Human Trophoblast Cells Are Permissive to the Complete Replicative Cycle of Human Cytomegalovirus

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Human trophoblast cells were permissively infected by human cytomegalovirus. The kinetics of viral immediate-early, early, and late gene expression was clearly delayed compared to that in fibroblasts. Productive infection was unequivocally proven by the detection of virion particles, infectious virus in trophoblast culture supernatant, and cell-to-cell spread of cytomegalovirus from infected trophoblasts to uninfected fibroblasts. These observations indicate that infected trophoblasts may be involved in maternofetal transmission of human cytomegalovirus.

Maternofetal transmission of human cytomegalovirus (HCMV) is the most common cause of congenital viral infection. The individual course of infection may vary between asymptomatic virus shedding, long-term sensorineural deficits, abortion or stillbirth, or congenital CMV syndrome, including thrombocytopenia, hepatosplenomegaly, and mental retardation (1). The factors determining the pathogenesis of this infection are widely unknown. Interestingly, in the murine system, the conditions of primary infection in the organism seem to influence the subsequent course of CMV infection (5). Thus, the mode of transplacental virus transmission during initial maternal viremia might be an important step in the pathogenesis of the congenital HCMV syndrome by determining the viral load in the fetal organism. In this context, a cell type of major interest is the trophoblast, which forms the interface between maternal blood and fetal tissue. In a guinea pig model, productive infection of the syncytiotrophoblast was found (3), which suggested that human trophoblasts might also be a target of HCMV infection. Actually, HCMV infection of the trophoblast has been described in placental tissue from congenitally infected fetuses (4, 8). However, productive infection of the trophoblast has not yet been demonstrated. Viral gene expression seemed to be restricted to immediate-early or early gene products (8, 9). In a recent study, late viral proteins were found in trophoblasts only after human immunodeficiency virus (HIV) coinfection (9), a situation that is absent in the vast majority of natural maternofetal HCMV transmissions. In the present study, we demonstrate by several lines of evidence that trophoblast cells can be permissively and productively infected by HCMV without any coinfecting agent.

**Infection of trophoblast cultures with HCMV.** Trophoblast cells were isolated from human term placenta by trypsin digestion, percoll gradient centrifugation, and immunoselection for HLA class I-negative cells as described elsewhere (2, 7). Cells were seeded and kept without further passaging in keratinocyte growth medium (Gibco, Eggenstein, Germany) supplemented with 20% fetal calf serum, glutamine, gentamicin, recombinant epidermal growth factor, and bovine pituitary extract. Cell cultures consisted of >95% trophoblasts as judged by the immunodetection of the trophoblast marker proteins human chorionic gonadotropin β (beta-hCG; antibody from Dako, Hamburg, Germany) and cytokeratin 8/18/19 (antibody from Monosan, Am Uden, The Netherlands). Below 5% were stromal cells expressing the mesenchymal cell marker vimentin (antibody from Dako). We first analyzed the susceptibility of these cells for various HCMV strains, including three clinical HCMV isolates and HCMV laboratory strains AD169 and Towne. Trophoblast cultures in 96-well plates were infected with cell-free virus preparations at multiplicities of infection (MOIs) between 0.1 and 1. After 48 h of infection, HCMV immediate-early antigen was detected in the nuclei of infected cells by immunostaining using monoclonal antibody (MAb) E13 (anti-pUL122/123). Infected cells were identified as trophoblasts by the detection of trophoblast marker beta-hCG (Fig. 1). With either HCMV strain, between 5 and 50% of cells were infected. This indicated that trophoblast cultures were readily susceptible to HCMV infection, irrespective of the virus strain used.

**Delayed kinetics of viral antigen expression in trophoblast cells.** Previous workers had suggested that trophoblast cultures were nonpermissive for the full replicative cycle of HCMV (6, 9). Recently, HCMV gene expression was reported to be restricted to immediate-early and early genes. Only after coinfection with HIV did these authors find unrestricted HCMV replication in trophoblast cultures (9). As abortion of HCMV infection after efficient early gene expression seems unlikely, we assumed that delayed kinetics of viral gene expression might account for the difficulties in detecting late viral gene products. Therefore, we analyzed immediate-early, early, and late gene expression in trophoblast cultures at various intervals after infection with cell-free preparations of strain AD169 at an MOI of 1. In particular, we detected the IE1 and IE2 proteins (UL122/123; MAb E13; Biosoft, Paris, France), the early protein p52 (pUL44; MAb BSS10; Biotest, Dreieich, Germany), and the late major capsid protein (pUL86; MAb
28-4, kindly provided by W. Britt) in acetone-fixed cells by an indirect immunoperoxidase technique (Fig. 2). Proteins of all phases of HCMV replication were detectable in up to 100% of cells. Characteristic cytopathogenic effects occurred, and the cell cultures were completely lysed 14 days after infection. However, the kinetics of viral gene expression was clearly delayed compared with that for standard fibroblast cultures. We detected immediate-early antigens at 2 days postinfection.

