Polarized Release of Human Cytomegalovirus from Placental Trophoblasts

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Human cytomegalovirus (HCMV) is a ubiquitous infectious pathogen that, when transmitted to the fetus in utero, can result in numerous sequelae, including late-onset sensorineural damage. The villous trophoblast, the cellular barrier between maternal blood and fetal tissue in the human placenta, is infected by HCMV in vivo. Primary trophoblasts cultured on impermeable surfaces can be infected by HCMV, but release of progeny virus is delayed and minimal. It is not known whether these epithelial cells when fully polarized can release HCMV and, if so, if release is from the basal membrane surface toward the fetus. We therefore ask whether, and in which direction, progeny virus release occurs from HCMV-infected trophoblasts cultured on semipermeable (3.0-μm-pore-size) membranes that allow functional polarization. We show that infectious HCMV readily diffuses across cell-free 3.0-μm-pore-size membranes and that apical infection of confluent and multilayered trophoblasts cultured on these membranes reaches cells at the membrane surface. Using two different infection and culture protocols, we found that up to 20% of progeny virus is released but that ≤1% of released virus is detected in the basal culture chamber. These results suggest that very little, if any, HCMV is released from an infected villous trophoblast into the villous stroma where the virus could ultimately infect the fetus.
pare the susceptibilities of apical and basolateral membranes and to investigate directional release of progeny virus.

Construction of suitable confluent tight-junction membrane cultures of villous trophoblasts has been a particular challenge, primarily because isolated primary trophoblasts do not replicate and culture integrity must be maintained for at least a week after virus challenge. However, such cultures are available and are completely impenetrable, over a 6-hour period, by high levels of HCMV (23). Upon HCMV infection of these trophoblast membrane cultures, we find that the majority of progeny virus remains cell associated, with some apical, and relatively little basal, release.

**MATERIALS AND METHODS**

Cells. (i) Isolation of term villous CT. Placentas were obtained after normal term delivery or elective cesarean section from uncomplicated pregnancies. Villous CT cells (>99.99% pure) were isolated by trypsin-DNase digestion of minced chorionic tissue and immunosorption onto immunoglobulin (Ig)-coated glass bead columns (Biotex, Edmonton, Alberta) as previously described (28, 55), using anti-CD9 (clone 50H.19 [30, 35]) anti-major histocompatibility complex class I (W6/32; Harlan Sera-Lab, Crawley Down, Sussex, England) and anti-major histocompatibility complex class II (clone 7H3) antibodies for immunostaining. Trophoblasts isolated from different placentas were cryopreserved for use in this study. Fully formed membrane cultures from all trophoblast preparations contained fewer than five vimentin-positive cells (nontrophoblasts) per insert as assessed by immunohistochemistry with vimentin antibody (Vim, clone V9; Dako Corporation, Carpenteria, Calif.).

(ii) Culture of CT on semipermeable insert membranes. Insert membranes (6.4 mm; 3-μm pores) precoated with fibronectin (Biocoat; Becton Dickinson) were soaked in Iscove’s modified Dulbecco’s medium (IMDM) (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS) (GIBCO) and 50 μg of gentamicin per ml for 1 h prior to cell plating. Trophoblasts were seeded three times as previously described (23) to ensure confluence as detected by low diffusion rates of radiolabeled compounds and high transepithelial electrical resistance (TER) (see below). Briefly, 2 × 10⁵ cells in 100 μl of 10% FBS-IMDM were seeded into soaked membrane inserts and incubated at 37°C in 5% CO₂ humidified atmosphere in specialized Falcon companion 24-well tissue culture plates (Becton Dickinson). Nonadherent cells were removed 4 h later by three gentle shaking washes with prewarmed IMDM, and the cultures were replenished with 200 μl (per insert) and 800 μl (per culture well) of 10% FBS-IMDM containing 10 ng of recombinant human epidermal growth factor (EGF) (Prepro-Tech, Rocky Hill, N.J.) (17, 34) per ml to promote syncytialization (33). On each of the third and seventh days of culture, freshly thawed and washed trophoblasts from the same placentation preparation were added to the insert cultures at doses of 1.25 × 10⁶ cells/ml in both chambers and were changed every 2 days. Syncytialization was confirmed by immunohistochemically staining fixed cells with desmoplakin antibody (Sigma Immunologicals, St. Louis, Mo.) to visualize desmosome-containing junctions (11) as previously described (55).

