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Detection of Human Cytomegalovirus Gene in Formalin-Fixed Paraffin-Embedded Fetal Tissues

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Cytomegalovirus (CMV) is an opportunistic pathogen that can cause infection at any time during the course of a lifetime and constitutes an important cause of intrauterine infection and death in newborns, especially if associated with human immunodeficiency virus (HIV) infection (1-3). It is generally assumed that intrauterine transmission occurs transplacentally during maternal viremia (4,5). Therefore, the placenta may act as a portal of entry for the virus, as well as acting as a barrier. Despite its important role very little is known about the interactions between the placenta and CMV. Since the initial description of acquired immunodeficiency syndrome (AIDS) in 1981, it has been proposed that CMV may play an important role in the transmission and progression of perinatal HIV infection. A number of mechanisms have been identified in vitro that could explain the adverse effects of coinfection with CMV on the course of HIV disease. CMV may activate HIV directly or as a result of a superantigen effect with increased cytokine production; it may permit HIV to infect cells otherwise resistant to infection (6,7); or it may suppress immune function in infected individuals (8), thereby facilitating the escape of HIV from host defense mechanisms.

In this study, we retrospectively evaluated a 34-week gestation fetus who was born into a full-blown AIDS mother with positive seral CMV Ig-M. The fetus died in utero but neither the placenta nor the fetus demonstrated any histological stigma of congenital viral infection at autopsy. Since detection of viral genome indicates the presence of virus in the infected cells, we applied a newly developed ligation-dependant polymerase

chain reaction (LD-PCR) method to detect the CMV DNA and RNA in various fetal tissues.

Definitions: CMV infection was defined by positive isolation of CMV from any site or DNA detection in fetal tissues by two consecutive PCR assays. Full-blown, symptomatic CMV infections were defined as CMV infections that were associated with either (i) a proven visceral disease, or (ii) a presumptive viral syndrome (9).

Controls: All the recommended precautions (10) to avoid contamination by PCR product carryover were taken, including physical separation of the pre- and post-PCR steps and use of filtered tips. Two negative and positive controls were included in each experiment.

Clinical history and post-mortem examination: Fetal death was diagnosed with a given maternal history of AIDS with CD4 count of 200 and positive seral CMV Ig-M. The delivery was induced on the same day and autopsy was performed 48 h after death. The gross examinations of the fetus and placenta at the autopsy and the subsequent histopathological examination of various tissues could not explain the cause of fetal death. LD-PCR was carried to detect the presence of CMV in placenta and various fetal tissues to rule out CMV infection-related fetal death. In addition, PCR was performed to detect cellular glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene to confirm the intactness of DNA.

Nucleic acid extraction: The formalin-fixed, paraffin-embedded (FFPE) specimens were sectioned on a microtome and 3-5 sections were placed in a 1.5 ml microcentrifuge tube (11). After deparaffinization with 1 ml of xylene by incubating at 60 °C for 10 min, the

sections were washed twice with absolute ethanol for removal of xylene. The specimens were then allowed to air-dry. The DNA was extracted by cell lysis in the presence of 40 μ l of 20 mg/ml proteinase K (Gibco BRL, USA), and 400 μ l of 1xPCR buffer (Promega, USA) for an overnight digestion at 60 °C. The DNA was purified by extraction with phenol/chloroform and precipitated in ice-cold ethanol. The precipitated DNA was then redissolved in Tris-EDTA (TE) buffer (Sigma, USA) and used as the PCR substrate.

LD-PCR assay: At autopsy, the representative tissues from each organ were sectioned and then embedded in paraffin after fixation in 10% phosphate-buffered formalin. The tissue sections were stained with hematoxylin-eosin and examined under light microscopy. Sections from the heart, lung, liver, pancreas, thymus, thyroid, spleen, bladder, large intestine, and placenta were available for PCR assay.

