Highly Sensitive Detection and Localization of Maternally Acquired Human Cytomegalovirus in Placental Tissue by In Situ Polymerase Chain Reaction

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Background. Transplacental transmission of human cytomegalovirus (CMV) can result in congenital malformations, although details on the mechanisms of transmission and the location of CMV in infected placentae need to be described.

Methods. Placental tissue from term (third trimester) deliveries was screened for CMV infection by polymerase chain reaction (PCR), in situ PCR (IS-PCR), and IS reverse-transcriptase PCR (IS RT–PCR).

Results. CMV DNA was detected in tissue samples from 11 placentae that had been determined to be negative for CMV during routine pathological examination. IS-PCR demonstrated the presence of CMV DNA in all cell types within placental villi, and IS RT–PCR further defined this result by identifying viral transcripts from all stages of replication. CMV DNA and RNA were shown to be highly concentrated in placental trophoblast cells. The infecting viruses were detected with primers specific for the major immediate early section of the genome (UL122/123), the UL21.5 virion gene, and the glycoprotein B (gB) gene and were determined to be predominantly genotype gB2. Therefore, maternal and fetal host factors, as well as viral load and possibly viral genotype, may all affect the outcome of placental CMV infection.

Conclusion. Placental villi are involved in the transfer of blood from maternal to fetal circulation. Infection and replication of CMV within placental trophoblasts suggests that these structures may be involved in the transmission of CMV.

In developed countries, human cytomegalovirus (CMV) is the most common viral cause of congenital infection and malformations [1, 2]. Primary CMV infection occurs in 0.15%–2.0% of all pregnancies, and the virus may be transmitted to the fetus in up to 40% of cases [1]. Prior immunity to CMV is not necessarily protective against reinfection, reactivation, or fetal disease [3, 4]. It is possible that other factors—such as viral strain variation, viral load, and host factors, such as the presence of neutralizing antibody—are associated with transplacental transmission and subsequent fetal infection [5–10].

Defining the mechanisms of placental tissue infection by CMV and the subsequent passage of the virus through the placenta is of critical importance in understanding the pathogenesis of congenital CMV infection. Gestational age at the time of infection appears to be important, because the clinical sequelae of congenital CMV infections are usually more severe when infection occurs early during gestation, although the risk of transmission is lowest early during pregnancy [1, 11, 12]. The involvement of placental villi in the transfer of blood and nutrients from maternal to fetal circulation suggests that these structures may be involved in the transfer of virus from maternal leukocytes.
to the fetus during gestation [13, 14]. Indeed, CMV infection causes villitis [15], and the 3 main cell types contained in placental villi can be infected with CMV [16–18].

Our understanding of the mechanisms involved in CMV placental infection would benefit from the development of a model of such infection. Animal models exist for infection with guinea pig CMV [11, 19, 20] and murine CMV [21, 22]; however, the species specificity of human CMV limits the use of models to humanized animal models, such as the SCID-hu mouse [23].

To overcome the lack of an appropriate model, in vitro models utilizing cultured human trophoblast cells have been established for the study of placental infection [16, 17]. Trophoblasts are a unique type of cell formed only during pregnancy. CMV is able to infect trophoblasts, which suggests that transmission from maternal leukocytes to the fetal circulation may occur via trophoblasts in vivo [16, 17, 24–27]. However, infection and replication in cell culture may not accurately reflect in vivo events. Molecular methods for detection of viral DNA in situ, such as in situ polymerase chain reaction (IS-PCR) and hybridization, can demonstrate cellular infection with CMV and are more sensitive than immunohistochemical techniques [28]. In the present study, in vitro examination of placental cell infection by IS-PCR is used to investigate term (third trimester) placenta from pregnant women who did not have specific risk factors for CMV infection. In particular, our experiments on human term placentae have identified cells involved in placental CMV infection as well as viral transcripts from all stages of viral replication.

