and from two infants 2 years of age (symptomatic congenital and asymptomatic perinatal HCMV infections). Whole blood samples were also collected from four non-pregnant women with serum anti-HCMV IgG antibody. Informed consent was obtained from all women and parents of infants.

Detection of HCMV-specific CD4+ T cells by intracellular cytokine staining

Heparinized whole blood (1 mL) was incubated with infected cell lysate antigen preparation for HCMV CF (Microbix Biosystem, Toronto, Canada), or an uninfected cell control (Microbix Biosystem). Staphylococcal enterotoxin B (Toxin Technology Inc., Sarasota, FL, USA) and PBS were used as positive and negative controls. Anti-CD28 antibody (Becton Dickinson, San Jose, CA, USA) was added to each sample (3 mg/mL) as a co-stimulator.

After 2 h, brefeldin A (Sigma, Saint Louis, MI, USA) (10 μg/mL) was added to block the transport of cytokines to the cell surface. After a 6-h incubation at 37°C, EDTA at a final concentration of 2 mM was added for 15 min to remove adherent cells; red blood cells were lysed, and cells were fixed with the fluorescence-activated cell sorter (FACS) lysing solution (Becton Dickinson, San Jose, CA, USA).

The fixed cells were washed with wash buffer, incubated with FACS permeabilization solution (Becton Dickinson) for 10 min, and stained in the dark for 30 min with monoclonal antibodies against two combinations of cytokine and cell surface markers. The combinations were CD4–peridinin chlorophyll A protein (PerCP)/CD69–phycoerythrin (PE)/interferon (IFN)–γ–fluorescein isothiocyanate (FITC), and CD4–PerCP/CD69–PE/tumor necrosis factor (TNF)–α–FITC. CD69–PE was used to detect activated T cells.

Samples were analyzed by use of a FACS Calibur (Becton Dickinson) and side-scatter gating with CD4 and CD69. Data for 10 000 gate events were analyzed by
use of CELL Quest (Becton Dickinson) and are shown as mean ±.

Statistical analysis

For the purpose of calculation, women are classified into three groups according to the conditions of pregnancy and HCMV infection (group 1—pregnant women with serum anti-HCMV IgG antibody; group 2—non-pregnant women with serum anti-HCMV IgG antibody; group 3—HCMV-seronegative women). We used the Mann–Whitney rank sum test to compare quantitative variables. A two-tailed P-value of 0.05 was considered significant. The statistical precision of test indices was determined by calculating the 95% confidence interval.

RESULTS

Screening of pregnant women for HCMV infection in Sapporo, 2000 (Table 1)

Anti-HCMV IgG antibody was found in 67.6%, and three of 173 pregnant women (1.7%) were positive for anti-HCMV IgM antibody. HCMV was isolated from urinary samples of three of 642 pregnant women (0.5%), and HCMV DNA was detected by PCR in the cervical swabs from two pregnant women.

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<th>Table 1. Screening of HCMV infection in pregnant women in Sapporo, Japan</th>
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<td>Serum anti-HCMV IgG (+)</td>
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<td>Serum anti-HCMV IgM (+)</td>
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<td>Isolation of HCMV from urine (+)</td>
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<td>HCMV DNA from cervical swab (+)</td>
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Detection of intracellular IFN-γ after HCMV antigen stimulation

HCMV antigen-stimulated detection of intracellular IFN-γ in one pregnant woman (positive for anti-HCMV IgG antibody and negative for urinary HCMV) is shown in Figure 1. After stimulation with uninfected control antigen, 0.01% of CD4+ T cells produced IFN-γ, while after HCMV antigen stimulation, 0.17% of CD4+ T cells produced INF-γ. The intra-assay variation was less than 0.01%.

Detection of intracellular TNF-α after HCMV antigen stimulation

The value for TNF-α obtained in the same sample is shown in Figure 2. After stimulation with uninfected control antigen, 0.03% of CD4+ T cells produced TNF-α, while after HCMV antigen stimulation, 0.26% of CD4+ T cells produced TNF-α. The intra-assay variation was less than 0.01%.

Frequencies of IFN-γ-positive cells after HCMV antigen stimulation

The frequencies of IFN-γ positive cells in samples from pregnant women with anti-HCMV IgG-positive serum were 0.13–0.63% (0.30% ± 0.23%) (Figure 3). The frequencies of IFN-γ-positive cells in urinary HCMV-positive samples were 0.29% and 0.13%. Whereas the frequency of IFN-γ-positive cells in the 2-year-old infant with symptomatic HCMV infection who had congenital hydrocephalus at birth was 0.23%, the corresponding value for an infant of the same age with asymptomatic perinatal infection was 0.01%.