(iii) HEL fibroblasts. Human embryonic lung (HEL) fibroblasts (supplied by J. Preiksaitis, Department of Medicine, University of Alberta) were propagated in Eagle’s minimum essential medium (MEM), supplemented with 10% FBS and 50 μg of gentamicin per ml. To assess stock and progeny viral titers of supernatants and cell lysates, 4 × 10⁵ HEL fibroblasts were plated in 100 μl of 10% FBS–MEM in 96-well tissue culture wells and cultured to confluence. To assess progeny virus in the basal compartment (see below), HEL fibroblasts were plated in 800 μl of 10% FBS-MEM at a concentration of 10⁶ cells per well in 24-well Falcon companion plates and grown to confluence. All cultures were used within 48 h of plating.

HCMV, (i) Virus stock preparations. HCMV laboratory strain AD169 and a clinical isolate, Kp7 (from J. Preiksaitis), were propagated in HEL fibroblasts. The infectious virus titer was assessed by expression of HCMV immediate-early (IE) antigen after an 18-h culture of serial dilutions in HEL fibroblast monolayers as previously described (22). Each IE antigen-positive nucleus was equated to one infection focus (IF) of virus, and the titer was determined within a linear dose-response concentration range as IFs per milliliter.

(ii) Virus challenge protocols. Challenge with AD169 or Kp7 at the multiplicities of infection (MOIs) indicated in the figure legends was carried out in 2% FBS-IMDM containing 10 ng of recombinant human EGF per ml for 24 h at 37°C in 5% CO₂. The virus challenge for each experiment was calculated as IFs = Mean number of nuclei per insert. The number of nuclei per insert was calculated in parallel insert cultures (enumeration only) by multiplying the mean number of nuclei counted in five 0.25-mm² fields by the total number of fields per insert (123 fields at a magnification of ×200). Trophoblast insert cultures were exposed to virus in two different ways.

In method 1, insert cultures were initially challenged with HCMV strain AD169 at an MOI of 10 after triple-seeded cultures achieved high TER and low [¹⁴C]dextran diffusion (see below) (23). After 24 h of challenge, both sides of the culture inserts were washed five times with serum-free IMDM and incubated with a pH 3.0 saline solution for 2 min to remove residual virus (8). The cultures were again washed with IMDM, resuspended in 2% FBS-IMDM, and further incubated as described in individual figure legends.

In method 2, after the first seeding and 3-day culture, trophoblasts were challenged with either AD169 or Kp7 for 24 h, followed by washing and a low-pH treatment as described above. The cultures were then replated with 2% FBS-IMDM and cultured for 24 h. Following further washing to remove residual virus, the second and third seedings of trophoblasts were carried out as described above.

(iii) Assessment of infection. Infection in trophoblast cultures is reported as percent IE antigen-positive nuclei, obtained by counting the total IE antigen-positive nuclei per insert and dividing by the estimated total nuclei per insert. Expression of the early-late HCMV antigen pp65 was assessed as the absolute number of pp65-positive foci, where each focus consisted of one or more closely associated positive nuclei. All trophoblast preparations were assessed for initial or reactivated HCMV infection by staining uninfected control cultures for IE and pp65 antigens as described below.