SUBJECTS, MATERIALS, AND METHODS

Study cohort. Placental tissue from 94 de-identified women delivering at term was collected from samples that had been sent for routine pathological analysis to the Department of Anatomical Pathology at the Prince of Wales Hospital (Sydney, Australia) between September 2000 and August 2001. Tissue sections from random sites of the placentae were examined; all sections included maternal and fetal surfaces and were from parts of the placentae that were normal with respect to gross anatomy. Negative control placental tissue was collected from 10 samples from CMV-seronegative mothers; these samples had been provided for cord blood-bank donations. Ethics approval for the collection of placental-tissue samples from de-identified subjects was obtained from the South Eastern Area Health Services Research Ethics Committee and the University of New South Wales Ethics Committee. Because subjects were de-identified for ethical reasons, the long-term outcome in infants was not assessed. Placental tissue samples were sent for routine histological assessment, including examination and staining for viral inclusions, inflammation, and other gross abnormalities.

Placental tissue samples. For fresh tissue samples, full-thickness sections were cut under sterile conditions and were stored directly at −80°C. Additional samples were stored in 10% buffered formalin (0.04 mol/L KH₂PO₄, 0.1 mol/L Na₂HPO₄, and 10% formalin) and were fixed for a period of 24–48 h. Where fresh tissue samples were not available, full-thickness sections were obtained prefixed in 10% buffered formalin.

 Extraction of DNA from placental tissue samples. A section measuring ~1 cm² was cut from an area that was typical of the overall placental tissue sample with respect to gross morphological appearance and was rinsed 3 times in PBS. The section was finely minced by use of a sterile scalpel and was resuspended in 1 mL of TE (50 mmol/L Tris [pH 8.0] and 1 mmol/L EDTA). Two milliliters of lysis buffer (200 mmol/L Tris–HCl, 1.5% SDS, 20 mmol/L EDTA, 200 mmol/L β-mercaptoethanol, and 54% sucrose) and 200 μL of proteinase K (20 mg/mL) were added, and the sample was incubated at 56°C for 3 h. An equal volume of phenol–chloroform (1:1) was added to the solution, and, after gentle agitation, the tubes were centrifuged at 2000 g for 5 min. The aqueous phase was retained and further purified by 2 rounds of addition of phenol–chlo-

PCR and CMV genotyping. Solution-phase PCR was conducted with primers specific for the CMV gB gene [7, 29]. The gB genotype was determined by subjecting the PCR products to heteroduplex mobility analysis and DNA sequencing, as described elsewhere [7]. Primers specific for the UL21.5 virion gene [30] and the major immediate early (MIE) section of the genome (UL122/123) were also used in the IS-PCR analysis. The MIE section of the CMV genome was amplified by nested PCR. The first round amplified a 416-bp region, with the following designed primers: MIE1517 (5′-AAGGTTCTGAGTG-GACATGTT-3′; sense) and MIE1932 (5′-CAGCCATTTGGGT-GTCTTAGG-3′; antisense). A combination of the following previously described primers were used for the second round to amplify a 249-bp product: MIE1661 (5′-GAGGCTTTGCGG AGATGAA-3′; sense) and MIE1909 (5′-GGCTGAGTCTTGTGGA TAAGA-3′; antisense) [31]. The thermocycling conditions for both rounds were as follows: initial denaturation at 94°C for 2 min; 30 cycles at 94°C for 30 s, 58°C for 40 s, and 72°C for 50 s; and final extension at 72°C for 3 min.

Preparation of placental tissue samples for IS-PCR. Formalin-fixed fetal/maternal transverse sections of placental tissue samples were embedded in paraffin by standard techniques, and 4-micron sections were cut onto silane-coated glass slides. The microtome blade was cleaned after each section was cut, to prevent cross-contamination. The slides were placed in a Hybaid
Omnislide IS-PCR thermal cycler and were heated to 70°C for 12 min, to remove the paraffin. After heating, the slides were immersed first in xylene for 15 min and then in 100%, 95%, and 80% ethanol solutions for 10 min each. The slides were then placed in 0.1 mol/L Tris (pH 8.0) until amplification [32].