Figure 1. Representative fluorescence-activated cell sorter (FACS) plot of CD4+ T-cell responses to HCMV antigen by intracellular cytokine (ICC) staining assay. This FACS plot illustrates ICC results for HCMV seropositive pregnant woman. Left: Negative control response when uninfected cell lysate is used. Right: Positive response when HCMV lysate is used. The percentage of cells positive for CD69 and IFN-γ is shown for each panel. FACS analysis was done with CD4 gating.
Figure 2. Representative FACS plot of CD4+ T-cell responses to HCMV antigen by ICC staining assay. The percentage of cells positive for CD69 and TNF-α is shown in the same sample as Figure 1.

A sample from a pregnant woman who was negative for anti-HCMV IgG at 15 weeks of gestation and was positive for anti-HCMV IgM antibody at 25 weeks of gestation showed following results. The frequency of IFN-γ-positive cells was 0.63%. The frequency of IFN-γ-positive cells was 0.16% in one normal pregnant woman who had serum anti-HCMV IgG antibody and was negative for urinary HCMV.

The frequencies of IFN-γ-positive cells in samples from non-pregnant women with anti-HCMV IgG-positive serum were 0.04–0.63% (0.37% ± 0.25%). In samples from pregnant women without anti-HCMV IgG antibody, the frequencies of IFN-γ-positive cells were 0.00–0.02%. No response to HCMV antigen stimulation was observed. The frequencies of IFN-γ-positive cells in samples from seropositive pregnant women were significantly higher than those from seronegative women (P = 0.011), but lower than those in samples from seropositive non-pregnant women.

 Frequencies of TNF-α-positive cells after HCMV antigen stimulation

The frequencies of TNF-α-positive cells in pregnant women with serum anti-HCMV IgG antibody were 0.04–0.34% (0.17% ± 0.13%) (Figure 4). The frequencies
of TNF-α-positive cells in two urinary HCMV-positive pregnant women were 0.29% and 0.08%. The frequency of TNF-α-positive cells was 0.12% in an infant with symptomatic congenital infection, but no TNF-α-positive cells were detected in an infant with asymptomatic perinatal infection. The frequencies of TNF-α-positive cells in two cervical swab PCR-positive pregnant women were 0.06% and 0.04%. The frequency of TNF-α-positive cells in anti-HCMV IgM-positive pregnant woman was 0.43%.

The frequencies of TNF-α-positive cells in samples from non-pregnant women with anti-HCMV IgG-positive serum were 0.20–0.83% (0.44% ± 0.27%). As in the case of IFN-γ, the frequencies of TNF-α-positive cells after antigen stimulation were 0.00–0.11%, in HCMV-seronegative pregnant women. The frequencies of TNF-α-positive cells in samples from seropositive pregnant women were significantly higher than those in samples from seronegative women (P = 0.023), but lower than those in samples from seropositive non-pregnant women.

DISCUSSION

A decrease in the prevalence of serum antibodies against HCMV has been reported in recent years as a consequence of improvements in the social and economic conditions in Japan in the last 20 years. The prevalence of serum antibody to HCMV was more than 90% among women of childbearing age in Japan, but recently it has been speculated that the seroprevalence is decreasing. In 1999, Nishimura et al reported that the prevalence of serum antibody to HCMV was 77.5% among all pregnant women. As antibody decreased to 67.7% among women under 25 years of age in their study, young women in Japan are at greater risk for primary infection during pregnancy. In the present study, a prevalence of HCMV IgG antibody in pregnant women accounted for 67.6% of the study population, and this result is lower than our previous ones. The incidence of congenital HCMV infection may increase in the future. To determine strategies of intervention for those at risk, and to prevent sequelae, accurate prenatal diagnosis of congenital HCMV infection is clinically important.

In practice, accurate clinical diagnosis of maternal primary HCMV infection is sometimes difficult, because most HCMV infection is asymptomatic and goes unnoticed. Serum anti-HCMV IgM as a parameter for serologic diagnosis of primary infection can be present in 10% of pregnant women with HCMV reactivation and continues to increase for about 18 months after onset of primary infection. HCMV can be reactivated more frequently in the late stage of pregnancy, because women often have some kind of immunosuppressive condition. It was reported that the urinary excretion rate of HCMV increased from 1% in early pregnancy to 13% in late pregnancy, as determined by PCR. Attempts have been made to diagnose primary infection by the avidity index for serum anti-HCMV IgG antibodies or by a new HCMV IgM immunoblotting method, but these methods do not yet provide for evaluation of possible prognostic markers in infected pregnant women and intrauterine HCMV infection.

