Permissive Cytomegalovirus Infection of Primary Villous Term and First Trimester Trophoblasts

D. G. HEMMINGS, R. KILANI, C. NYKIFORUK, J. PREIKSAITIS, AND L. J. GUILBERT*

Department of Medical Microbiology and Immunology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

Received 4 December 1997/Accepted 2 March 1998

Forty percent of women with primary cytomegalovirus (CMV) infections during pregnancy infect their fetuses with complications for the baby varying from mild to severe. How CMV crosses the syncytiotrophoblast, the barrier between maternal blood and fetal tissue in the villous placenta, is unknown. Virus may cross by infection of maternal cells that pass through physical breaches in the syncytiotrophoblast or by direct infection of the syncytiotrophoblast, with subsequent transmission to underlying fetal placental cells. In this study, we show that pure (>99.99%), long-term and healthy (>3 weeks) cultures of syncytiotrophoblasts are permissively infected with CMV. Greater than 99% of infectious progeny virus remained cell associated throughout culture periods up to 3 weeks. Infection of term trophoblasts required a higher virus inoculum, was less efficient, and progressed more slowly than parallel infections of placental and human embryonic lung fibroblasts. Three laboratory strains (AD169, Towne, and Davis) and a clinical isolate from a congenitally infected infant all permissively infected trophoblasts, although infection efficiencies varied. The infection of first trimester syncytiotrophoblasts with strain AD169 occurred at higher frequency and progressed more rapidly than infection of term cells but less efficiently and rapidly than infection of fibroblasts. These results show that villous syncytiotrophoblasts can be permissively infected by CMV but that the infection requires high virus titers and proceeds slowly and that progeny virus remains predominantly cell associated.

Cytomegalovirus (CMV), a member of the Herpesviridae family, is endemic and results primarily in subclinical infections in normal healthy individuals (reviewed in reference 32). However, the virus causes severe disease and death in immunocompromised hosts and can be transmitted to the fetus during pregnancy. Excluding rubella epidemics, CMV is the most common congenital infection in the world, occurring in 0.5 to 2.0% of all live births (50). Ten to fifteen percent of infected infants show severe symptoms at birth (14, 41). Thirty to sixty percent of infants born with mild or clinically asymptomatic infections develop neurological deficits of various degrees later in life (41, 55). Approximately 40% of mothers with a primary infection during gestation transmit CMV to their infants, compared to <0.5% transmission during a recurring infection (65). A primary infection in the first trimester of pregnancy may result in more severe fetal consequences than one occurring in the third, but this timing is not associated with an increased risk of transmission (9, 10, 31, 42, 54).

Human CMV replicates in vivo in a variety of human cells including epithelium (56). In vitro it preferentially replicates in human fibroblasts, although low levels of replication occur in other cell types (52). Several patterns of infection occur, dependent on the cell types and virus strains involved (20, 45, 60), including (i) permissive infections during which infectious virus is produced and cytopathic effects are observed, (ii) persistent permissive infections during which virus is produced but cell loss is lower than cell replacement by proliferation, allowing the culture to survive indefinitely, or (iii) abortive infections during which immediate-early (IE) antigen, but not infectious virus, is produced.

Although the pathogenesis of CMV transmission to the fetus during pregnancy is unknown, congenital CMV infections are commonly associated with chronic villitis (38, 47) and infection of the placenta (1, 7, 18, 33, 35, 37, 38, 43, 47, 48, 51). Thus, passage likely occurs through the placenta (5, 6), which may also act as a viral reservoir (22). Since only 40% of pregnant women with primary CMV infections give birth to infected infants (54), an effective fetal barrier, either physical or immunological, must exist. Within the villous placenta lies a physical barrier of fetal cells consisting of continuous, mitotically inactive, multinucleated syncytiotrophoblasts (ST) that are in direct contact with maternal blood (6). In order to reach the fetus in the third trimester of pregnancy, molecules, cells, or organisms must cross the ST and the endothelial cells of fetal blood vessels. Underlying the ST in the first and second trimester are immature, mitotically active, mononuclear cytotrophoblasts (CT) that fuse into the ST (4). Connective tissue containing placental fibroblasts and macrophages can intervene between these CT and fetal blood vessels. Whether virus crosses the ST by direct infection or through sites of damage is not known.