For LD-PCR, the formalin-fixed, paraffin-embedded (FFPE) specimens were sectioned on a microtome and 3-5 sections were placed in a 1.5 ml microcentrifuge tube (12). After deparaffinization with 1 ml of xylene by incubating at 60 °C for 10 min, the sections were washed twice with absolute ethanol to remove xylene (Sigma Chemical Co., St. Louis, MO). The sections were then dried by placing them on a hot block at 65 °C for 20 min. The deparaffinized tissue sections were lysed by incubating at 100 °C for 30 min in 250 ml lysis solution containing 5 mol/L guanidinium thiocyanate (GTC) (Sigma), 400 mmol/L Tris-HCl (pH 7.5), 80 mmol/L EDTA, 0.5% Sodium-N-Lauroylsarcosine (Sigma), and 0.5% bovine serum albumin (Sigma), and then incubated at 65 °C for an additional 30 min. This process lysed the cell and released and denatured viral DNA and RNA. Then 80 μ l of lysate was added to 120 μ l of hybridization buffer (0.5% bovine serum albumin, 80 mmol/L EDTA, 400 mmol/L Tris-HCl (pH 7.5), and 0.5% sodium-N-lauroylsarcosine (Sigma)), including 20 μ g streptavidin-coated paramagnetic beads (Dyna, Oslo), 10^{11} molecules of phosphorilated hemiprobe 1 and hemiprobe 2, and 10^{12} molecules of each capture probe. The probes are designed to complement the region of immediate early (IE) gene of CMV (Table 1). If viral DNA or RNA were present, the capture probes would bind to the viral sequences and both hemiprobos would hybridize to the target adjacent to each other. The target-probe complexes were captured on the beads, by binding biotin

of the streptavidin of the capture probe on the beads. The beads were then washed twice with 150 μ l of washing buffer (10 mmol/L Tris-HCl (pH 7.5), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, and 0.5% Nonidet P-40 (Sigma)). During each wash, the tubes were placed onto the magnetic separation stand (Dyna) to allow the beads to move to the side of tubes, enabling the supernatant to be removed by pipetting. The hybrids were then resuspended in 20 μ l of the ligation mixture (66 mmol/L Tris-HCl (pH 7.5), 1 mmol/L ATP, 1 mmol/L $MnCl_2$, 1 mmol/L dithiothreitol, 5 units of T4 DNA ligase (Boehringer Mannheim) and incubated at 37 °C for 60 min. A 10- μ l volume of ligation product including paramagnetic beads was transferred to 20 μ l of PCR mixture containing 0.66 μ mol/L PCR primer 1 and 0.66 μ mol/L PCR primer 2, 0.5 units of Taq DNA polymerase (Boehringer Mannheim), 0.2 mmol/L dATP, 0.2 mmol/L dCTP, 0.2 mmol/L dGTP, 0.2 mmol/L dTTP, 1.5 mmol/L $MgCl_2$, 10 mmol/L Tris-HCl (pH 8.3), and 50 mmol/L KCl. PCR amplification was carried out on a GeneAmp PCR System 9600 Thermocycler (Perkin-Elmer, CT) for 35 cycles, each consisting of denaturation (30 s at 95 °C), primer annealing (30 s at 55 °C), and primer extension (1 min at 72 °C). After the first PCR run, 5 μ l of each reaction were transferred into 25 μ l of a second PCR mixture containing the same components except that 0.66 μ mol/L PCR primer 1 and 0.66 μ mol/L PCR primer 3 were used. A 10 μ l volume of the second PCR reaction product was then analyzed by electrophoresis through a 6% polyacrylamide gel and visualized by ultraviolet fluorescence after staining with ethidium bromide (0.5 μ g/ml). The presence of a 90 base-pair band for the second PCR product was considered a positive result.