**Amplification of DNA by IS-PCR.** Before amplification, tissue sections were subjected to protease digestion by use of proteinase K (1 µg/mL) at 37°C. The digestion time (5–15 min) was optimized for each tissue section. The slides were washed in 0.1 mol/L Tris (pH 8.0) for 10 min, the edges were dried with a paper towel, and a Geneframe (AB Gene) was attached to each slide. A reaction mixture containing 40.7 µL of deionized water, 7.5 µL of 10× buffer (500 mmol/L KCl and 100 mmol/L Tris [pH 8.3]), 4.5 µL of MgCl₂(25 mmol/L), 19 µL of a dNTP mix (dATP, dCTP, and dGTP; 1.0 mmol/L each), 18 µL of dTTP (1.0 mmol/L), 1.0 µL of digoxigenin (DIG)–11-dUTP (1.0 mmol/L), 1.5 µL each of the sense and antisense primers (10 pmol/µL), and 0.3 µL of Taq DNA polymerase (5 U/µL; Promega) was prepared in a separate pre-PCR laboratory, to avoid contamination. The reaction mixture was placed on the section, and a coverslip was carefully laid on top of the Geneframe; that no air bubbles were present was ensured. The slides were then placed on the IS-PCR thermal cycler. The thermocycling conditions were as follows: initial denaturation at 94°C for 5 min; 20 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and final extension at 72°C for 10 min. A positive control (CMV-infected tissue), a negative control (CMV-uninfected tissue), and a no-DIG control (CMV-infected tissue without DIG in the reaction mixture) were included in each experiment.

**Detection of amplified products.** After amplification, the Geneframes and coverslips were removed, and the slides were washed in 1× standard saline citrate (SSC) buffer for 10 min. The slides were then washed in buffer 1 (100 mmol/L Tris and 15 mmol/L NaCl [pH 7.5]) for 10 min, after which they were immersed in a blocking buffer (100 mmol/L Tris, 150 mmol/L NaCl, 0.6% Triton X-100, and 5% normal goat serum [Vector]) for 30 min. A 1:500 dilution of anti–DIG–alkaline phosphatase Fab fragments (Roche Diagnostics) was prepared in the blocking buffer, and 100 µL was placed on the sections before incubation at room temperature for 1 h. The slides were washed in buffer 1 twice for 10 min and then in buffer 3 (100 mmol/L Tris, 150 mmol/L NaCl, and 50 mmol/L MgCl₂ [pH 9.0]) once for 5 min. A 1:57 dilution of nitroblue tetrazolium;5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics) (0.33 g/mL and 0.16 g/mL, respectively) substrate solution was prepared in buffer 3, and 100 µL was placed on the sections, along with 50 µL of levamisole (1 mmol/L) (Sigma Aldrich). The slides were placed in the dark, and color development was monitored. Once dark blue/purple staining was observed within cells, the reaction was stopped by removal of excess substrate, and the slides were washed in 1× SSC buffer for 10 min and then in deionized water.

**Eosin counterstain of amplified products.** The slides were immersed first for 30 s in a 70% ethanol solution and then for 30 s in a 95% ethanol solution before being transferred to an eosin solution (6% wt/vol) in ethanol (90%). Immersion time varied from 10 to 30 s, according to the desired level of counterstaining. The slides were dipped 15 times in 3 changes of 100% ethanol and then were dipped 15 times in 3 changes of xylene. The slides were air-dried, and the sections were mounted by use of an organic mountant and a coverslip and were sealed. Sections were viewed under a light microscope.

**Reverse-transcriptase IS-PCR (RT IS–PCR).** Tissue was prepared as described for DNA IS-PCR, except that RNase-free solutions and equipment were used. Cell permeabilization of sections was done by chemical digestion with 0.1% Triton X-100 in diethyl pyrocarbonate (DEPC)–treated water for 15 min, followed by a 5-min wash in 0.1 mol/L Tris (pH 8.0). The edges of the slides were dried around the section before the addition of 15 µL of RNase-free DNAse I enzyme (1 mmol/L; Roche Diagnostics). A coverslip was placed over each section, to prevent evaporation, and the slides were incubated at 37°C for 16 h. After incubation, the coverslips were removed, and the slides were washed for 5 min in 0.1 mol/L Tris (pH 8.0).