The role of ST in transmission of CMV across the placental barrier is unclear. Results from in vivo studies are difficult to assess because placentas obtained from stillbirths, symptomatic congenitally infected infants at term, or those with chronic villitis at term tend to be preferentially studied (18, 33, 37, 48, 51). Such term placentas, and the trophoblast in particular, rarely display the inclusion bodies characteristic of permissive CMV infections (18, 33, 37, 38, 43, 51). Immunohistochemical analysis of sections from term placentas displaying chronic villitis revealed IE (37, 51) but not early nuclear (37) or late (p150) (51) antigens, suggesting abortive infections (51). In situ hybridization revealed CMV DNA primarily in stromal cells and rarely in the trophoblast of term placenta with chronic villitis (47). Term placentas perfused in vitro and challenged with high titers of a CMV laboratory strain for up to 9.5 h were nonpermissive within this short experimental time frame (36).

In contrast, placentas from first or second trimester abor-
tions contain nuclear inclusions frequently in stromal cells (48) and more rarely in trophoblasts (18), with some expression of pp65 antigen in the trophoblast (61), indicating that a permissi-
ve trophoblast infection during the first half of gestation is possible. In vitro infections of first trimester placentals explants
show permissive infections by both morphological and immu-
nohistochemical criteria (2, 3). In guinea pigs, detection of intranuclear inclusions and expression of CMV antigens in ST
at all stages of gestation indicate permissive infections are also possible in this animal model (22). Highly purified term trophoblasts express IE antigens after CMV challenge but do not release virus into culture supernatants unless coinfected with either human immunodeficiency virus type 1 (HIV-1) (59) or human T-cell leukemia virus type 1 (HTLV-1) (58). These results are compatible with the in vivo findings of infrequent nonpermissive trophoblast infections at term. However, it remains difficult to explain the 40% transmission rate resulting from primary maternal infections or the more frequent indications of permissive infections in first trimester trophoblasts on the basis of such coinfections.

Although apparently straightforward, the development of an effective culture model of ST infection by CMV must address two interdependent problems: fibroblast contamination and long-term culture viability. Placental fibroblasts are likely preferred targets for this virus not only because laboratory strains are passaged in fibroblasts but also because placental trophoblasts, unlike primary villous trophoblasts (66), proliferate in culture. CMV replicates more rapidly in proliferating than quiescent cells (57) and would be predicted to replicate more slowly in trophoblasts than fibroblasts. Permissive infection by CMV also requires viable (healthy) cultures (32). The possibility of slow virus replication dictates that cultures must be viable longer than 2 weeks. However, primary trophoblasts have rarely been cultured for longer than 7 days because of fibroblast overgrowth or loss of viability (15, 16, 29, 65). We have developed culture models of highly purified (>99.99% [28]) term trophoblasts that maintain viability for greater than 3 weeks in culture and have modified this model to obtain highly purified first trimester trophoblasts. We demonstrate using these models that CMV laboratory strains and a clinical isolate permissively infect term and first trimester trophoblasts, but virus replication is slow and infectious virions remain cell associated.

MATERIALS AND METHODS

Cells. (i) Isolation and purification of human term villous CT. Placentas were obtained after normal term delivery or elective cesarean section from uncomplicated pregnancies. Villous CT (>99.99% pure) were isolated by trypsin-
DNase digestion of minced chorionic tissue and immunosorption onto immu-
noglobulin (Ig)-coated glass bead columns (Biotech, Edmonton, Alberta, Canada) as previously described (28, 65), using anti-CD9, anti-major histocompatibility
class II (clone 7H3) antibodies for immunoelimination. As previously described (28, 65), using anti-CD9, anti-major histocompatibility
class II (clone 7H3) antibodies for immunoelimination. Placental chorionic tissue was separated microscopically from fetal material
obtained from elective abortions performed at 10 to 15 weeks of gestation. Chorionic villi were isolated as described previously (65), with the following modifications: 10 ml of tissue was harvested per placenta; and trypsin-DNase digestion was performed at the same concentration for the same number of times as with term placentas, but with 1 ml volumes of tissue homogenate reduced to 0.5 ml at reduced time of 5 min. Cell purification was carried out on glass bead columns as described above. CT preparations from first trimester placentas used in this study contained fewer than 0.02% vimentin-positive cells. Culture and induction of syncytialization of first trimester villous CT were performed as described for term CT.