(iv) Monitoring of infectious progeny virus. Progeny virus was monitored over time in three compartments: the supernatant of the culture insert (apical), the cell lysate (cells), and the supernatant in the underlying culture well (basal). At various times after viral challenge, each insert was carefully washed on both sides with IMDM, replenished with medium, and placed into a well containing confluent HEL fibroblasts. After 24 h, the insert was removed and the HEL culture was incubated for a further 18 h before fixing and staining for IE antigen (basal compartment). Insert culture supernatants were removed at various times after virus challenge and frozen at −80°C until they were assessed for viral titer (apical compartment). Adherent cells were washed three times with phosphate-buffered saline (PBS) and incubated with a pH 3.0 saline solution for 2 min to remove external virus. After further washing, the cells were lysed in 100 μl of 2% FBS-IMDM by freezing and thawing three times and were stored at −80°C for assessment of viral titer (cell lysate). Virus titers in culture supernatants or cell lysates were assessed on confluent HEL cultures as described above and calculated as IFs per compartment. Monitoring of progeny virus as described above began in individual experiments only after demonstration of high TER (>60 Ω × cm²) and low [¹⁴C]dextran diffusion (<1.0 pmol/h/cm²) across infected insert cultures.

Immunohistochemical staining. Infected and uninfected cultures were washed twice with PBS, fixed in ice-cold methanol for 10 min at −20°C, and washed three times with PBS. Membranes were fully immersed and thoroughly washed in staining reagents. Endogenous peroxidase activity was neutralized by a 30-min incubation at room temperature with 3% H₂O₂, which was followed by a 1-h blocking incubation at room temperature in 10% nonimmune goat serum (Zymed/Intermedico, Markham, Calif.). Primary antibodies detecting either HCMV IE antigen (detecting p72; Specialty Diagnostics, Dupont) or HCMV pp65 antigen (detecting pp64/pp65; Becton, Dreieich, Germany) and their respective isotype controls, IgG2a (Zymed/Intermedico) and IgG1 (Dako Corporation) were added, and the plates or inserts in plates were sealed with Parafilm and incubated overnight at 4°C. After thorough washing with PBS, secondary antibody (biotinylated goat anti-mouse IgG) and streptavidin-peroxidase conjugate (Streptavidin Biotin System, Histostain-SP Kit; Zymed) were added according to the manufacturer’s instructions. Following a PBS wash, either Ni-DAB substrate (95 mg of diaminobenzidine, 1.6 g of NaCl, 0.136 g of imidazole, and 2 g NiSO₄, made up to 200 ml with 0.1 M acetate buffer [pH 6.0]), yielding a dark brown precipitate, or aminoethyl carbazole substrate, yielding a red precipitate, was added and left for 2 to 5 min. The wells or inserts were then washed with double-distilled water. All insert cultures were counterstained with hematoxylin (Sigma) to visualize nuclei and Stat Stain (WWR, Mississauga, Ontario, Canada) to visualize cytoplasm. All HEL cultures were counterstained with hematoxylin after tissue dry, the membranes were cut out of each insert and mounted in GVA mounting medium (Zymed) on a glass slide. Photographs were taken within a week of mounting.

TER. TER was measured in an Endohm tissue resistance measurement chamber (World Precision Instruments, Inc., Sarasota, Fl.) and monitored with a
RESULTS

HCMV diffuses through 3.0-μm pores of cell-free insert membranes. Because of its large size (200 nm) (reviewed in reference 31), confirmation of HCMV diffusion through membrane insert pores was essential. Infectious virus was not detected in the basal chamber after a 2-h apical incubation over 0.45-μm-pore-size membranes (data not shown). However, infectious virus readily diffused through 3.0-μm pores. In the first hour after virus addition to the upper chamber, 5.69% ± 4.53% (n = 3 independent experiments) of the infectious virus diffused through 3.0-μm-pore-size membranes to infect HEL fibroblasts cultured in the basal chamber. Because of absorption and/or heat inactivation (53), infectious virus is rapidly lost from supernatants in cell-free tissue culture wells (94.5% ± 0.8% in the first hour [data not shown]). Thus, the above initial diffusion rate is a minimum estimate suggesting that relatively free diffusion occurs. All subsequent experiments were carried out using 3.0-μm-pore-size insert membranes.