The reaction mixture was prepared in a separate pre-PCR laboratory and consisted of 42 µL of DEPC-treated water, 7.5 µL of 10× buffer, 7.5 µL of MgCl₂(25 mmol/L), 1.5 µL of a dNTP mix (dATP, dCTP, dGTP, and dTTP; 10 mmol/L each), 1.0 µL of DIG–11-dUTP (1.0 mmol/L), 1.5 µL of each primer (10 pmol/µL), 3 µL of oligo(dT) (10 pmol/µL), 7.5 µL of dithiothreitol (0.1 mol/L), 0.5 µL of Taq DNA polymerase (5 U/µL; Promega), and 1.5 µL of AMV reverse transcriptase (10 U/µL; Promega). A Geneframe was placed around each section before addition of the reaction mixture and a coverslip. The slides were then placed on the IS-PCR thermal cycler. The thermocycling conditions were as follows: an initial reverse-transcription step at 42°C for 40 min; initial denaturation at 95°C for 5 min; 20 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min; and final extension at 72°C for 7 min. Detection and eosin counterstaining of amplified products was done as described for DNA IS-PCR.

**Staining of tissue sections with cytokeratin and vimentin, to detect cytotrophoblasts.** Tissue sections were embedded in paraffin and as described for DNA IS-PCR. Slides were placed in citrate buffer (pH 6.0) and heated for 40 min in a 100°C water bath. The slides were transferred to buffer A (0.1 mol/L Tris [pH 8.0]) for 5 min and then immersed in a 3% hydrogen peroxide blocking solution before being washed in distilled water for 2 min and in buffer A for 5 min. Trophoblasts were detected by the addition of 100 µL of anti-cytokeratin or anti-vimentin primary antibody (ICN Biomed-
In situ polymerase chain reaction of placental tissue samples after digoxigenin–11-dUTP incorporation during amplification with primers specific for the cytomegalovirus (CMV) gB gene. A, CMV-positive tissue. B, CMV-negative control tissue. Original magnifications are shown.

Identification of placental cells infected with CMV. To obtain a greater understanding of the mechanisms involved in the transplacental transmission of CMV, the CMV-infected cells identified by IS-PCR were characterized (figure 3). IS-PCR analysis of the CMV-positive placental tissue indicated that all villus cell types—namely, trophoblasts (cytotrophoblasts and syncytiotrophoblasts), stromal fibroblasts, and endothelial cells of fetal capillaries—contained CMV DNA (figure 4).

Detection of CMV RNA in placental tissue by IS-PCR. The placental tissue samples that were positive by both solution-phase PCR and IS-PCR were further tested for the presence of immediate early, early, and late CMV transcripts. Transcripts of the MIE section, UL21.5, and gB gene products were present in maternal trophoblasts on the outer surfaces of villi and in fetal endothelial cells within villi (figure 5). The identification of CMV transcripts—especially immediate early transcripts, which are essential for replication—indicates the potential for a full cycle of viral replication within these cell types. Comparison of transcripts from MIE (immediate early–early), gB (early-late), and UL21.5 (early-late) indicated that, in all placental tissue samples, the quantity of early-late transcripts (gB and UL21.5) was greater than that of immediate early transcripts (MIE).
DISCUSSION

CMV DNA and immediate early, early, and late transcripts were found within syncytiotrophoblasts, endothelial cells of fetal capillaries, and stromal fibroblasts, a finding that suggests that the villus cell types of the placenta can support CMV replication. The presence of CMV DNA does not necessarily constitute active placental infection, because CMV can be isolated from different leukocyte populations without evidence of replication [14, 33, 34]. CMV DNA was detected by solution-phase PCR in 11.7% of placental tissue samples. It is possible that PCR detects transiently infected maternal leukocytes present in the lacunae of the placenta [34, 35], which may account for the single placental tissue sample that was positive by solution-phase PCR but negative by IS-PCR. However, all of the remaining placental tissue samples were positive by both solution-phase PCR and IS-PCR, which shows that these molecular techniques have a high degree of sensitivity, because all of the placentae had been previously determined to be negative for CMV immunoperoxidase, with no CMV inclusion bodies, during routine analysis.

To determine the relative roles played by different cell types during CMV infection in vivo, the placental tissue samples that were positive by IS-PCR were further analyzed. CMV DNA and RNA was found in the majority of trophoblasts surrounding placental villi, although stromal fibroblasts and endothelial cells lining the fetal blood vessels within the villi also contained CMV DNA and RNA (figure 4). The high ratio of infected trophoblasts to other infected villus cell types suggests that CMV may be selective for trophoblasts and/or that these cells have mechanisms by which they prevent the passage of CMV from maternal to fetal circulation.