(ii) Isolation, purification, and culture of PF. Placental fibroblasts (PF) were isolated from first trimester chorionic cell suspensions prior to antibody treat-
ment of the column purification by plating the suspensions in low in 10% FBS–IMDM culture dishes for 60 min, followed by removal of nonadherent cells and culture in 10% FBS–IMDM. Adherent cells grown to confluence were lifted by treat-
ment with 0.05% trypsin-EDTA (GIBCO), washed in 10% FBS–IMDM, and further propagated in 100- to 20-mm tissue culture dishes. Confluent cultures were passaged at least five times to ensure >99% purity, as assessed by immu-
nohistochemical staining for vimentin, and cryopreserved in 10% dimethyl sul-
foxide in FBS. Before experimental use, PF were thawed and cultured in 10% FBS–IMDM and 50 μg of gentamicin per ml until confluent and passaged at least three times.

(iv) HEL cells. Human embryonic lung fibroblasts (HEL cells) were propa-
gated in Eagle’s minimum essential medium (MEM) supplemented with 10% FBS and 50 μg of gentamicin per ml. For CMV infection assays, the cells were plated at 10% FBS–MEM at a concentration 4 × 10^4 per 100 ml in 96-well tissue culture plates. All experiments were carried out on confluent cultures with changes of media every 96 h.

(v) Determination of cell numbers in culture. The number of trophoblasts and fibroblasts in microplates was determined by counting the number of viable cells after 45 min at 2,500 rpm in a GCL-2 Sorvall centrifuge, the wells were washed five times with fresh 2% FBS–MEM. The cultures were fixed in ice-cold methanol and immu-
nohistochemically stained for CMV IE antigen as described below. Each IE-positive nuclear is equated to an infection focus (IF) of infectious virus, and the titer of virus was determined within a linear dose-response concentration range as IF/million.

(iii) Isolation protocols. Infection with each strain or isolate at various multi-
plies of infection (MOIs) was carried out in serum-free IMDM for 2 h at 37°C in
5% CO₂, MOI is the ratio of IF of inoculating virus to the total number of cells in culture to be infected. The cell number was determined at all times of culture as described above. The cultures were infected as follows: EGF-treated (+ EGF) term trophoblasts at day 5 of culture, non-EGF-treated (- EGF) term trophoblasts at day 1 of culture, + EGF first trimester trophoblasts at day 3 of culture, and PF and HEL cells at confluence. The cells were then washed five times with serum-free IMDM and incubated in fresh 2% FBS–IMDM with or without EGF for various times postinfection, and the media were changed every 96 h. HEL infection was carried out as described above in serum-free MEM, followed by incubation in 2% FBS–MEM. All IE-positive nuclei strongly stained and were scored as IF, as in Fig. 1A there are three pp65-positive nuclei in the field. However, the only strongly pp65-positive nuclei were scored since these were often surrounded by nuclei that stained more weakly for pp65 antigen (e.g., in Fig. 1B there are three pp65-positive nuclei in the field). All placental preparations were tested for initial or reactivated CMV infection by including uninfected control cultures stained with IE and pp65 antibodies as described above.

(iii) Determination of infectious virus titers in supernatants or cell lysates. Supernatants were removed from cultures at various times postinfection and frozen at −80°C until assayed for virus titer on HEL cells. Adherent cells were washed three times with phosphate-buffered saline (PBS) and lysed in 100 μl of 10% FBS–IMDM by freezing and thawing three times (lysat). Viral titers in culture supernatants or cell lysates were assayed on HEL cultures as described above, and IF/million of transferred supernatant or cell lysate was determined. Infectious virus found in supernatants were not from residual inoculum since in all cases, none was found 24 h after challenge (data not shown).
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. Endogenous peroxidase activity was neutralized by a 30-min incubation at room temperature with 3% H2O2, followed by a 1-h incubation at room temperature in 10% nonimmune goat serum (Zymed/Intermedico, Markham, Calif.) to block nonspecific sites. Primary antibodies detecting either CMV IE (detecting p72; Specialty Diagnostics, Dupont) or CMV pp65 (detecting pp64/pp65; Biotest, Dreieich, Germany) antigens, and their respective isotype controls, IgG2a (Zymed/Intermedico) and IgG1 (Dako Corporation, Carpinteria, Calif.), were added; the 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 steps were repeated as described above, using aminoethylcarbazole (AEC) as a substrate, yielding a red precipitate. The cells were counterstained with hematoxylin, and photographs were taken immediately.