Progeny virus in apical, cellular and basal compartments of infected triple-seeded trophoblast cultures. To determine if virus can reach the basal compartment of a confluent infected trophoblast culture, the distribution of progeny virus in apical, cellular, and basal compartments was investigated. Confluent trophoblast layers on 3.0-μm-pore-size semipermeable insert membranes were prepared by seeding primary CT three times, interspersed with 3- to 5-day incubations in EGF (23). The cultures were infected when high TER and low [14C]dextran transeptal diffusion were evident (data not shown). Data on infectious virus compartmentalization were gathered only while TER remained high (54.2 ± 12.4 Ω × cm2; n = 5) and [14C]dextran transeptal diffusion remained low (0.18 ± 0.01 versus 9.12 ± 0.70 pmol/h/cm2; n = 5) compared to cell-free inserts. In four independent experiments only 0.02% ± 0.03% of infectious virus was captured in the basal culture compartment, with 8.6% ± 10.8% in the apical compartment (data not shown). As previously found in solid-substratum cultures (22), most (91.3% ± 10.8%) progeny virus was found in the cell lysates. On the date of assessment of progeny virus distribution, <1% HCMV IE antigen-positive nuclei and fewer than 100 pp65-positive foci containing 5 to 10 nuclei per focus were detected in any experiment performed.

Infected trophoblasts were found directly adjacent to the insert membrane. Trophoblasts cultured on insert membranes exist in multiple layers with an average thickness of 2.7 cells (23). Thus, the very low levels of progeny virus detected in the basal compartment could be explained by a superficial infection that never reached the layer of trophoblasts in direct contact with the semipermeable insert membrane. To determine if infected cells were in direct contact with the membrane, IE antigen-positive (data not shown) or pp65-positive nuclei were spatially localized by confocal microscopy. Figure 1 shows that pp65-positive nuclei can be detected in the first 0.5 μm above the insert membrane. In this experiment, 26.5% ± 15.0% of pp65-positive nuclei were found at the membrane surface. This suggests that the infection spread laterally down through the trophoblast layers and reached cells attached directly to the insert membrane. Further, these data imply that if progeny virus is released basally, approximately one-fourth of the virus released would be proximal to membrane pores and therefore free for diffusion into the basal chamber.

Viral challenge after the first trophoblast seeding. To increase the probability of virus-producing cells being adjacent to the insert membrane, trophoblasts were infected after the first of the three seedings required for confluency. At this stage of membrane culture formation, the cells are subconfluent and more closely resemble a monolayer (23). The culture was then brought to confluency with the usual second and third seedings. The time course of a representative experiment carried out with this protocol (out of a total of four performed) is depicted in Fig. 2. The low TER (25 Ω × cm2) observed 2 days after viral challenge illustrates the lack of tight-junctioned confluence in these cultures 1 day after the second seeding of trophoblasts (Fig. 2B). From the day after the third and final seeding of trophoblasts (day 5 after virus challenge) until the end of the experiment, the TER remained high (in the range of 70 to 90 Ω × cm2), indicating maintenance of a tight-junctioned and confluent culture (23). A much higher initial infection level was detected in these cultures compared to cultures infected after the third seeding of trophoblasts (>10% compared to <1%). The 10-fold increase in infection level in trophoblasts located in close proximity to the filter did not alter the progeny virus distribution pattern observed in trophoblast cultures infected after the third seeding. In the experiment depicted, >80% remained cell associated at the three time points tested, with <0.25% at any time found in the basal chamber (Fig. 2C). A summary of the results of four independent experiments with three different preparations of trophoblasts is presented in Fig. 3. The distribution of progeny virus, using median percentages, was 13.7% in apical supernatants, 83.7% in cell lysates, and 0.07% in the basal compartment. The data presented are from the final day of each experiment, just before TER fell and transeptal diffusion increased, both
indications of loss of culture integrity. As noted in the legend to Fig. 3, this final day varied in different experiments, depending on the trophoblast preparation. Increasing the virus challenge from an MOI of 10 to 20 did not alter the results (Fig. 3).

Very similar results were obtained with a clinical isolate of HCMV (Kp7) (Fig. 4). The infection frequency was consistently higher than that found with AD169 (28.6% ± 1.06% on day 8 and 45.3% ± 0.35% on day 10 for Kp7 compared to 10 to 12% for AD169) (Fig 2A). The total progeny virus produced exceeded by 10-fold that produced by AD169 infections (4.86 × 10^5 PFU/culture on day 10 for Kp7 compared to 2.57 × 10^4 for AD169 on approximately day 11) (Fig 3). By day 13 (Fig. 4), total progeny virus exceeded 8.07 × 10^6 PFU/culture. However, as with AD169, most (>98% on day 13) of the progeny virus was cell associated and most released virus was into the apical compartment, with only 0.4% of the total being released into the basal compartment.