The detection of viral DNA in the syncytiotrophoblastic and trophoblastic layers surrounding placental villi (figure 4) has clinical significance, because trophoblasts are the first layer of cells exposed to maternal blood. Trophoblasts are epithelial in nature but have unique characteristics, including reduced HLA class I expression, no HLA class II expression, and no reactivity to anti-vimentin antibodies [36]. This suggests that there may be trophoblast-specific, receptor-mediated attachment and entry of CMV. Trophoblasts are positive for annexin II [37], and CMV utilizes annexin II as a receptor for attachment to host cells [38, 39], along with several other cell-binding partners, including epidermal growth factor receptor, Toll-like receptors, and heparan sulfate proteoglycans (reviewed in Compton [40]). It is, therefore, possible that annexin II is involved in allowing attachment of CMV to trophoblasts and transfer into the stromal layer.

Previous studies have shown that, in first trimester chorionic villi, syncytiotrophoblasts do not become infected with CMV but that clusters of underlying cytotrophoblasts express viral proteins [16]. The samples studied here were from term placentae, and IS RT–PCR showed evidence of immediate early (MIE) and early-late (gB and UL21.5) transcripts in all pla-
Figure 5. Reverse-trascriptase in situ polymerase chain reaction (RT IS–PCR) of placental tissue. A, RT IS–PCR with primers specific for the gB gene. Arrows indicate cytomegalovirus-positive cells, with staining most prevalent in trophoblasts and stromal fibroblasts. B, Control IS-PCR (with no RT) for panel A, performed under identical conditions with no staining observed. C, RT IS–PCR with primers specific for the UL21.5 virion gene. Staining is predominantly in trophoblasts, with associated staining in the stromal layer as well. D, Control IS-PCR (with no RT) for panel C. E, RT IS–PCR with primers specific for the major immediate early section of the genome (UL122/123). Similar staining patterns are observed. F, Control IS-PCR (with no RT) for panel E. Original magnifications are shown.
cental tissue samples (figure 5). The presence of these transcripts indicates the potential for a full cycle of viral replication, and previous studies have demonstrated viral replication in first trimester and term trophoblasts in vitro [17, 18, 41].

We have previously studied the distribution of genotypes in viral isolates from congenital and perinatal CMV infections and found genotypes gB1 and gB3 to be more common than others. The gB3 genotype was predominant (33%), and genotypes gB1 and gB2 were present in similar numbers (28%) [7]. In contrast, the present study found the gB2 genotype to be predominant in placental tissue from this population, despite the prevalence of other genotypes in congenital and perinatal infections [6, 7]. The sample numbers here were small, and the samples were obtained from a single population, so no definitive conclusion can be drawn. It has been shown that laboratory-adapted and cell culture–adapted CMV isolates are not transmitted to polymorphonuclear leukocytes [42] and that gB genotypes have specific cell tropisms in transplant recipients [43]. A prospective study of congenital CMV infection is currently being conducted, to assist in determination of whether there is an association between genotype and congenital CMV infection, although the rate of such infection (0.5%) means that a large-scale collection of placental tissue from different populations is required to allow definitive conclusions [44]. The screening of mothers from a well-defined population will also assist in determination of the CMV genotypes that circulate in the general childbearing population.

Maternal CMV infection during pregnancy and subsequent transmission to the fetus can result in the development of congenital disease. In the present study, the presence of CMV in placental villi was demonstrated, and our IS-PCR and IS RT–PCR results indicated that trophoblasts and the stromal layer were actively infected. More importantly, the endothelial cells that line the fetal blood vessels were also infected, which suggests passage of the virus from the trophoblastic layer into the stromal layer and then into the endothelial cells of the fetal blood vessels. Such a mechanism supports the theory that CMV-infected maternal leukocytes are involved in the delivery of the virus to the placenta [16, 17]. The demonstration of infected cells provides direction for further dynamic studies of placental infection as well as in vivo correlates for the findings of previous in vitro studies [16, 17].

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References