Measurement of DNA. The assay was a modification of the method described by Cesaroni et al. (11). Cells cultured in 96-well plates were washed twice with PBS, 100 μl of double-distilled H2O was added to each well, and the plates were frozen and thawed three times to lyse the cells. The samples were transferred to 96-well V-bottom plates (Nunc) and mixed with equal volumes of Hoechst dye solution (1 μg of Hoechst 33258 [Sigma Chemical, St. Louis, Mo.] per ml, 10 mM Tris, 1 mM EDTA, 2.1 M NaCl [pH 7.4]), and the fluorescence was measured on an LS-5 luminescence spectrometer (Perkin-Elmer, Norwalk, Conn.), using calf thymus DNA as a standard to calculate the amount of DNA per well in nanograms/milliliter.

RESULTS

Villous trophoblasts from term placentas are infected with cell-free CMV. Primary villous CT cultured with EGF (designated +EGF) form within 4 days a continuous cell layer that is predominantly multinuclear ST-like; Fig. 1), whereas cells cultured without EGF (designated -EGF) form a continuous layer of predominantly mononuclear cells (CT-like) (65). When +EGF cultures were challenged with AD169 and examined for CMV IE or pp65 (early-late) antigens and desmoplakin 12 days after challenge (Fig. 1A and B), both CMV antigens are expressed. Each multinucleated (syncytialized) cell, demarcated by desmoplakin staining, was generally IE positive in all nuclei or none (Fig. 1A). Characteristic cytopathic manifestations of CMV infection such as enlarged cells with nuclear inclusions (24, 27, 44) were also observed in all infected trophoblast cultures (data not shown).

Because trophoblasts do not proliferate in vitro (4, 19), cultures lose 20 to 50% of their DNA content over a 1-month period. Virus challenge of trophoblasts did not increase this loss of DNA either in the presence or in the absence of EGF over a 3-week culture period (two independent experiments with different placental preparations [data not shown]). To determine the kinetics of infection, +EGF cultures were challenged with strain AD169 and examined for CMV IE or pp65 antigens in trophoblast cultures by double immunohistochemical staining. Column-purified villous CT were induced to syncytialize by the addition of 10 ng of EGF per ml and challenged on day 5 of culture with CMV strain AD169 at an MOI of 1.0. At day 12 postinfection, cultures were immunohistochemically stained for CMV antigens with Ni-DAB substrate and for desmoplakin with AEC substrate. (A) Infected culture stained for CMV IE antigen and desmoplakin; (B) infected culture stained for pp65 and desmoplakin; (C) infected culture stained for desmoplakin. Bar, 25 μm.

FIG. 1. Detection of desmoplakin and nuclear expression of CMV IE or pp65 antigens in trophoblast cultures by double immunohistochemical staining. Column-purified villous CT were induced to syncytialize by the addition of 10 ng of EGF per ml and challenged on day 5 of culture with CMV strain AD169 at an MOI of 1.0. At day 12 postinfection, cultures were immunohistochemically stained for CMV antigens with Ni-DAB substrate and for desmoplakin with AEC substrate. (A) Infected culture stained for CMV IE antigen and desmoplakin; (B) infected culture stained for pp65 and desmoplakin; (C) infected culture stained for desmoplakin. Bar, 25 μm.

Because trophoblasts do not proliferate in vitro (4, 19), cultures lose 20 to 50% of their DNA content over a 1-month period. Virus challenge of trophoblasts did not increase this loss of DNA either in the presence or in the absence of EGF over a 3-week culture period (two independent experiments with different placental preparations [data not shown]).