DISCUSSION

A previous observation that progeny virus from HCMV-infected trophoblasts remains predominantly cell associated when cultured on nonpermeable plastic dishes (22) suggested that, although they are infectible, placental trophoblasts may not readily transmit virus to underlying fetal tissue. However, trophoblasts are polarized epithelial cells that may not manifest full transport and secretion functions when cultured on nonpermeable plastic. We have examined this important question more physiologically with a villous trophoblast semipermeable membrane culture model that allows trophoblast polarization and both apical and basal diffusion of secreted progeny virus (23). The trophoblasts in this model differentiate into a syncytial patchwork that forms an effective physical barrier as evidenced by high TER and low transepithelial diffusions of both high-molecular-weight ([14C]dextran) and low-molecular-weight ([3H]inulin) molecules as well as organisms such as HCMV. Using this model, we first found that the trophoblast layer could be infected by HCMV, albeit at lower efficiency than comparable cultures on solid plastic surfaces (up to 10-fold lower [D. G. Hemmings and L. J. Guilbert, unpublished data]). Interestingly, when cells were challenged with HCMV lab strain AD169 3 days after the first of three trophoblast seedings, the infection level was comparable to that observed in cultures on solid surfaces. This observation is in accord with findings that primary trophoblasts become more resistant to infection as they age in culture, suggesting that optimal infection occurs at an immature differentiation state (Hemmings and Guilbert, unpublished data). Second, we found that the infection in semipermeable membrane cultures

FIG. 1. Infection reaches triple-seeded trophoblasts directly adjacent to the insert membrane. Trophoblast insert cultures were infected with HCMV as described in Materials and Methods. At 11 days after viral challenge, cultures were prepared for confocal analysis as described in Materials and Methods (HCMV pp65-positive nuclei stained red with Alexa Fluor 546, and all nuclei stained green [false color] with DAPI; the insert surface also stained green). (A) A 0.5-μm optical section at the insert surface (green) showing the 3.0-μm pores (solid arrow) in focus and pp65-positive nuclei (open arrow). Bar, 12 μm. (B and C) Optical sections of the same field at different depths relative to the insert surface, i.e., 11.1 μm above (B) and 0.5 μm above (C), Bars, 30 μm. Arrows indicate a pp65-positive nucleus in the Alexa Fluor 546 scan (a), the DAPI scan (b), and the combined Alexa Fluor 546 and DAPI scans (c). Panels b are phase-contrast tungsten light images showing the insert membrane in focus in panel C but not in panel B.
progressed from immediate-early to late productive stages with some release of progeny virus. Although the amount of progeny virus released relative to the total produced was somewhat greater in membrane than solid-surface cultures, the majority remained cell associated after infection with either the lab strain AD169 or the clinical isolate Kp7. Taken together, these findings show that polarized and differentiated villous trophoblasts can be productively infected by HCMV with progeny virus remaining predominantly associated with the cellular compartment. This is the first study examining viral infection of polarized trophoblasts.

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trophoblasts in a polarized manner predominately basal (23, 42), and HCMV can diffuse through the 3.0-μm pores of the membrane (this paper). Two permutations of the culture model were developed to address the issue of basal release: (i) infection of the upper layer of trophoblasts after 12 days of culture (a completely formed, confluent, and differentiated membrane culture) and (ii) infection of the trophoblasts directly adjacent to the membrane after the first seeding and before full differentiation to syncytium, followed by two additional seedings and culture periods to form the confluent and differentiated culture. Both approaches gave the same result: there was very little release of progeny virus into the basal chamber (<0.4% of AD169 or Kp7 total progeny virus). To interpret results from the first model, we determined by confocal microscopy whether the infection spread from the most apical cell layer through two or three layers to cells adjacent to the semipermeable membrane. Eleven days after infection, almost all infection foci consisted of vertical zones of infection reaching from the top of the culture to the membrane surface. Thus, progeny virus was available for basal secretion in both models at the membrane-most layer of cells. Once basally released from membrane-proximal cells, progeny virus has access to 3.0-μm pores, since ~10% of the surface area of the membrane consists of pores (23) and most cells (average size, 25 μm) encounter multiple pores. A theoretical basal release can be calculated from the data in Fig. 3 as being equal to apical release of progeny virus. Detectable infectious virus actually reaching the basal chamber is <1% of this theoretical value. Thus, progeny virus is available at the membrane-most cell layer, as is the physical means for its diffusion through the membrane upon secretion, but basal secretion or diffusion of HCMV occurs minimally, if at all.