Methods involving umbilical blood and amniotic fluid for diagnosis of intrauterine HCMV infection have recently been reported. Excellent detection ability has been reported with quantitative PCR assay of amniotic fluid and other materials. The quantitative determination of HCMV DNA in amniotic fluid of at least 10^5 genome equivalents gave 100% certainty of detection of an infected fetus. Higher viral loads were associated with fetuses or newborns with symptomatic infections. Timing of amniocentesis and discretion in the interpretation of results remained as practical problems. About 50% of pregnant women with a suspicion of primary HCMV infection refused amniocentesis.

Macrophages and natural killer (NK) cells are considered the main immunomodulating cells that control the activities of infecting cells in primary and latent HCMV infection, and express antiviral activity by producing IFN-γ and TNF-α. CD4+ and CD8+ T cells are also activated by HCMV infection, and produce interleukin 2 (IL-2) and IL-12. Asanuma et al measured serum levels of soluble IL-2 receptor (sIL-2R) in infants with liver dysfunction due to perinatal HCMV infection, and found that serum sIL-2R, one of the indicators of T-cell activation, was correlated with the severity of liver dysfunction. It is presumed that these cytokines are involved not only in the development of persistent HCMV infection and reactivation of HCMV infection but also in infectious transmission to the fetus. Numazaki et al found that serum levels of sIL-2R and IFN-γ were elevated during pregnancy in mothers who gave birth to babies with congenital HCMV infection. Based on their findings, they underlined the possibility of clinical application for the perinatal diagnosis of HCMV infection.

Methods for the analysis of T-cell responses to specific virus antigen have traditionally relied on the limiting dilution assay and enzyme-linked immunospot (ELISPOT) assay. These techniques suffer from the drawback that they do not enable analysis of single-cell responses in the context of unselected cellular backgrounds. In addition, these methods do not allow the assessment of the expression of more than one cytokine per cell, unless T-cell clones are employed.

Waldrop et al in 1997 described a highly sensitive flow cytometric technique to detect single-cell expression of cytokines, and to simultaneously quantify and phenotypically characterize virus-specific T cells. The sensitivity of this method for enumerating cytokine precursor frequencies appears to be superior to those of the limiting dilution assay and ELISPOT assay. Whereas the detection rate for active T-cell precursors in HCMV and mycobacteria accounts for only 0.0001–0.001% of
peripheral mononuclear cells as determined using limiting dilution and ELISPOT assays, the flow cytometric assay detects 0.001–0.2% of specific CD4+ cells. This increased sensitivity of the flow cytometric assay is explained by the highly efficient capture of cytokine produced within the cytoplasm of the secretion-inhibited responding cell and the relatively short-term incubation period, which mitigates against the potential negative effect of activation-induced apoptosis. Furthermore, an assay using whole blood instead of peripheral blood mononuclear cells was reported by Suni et al in 1998. It is desirable to be able to measure the in vivo effects of immunomodulators on cellular immunity. In the present study, therefore, the whole blood method of flow cytometric assay was employed.

The methods of screening for HCMV infection should be compared with the frequencies of IICMV-specific CD4+ T cells. A pregnant woman with serum anti-HCMV IgM antibody showed a higher frequency, and the frequency of HCMV-specific CD4+ T cells might be associated to some extent with virus quantity in the body. As pregnancy proceeds, HCMV becomes reactivated at the cervix. There was no significant difference in HCMV-specific CD4+ T-cell numbers when virus-shedding women were compared to HCMV-seropositive women who were not excreting virus in their urine. A low frequency of HCMV-specific CD4+ T cells in samples of the cervical DNA- and urinary virus-positive specimens suggests the possibility of local reactivation of viruses from latent infection without systemic immunologic changes.

We also evaluated specific cellular immunity in children with congenital HCMV infection. The frequencies of HCMV-specific CD4+ T cells detected by ICC, both IFN-γ and TNF-α, were higher in children with symptomatic congenital HCMV infection than in children with asymptomatic congenital infection. Categorizing findings obtained by the ICC assay may help to determine the prognosis of children with congenital HCMV.

In summary, the flow cytometric assay for HCMV-specific CD4+ T cells was found to be applicable in pregnant women with some kind of immunosuppressive condition. The flow cytometric assay may be useful as a tool for real-time monitoring of HCMV-specific cellular immunity in pregnant women and have clinical application for the transmission of maternal immune activity against HCMV infection to the fetus. Further large numbers of patients with HCMV infection need to be evaluated by this assay for it to be established as a useful diagnostic tool.

ACKNOWLEDGEMENTS

The authors would like to thank Emeritus Professor Shunzo Chiba, Professor Ryuichi Kudo and the staffs of the Department of Perinatal Medicine, Sapporo Medical University School of Medicine for their cooperation in sampling.

REFERENCES