For PCR to detect GAPDH gene (13), tissue sections were deparaffinized as described above. The tissue sections were digested in 400 μ l of 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, and 40 μ l of 20 mg/ml proteinase K (Gibco BRL, USA) overnight at 60 °C. The DNA was extracted by addition of 400 μ l of phenol and chloroform, followed by ethanol precipitation. PCR was carried out in 50 μ l reaction containing 1.5 mmol/L $MgCl_2$, 200 μ mol/L each dNTP, 25 pmol/L each primer (forward: TCACTGCCACCCAGAAGACT, reverse: TTCTAGACGGCAGGTCAGGT), 3 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer), 10 mmol/L Tris-HCl (pH 8.3), and 50 mmol/L KCl. Amplification was carried out for 40 cycles (94 °C for 1 min, 55 °C for 30 s, 72 °C for

1 min), after a preheat step of 95 °C for 10 min with a Perkin-Elmer 9600 thermal cycler. These cycles were followed by final extension step of 72 °C for 5 min. The PCR products were electrophoresed on an 8% polyacrylamide, non-denaturing gel, stained with ethidium bromide, and evaluated under UV light. The sizes of the PCR products were estimated according to the migration pattern of the *Msp I* digest of pBR322 DNA marker (BioLabs, USA).

Autopsy and histopathologic findings: The fetus was a 2005 g, 34 week gestational fetus with a maternal history of both clinical HIV and CMV infections. After gross examination of the fetus no anomaly was found. Various different tissue sections were obtained at the autopsy including brain, lung, spleen, liver, heart, pancreas, thymus, thyroid, bladder, large intestine, placenta and kidney. No inflammation or viral inclusion bodies was found, whereas histopathological examination showed autolysis and congestion. The presence of extensive hemosiderin deposition in extraplacental membranes, and histology of subacute abruption are consistent with acute uteroplacental vascular events at least 24-48 h prior to delivery. The timing of the brain injury (periventricular leucomalacia) is also consistent with this interval. LD-PCR findings: FFPE specimens (including heart, lung, liver, pancreas, thymus, thyroid, spleen, bladder, large intestine, and placenta) were tested

for the presence of CMV DNA by LD-PCR. CMV infection was defined by positive isolation of CMV from any site or DNA detection in fetal tissues by two consecutive PCR assays. All specimens were found to be negative except placental tissue, which would explain the histological findings of placental vascular compromise. The GAPDH DNA was detected in all specimens indicating that DNA in these tissues was of good quality for PCR.

CMV infection acquired in utero may take many forms. In approximately 90% of cases it is asymptomatic, but in some, mainly those who acquire the virus from a mother with primary infection, classic cytomegalic inclusion disease (CID) occurs (14). This variety of disease manifestations depends partly on the age of the infected host but even more on the host's resistance. Reactivations during pregnancy, rising markedly as term approaches, may be due to hormonal factors, but immunosuppression is generally the most powerful trigger. Patients with AIDS are particularly vulnerable. About 1% of mothers infected with CMV before conception transmit the virus to their children in utero, and approximately 1% of all babies become infected in utero (15). Higher titers of virus are transmitted to babies from mothers with primary than with recurrent infection. For primary maternal infection during pregnancy, the transmission rate is nearly 50%. In the presence of HIV infection, the transmission of CMV could be even higher (16). The preexisting immunity

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Probes (no. of nucleotide)	Sequence (5' to 3')
PCR primer 1 (18)	GTTAGCAGATACACAGAC (forward)
PCR primer 2 (18)	CAAGAGCAACTACACGAA (reverse)
PCR primer 3 (18)	TTCTCGATTAGGTTACTG (reverse)
Capture probe 1 (45)	Biotin-AAGATACACTGATAACCTCAGGCTTGGTTATCAGAGGCCGCTTGG
Capture probe 2 (45)	Biotin-AAGAGAGGCGGTGGGTTCTCAGCACCATCTCTCTCTCTGG
Hemiprobe 1 (44)	<u>CTGACACCAGCGGTGGCCAAAGTGTGTCTGTGTATCTGCTAAC</u>
Hemiprobe 2 (62)	<u>CAAGAGCAACTACACGAA</u> <i>TTCTCGATTAGGTTACTGCGGGTGACACCAGAGAATCAGAGGAG</i>
Full probe (120)	<u>CAAGAGCAACTACACGAA</u> <i>TTCTCGATTAGGTTACTGCGGGTGACACCAGAGAATCAGAGGAGCTGACACCAGCGGTGGCCAAAGTGTGTCTGTGTATCTGCTAAC</i>