In this regard, it should also be noted that the experimental protocol was biased toward detection of basally released progeny virus. The apical supernatant and cell lysate were frozen and thawed (the latter at least three times) prior to assessment of infectious virus titer, but basal chamber detection was continuous. Virus activity is rapidly lost due to absorption and heat inactivation, and at least 10% of activity is lost on every freeze-thaw cycle (data not shown). Any correction would place more progeny virus in the lysate and apical supernatant and therefore, on a relative basis, less in the basal chamber.

It is very likely that even the small amount of virus detected in the basal compartment derives from leakage of apically released progeny virus through holes or breaks in the cell layers. In a culture model of HCMV-infected Caco-2 epithelial cells grown on 3.0-μm-pore-size insert membranes, most virus remained cell associated and some was released apically, but no basal release was observed until after the cellular monolayer began to deteriorate (20 days after viral challenge) (27).

In a parallel fashion, we find that basal release from trophoblasts occurs late in culture at the time of cellular deterioration (Fig. 2). Further, we find that loss of membrane insert culture integrity almost invariably begins at the outer edge, leaving the center of the trophoblast culture intact, and that most infected HEL fibroblasts in the lower chamber are in a ring directly below the edge of the culture insert (data not shown). These observations suggest leakage, predominantly from the outer edge of the membrane culture, as the source of basal compartment virus.

The lack of detectable infectious virus in the basal chamber could be due either to the absence of basal release from the infected trophoblasts or to the presence of an intact trophoblast basement membrane (TBM) functioning as a barrier. Our results do not distinguish between these two possibilities. We have previously reported an electron-dense layer between trophoblasts and the insert membrane (23). In some cases this layer covers, but does not enter, the pores (data not shown). It has recently been shown that one component of the TBM is perlecan, a heparan sulfate proteoglycan (40), to which HCMV can bind. Perlecan could allow the TBM to absorb virus particles until removal by fetal macrophages. Future studies by electron microscopy to determine if progeny virus is sequestered in vacuoles in the trophoblast as in macrophages [14] or released and trapped in the electron-dense layer are therefore warranted. Burton and Watson (6) suggest that the basement membrane is an important placental barrier, since transient trophoblast damage down to the TBM followed by repair is often observed without concomitant fetal consequences (5, 54). Lack of basal release from infected trophoblasts and/or the presence of a TBM acting as an effective barrier may explain why vertical transmission does not occur more frequently in the first trimester than in the third trimester (9, 29) even though first-trimester trophoblasts are more readily infected (22).

These studies support the idea that in utero HCMV transmission is not caused solely by villous trophoblast infection. The trophoblast, like other epithelia, continuously renews its outer surface. Recent studies by Huppertz et al. suggest that ST renewal begins with CT fusion to ST and ends (with a half-life of 26 days) with the shedding of apoptotic bodies into the maternal circulation (24, 25). Our in vitro results show that...
mature membrane cultures retain progeny virus without concomitant cell damage for at least 20 days (Figs. 2 and 4). As previously seen in guinea pigs (19), the trophoblast may be acting as a sink for the virus, retaining it until the trophoblast is sloughed off into maternal circulation through normal trophoblast turnover. An infection of the outer ST layer in the absence of collaborative events would then be of little consequence. Such events as lateral spread to underlying CTS and consequent loss of trophoblast renewal capacity are under investigation.

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