FIG. 1. Multinucleated trophoblast cell 5 days after infection with HCMV strain AD169 at an MOI of 1. (A) Phase-contrast micrograph. Magnification, \( \times 320 \). (B) Double immunofluorescence staining to detect HCMV immediate-early antigens (FITC) and trophoblast marker beta-hCG (TRITC). Magnification, \( \times 320 \).
(p.i.), early antigen at 4 days p.i., and late antigen at 6 days p.i., except for very few cells that had slightly faster kinetics (Fig. 2). Viral antigens were never detected in mock-infected cultures. From these experiments, it was evident that trophoblast cells were permissive to all phases of HCMV gene expression and that infection was cytopathogenic and lytic.

**Infected trophoblast cells produce infectious CMV.** Finally, we asked whether late-stage-infected trophoblasts produce infectious HCMV particles. First, electron microscopic analyses were performed to visualize virion particles in late-stage-infected cells. Two days after isolation, trophoblast cultures were infected with HCMV strain AD169 at an MOI of 1 and cultured for an additional 6 days. Cultures were then prepared for transmission electron microscopy and examined at magnifications of 12,000 to 20,000. In multinucleated syncytiotrophoblast cells, we detected alterations of the nuclear ultrastructure that are characteristic of late-stage CMV infection. In so-called replication compartments, numerous herpesvirus capsids with or without DNA were visible (Fig. 3A).4 Coated particles at a lower frequency were detectable in the cytoplasm of these cells (data not shown). These ultrastructural data demonstrated the formation of HCMV particles in late-stage-infected trophoblasts.

To analyze whether infectious virions were actually released from those cells, we performed single-step growth curves (Fig. 3B). Trophoblast cultures and fibroblast cultures were infected with HCMV strain AD169 at an MOI of 1. After 2 h of incubation, cultures were washed five times to completely remove residual input infectivity. Subsequently culture supernatants were collected daily, made cell free by centrifugation, and stored at −80°C for determination of the infectious titer. Titers were determined by limiting dilution analysis in fibroblast cultures. Single-step growth curves in infected fibroblast cultures displayed viral replication beginning at day 2 p.i., with a peak at day 5 p.i. In contrast, production of infectious HCMV in infected trophoblast cultures was delayed, beginning at day 5 p.i. and peaking at day 11 p.i. The titer was 3 logs lower than that in identically infected fibroblast cultures; however, HCMV was clearly detectable in three independent experiments. This result strongly indicated that infectious virus was released from late-stage-infected trophoblast cultures.

To formally prove that late-stage-infected trophoblast cells can transmit infectious HCMV to adjacent cells, we performed a modified infectious center assay (Fig. 3C). Freshly isolated trophoblast cells were seeded sparsely on 6-well culture plates and infected with HCMV strain AD169 at an MOI of 1. Five days after infection, noninfected fibroblasts were seeded into the same culture, resulting in coculture of a few infected trophoblasts with abundant noninfected fibroblasts. After an additional day of coculturing, cells were fixed and stained for cell marker proteins and viral antigens to detect whether late-stage-infected trophoblasts were capable of transmitting HCMV to adjacent fibroblasts.

The simultaneous detection of cellular and viral proteins was
done by a quadruple staining procedure, combining immuno-
fluorescence and immunocytochemistry as follows: (i) detection of HCMV major capsid protein (MAb 28-4) by the PAP technique (Dako) with dianobenzidine as a chromogen, resulting in brown nuclear staining; (ii) detection of HCMV immediate-early proteins (MAb E13) by the APAAP technique (Dako) with fast blue as a chromogen, resulting in blue nuclear staining; (iii) detection of mesenchymal marker vimentin (MAb from Dako) by indirect fluorescein isothiocyanate (FITC) immunofluorescence, resulting in green cytoplasmic fluorescence; and (iv) detection of trophoblast marker beta-
hCG (polyclonal antibody from Dako) by indirect tetram-
ethyl rhodamine isothiocyanate (TRITC) immunofluores-
cence, resulting in red cytoplasmic fluorescence. The TRITC-
conjugated anti-rabbit immunoglobulin antibodies of step 4
additionally bound to the secondary rabbit antibodies of step 2,
thus yielding red nuclear fluorescence of HCMV-infected cells,
which could easily be distinguished from the cytoplasmic tro-
phoblast staining of step 4.

In three independent experiments we could consistently de-
tect infectious foci in the infected trophoblast-noninfected fi-
broblast cocultures. In the center of these foci we regularly
found a trophoblastic cell, as identified by beta-hCG staining.
This trophoblast expressed late viral structural antigen, while the adjacent fibroblasts expressed only viral immediate-early
antigen. This assay unequivocally proved that late-stage-in-
fected trophoblast cells can transmit infectious HCMV to ad-
jacent mesenchymal cells.

In summary, our results demonstrate the permissive produc-
tive infection of trophoblast cells by HCMV. It is noteworthy
that the kinetics of gene expression was delayed in all phases of
viral replication at least twofold compared with that in stan-
dard fibroblast cultures. Our infected trophoblast-noninfected
fibroblast coculture model resembles an in vivo situation that
might occur during transplacental transmission of HCMV,
thus supporting the hypothesis that infected trophoblasts may
play a major role in maternofetal transmission of HCMV.

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