Note: 1) Underlines indicate sequences complementary to the immediate early gene of CMV; *italic bold* letters indicate the binding regions for PCR primer 1; *bold* letters indicate the binding region for PCR primer 2; and *italic* letters indicate the binding region for PCR primer 3. 2) PCR Primer 1 and PCR Primer 2 were used for the first PCR reaction on ligated full probes; and PCR Primer 1 and PCR Primer 3 were used for the second PCR reaction.

present in recurrent cases confers considerable protection against disease in the fetus. The overall incidence of babies born with asymptomatic CMV infection is 0.5-2%, and 1 in 2000 has signs of CID (17). Transmission of CMV can occur at any stage of pregnancy, and severe damage to the developing fetus occurs when CMV circulates in the mother's blood, and reaches the uterus to infect the placenta and the baby. Placental and fetal infections frequently cause stillbirths, or maldevelopment of the fetus, depending on the time of infection. The virus damages host cells by entering the cell with specific surface viral proteins that bind to particular host proteins. Both HIV and CMV viral proteins directly damage the host cell's integrity and promote cell fusion. CMV replicates within cells and makes viral aggregates (inclusion bodies), which produce cytopathic-cytoproliferative inflammation in the cell (14).

Acute CMV infection could also lead to tissue necrosis due to vascular compromise. The congenital infection is not always devastating; however, immunosuppression can predispose the fetus to serious CMV infections that primarily affect the lungs, gastrointestinal tract, kidneys, or retina. Grossly, anatomic changes are minimal, consisting chiefly of slight enlargement of the involved organs, particularly the liver and spleen. None of the studies provide incidence data on death in utero in CMV infected mothers. Coinfection of CMV with HIV in AIDS patients may enhance CMV transmission and more severe tissue damage.

Although transplacental infection with CMV is now the commonest viral cause of prenatal damage to the fetus, it may be difficult to demonstrate CMV in chronic villitis cases by histological methods. Although CMV infection is usually indicated by characteristic inclusion cells, it is quite difficult to detect on routine examination of histological sections. Placental CMV infection documented by pathognomonic viral inclusions is a rare finding (17) and has been variously reported to be from 10% to 34% (18). In most instances by the time chronic villitis is found during histological examination of the placenta, it is too late to carry out microbiological studies. Since the

presented case had none of the characteristic findings of CID and did not manifest any of the other usual symptoms of congenital CMV infection, we think that an important proportion of the CMV placentitis cases remain undiagnosed. Nakamura (19) detected cytomegalic inclusion cells and positive immunoreactivity with monoclonal antibody only in a single case among 44 cases with chronic villitis. According to Saetta's findings an important proportion of the cases diagnosed as chronic villitis of uncertain origin are in fact due to CMV infection (20).

In this study, we applied a PCR assay to detect CMV DNA in various tissue and placental sections that had been fixed with formalin. The advantage of this technique over other diagnostic methods is that it is much more sensitive and is able to detect CMV in formalin-fixed placenta tissue. However, not all of the fetal tissues were positive, probably due to the postmortem autolysis. In conclusion, placental CMV infection documented by pathognomonic viral inclusions is a rare finding. Even in cases of proven intrauterine CMV infection, as in our case, the placenta and fetal tissues may appear normal on routine histologic examinations. Our data suggest that PCR for detection of CMV DNA provided the best marker for the diagnosis and developing preemptive therapy strategies. The method that we applied for the detection of CMV DNA is at least as sensitive as virus culture, but much faster. Further studies with our technique and other quantitative methods may help clarify the role of these techniques in the diagnosis and management of CMV infection. In conclusion, our results support the data that PCR detection of CMV DNA is the earliest and most sensitive technique for the diagnosis of CMV infection.

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