To determine the kinetics of infection, +EGF cultures were challenged with strain AD169 and examined for CMV IE or pp65 antigens in trophoblast cultures by double immunohistochemical staining. Column-purified villous CT were induced to syncytialize by the addition of 10 ng of EGF per ml and challenged on day 5 of culture with CMV strain AD169 at an MOI of 1.0. At day 12 postinfection, cultures were immunohistochemically stained for CMV antigens with Ni-DAB substrate and for desmoplakin with AEC substrate. (A) Infected culture stained for CMV IE antigen and desmoplakin; (B) infected culture stained for pp65 and desmoplakin; (C) infected culture stained for desmoplakin. Bar, 25 μm.
The infected cells in culture are predominantly trophoblasts. Placental fibroblasts, common contaminants of primary trophoblast cultures (29), can be infected with strain AD169 as efficiently as HEL cells (data not shown). It was therefore possible that the rather low frequency of infection observed in term trophoblast cultures could be attributed to contaminating fibroblasts. Fibroblasts, as well as other possible contaminating villous stromal cells such as macrophages and endothelial cells, can be immunohistochemically distinguished from trophoblasts by the former cells’ expression of the intermediate filament protein vimentin. Analysis of the seven preparations of placental trophoblasts used in this study for vimentin-positive cells between days 10 and 12 after infection showed 1.14 ± 1.17 (mean ± standard deviation [SD]) positive cells in +EGF microwell cultures and 1.08 ± 1.56 positive cells in -EGF cultures. Since there are between 4,000 and 16,000 cells in these cultures (see Materials and Methods), the average contamination frequency is between 0.03 and 0.007%. In an experiment using only one of these preparations (chosen for its unusually high number of vimentin-positive cells in the presence of EGF), the number of vimentin-positive cells did not exceed 10 per microwell over a 20-day infection period (Fig. 3A). Thus, it is unlikely that a significant fraction of the 15% IE-positive cells or the 2 to 3% pp65-positive nuclei observed 3 weeks after virus challenge were fibroblasts. Double staining of the cultures for IE antigen and vimentin 12 days postinfection confirmed this prediction: greater than 99% of IE-positive cells (in this experiment, 495 of 496) were vimentin negative and thus trophoblasts (Fig. 3B). Interestingly, most of the vimentin-positive cells were not IE positive (e.g., the vimentin-positive cell in Fig. 3B is IE negative).

Trophoblasts are permissively infected, but most progeny virus remains cell associated. A permissive infection of trophoblasts was demonstrated by the presence of infectious progeny virus, titered on HEL cells in culture supernatants (Fig. 4 and 5C). However, exact times and extent of virus release into culture supernatants varied between trophoblast preparations, with some (Fig. 5D) releasing no detectable virus. Differences in virus release were not due to fibroblast contamination, since experiments in which there was appreciable release (mean of 531 ± 450 IF/mล) between days 8 and 20 after infection had microwells containing 1.03 ± 1.23 vimentin-positive cells, while those with very low release (mean of 1.08 ± 1.56 positive cells in -EGF cultures).
The adherent layer was washed with PBS, and the cells were lysed in 100 times (horizontal axis) after challenge, 100 AD169 at an MOI of 1.0 as described in Materials and Methods. At the indicated placentas were cultured 5 days with EGF and challenged with CMV strain cell lysates as a function of time after challenge. Villous trophoblasts from term HEL IF assay (see Materials and Methods). Each point is the mean medium (Lysate). Viral titer (IF/milliliter; vertical axis) was calculated from the variability and low titers of infectious virus well.

To determine whether the difference in infection frequency of out (CT-like) EGF were compared (Fig. 5). The differences in infection frequencies of trophoblasts were reproducible between trophoblast preparations (Fig. 5A and C), but late release of infectious virus was not reproducible between preparations (Fig. 5D). This finding suggests that the infection progresses laterally within foci until virus is released into culture supernatants. First trimester cultures were double stained for vimentin and IE antigen (to detect infected fibroblasts). As noted above for term cells, >99% of IE-positive cells were vimentin negative and thus trophoblasts (data not shown). Infectious virus production also occurred sooner in first trimester (Fig. 6B) than term (Fig. 4 and data not shown) cells. Although the ratio of cell-associated to supernatant virus was only 6 on day 3 of culture, it increased to approximately 1,000 on days 6 and 9 (Fig. 6B). Thus, virus production in first trimester trophoblasts, as with term cells, is cell associated, but more cells are infected and the infection progresses faster.

**CMV infects a smaller fraction of trophoblasts than fibroblasts, and trophoblasts require a higher virus challenge.** The foregoing CMV infection experiments were carried out at fixed inoculum levels of CMV for each cell type. To compare the initial interaction efficiency of virus with trophoblasts and fibroblasts, the fraction of IE-positive cells was measured 24 h after challenge with various levels of virus, expressed as MOI (allowing for multinucleated cells) for confluent HEL cell and +EGF term and first trimester trophoblast cultures (Fig. 7). The results show that EGF-treated term and first trimester trophoblasts require >100-fold-higher ratios of virus to cells for infection than fibroblasts. Increasing virus challenge increased the fraction of IE-positive fibroblasts at 24 h to 100% at an MOI of 3.5. However, fewer than 20% first trimester trophoblasts were infected at an MOI of 16, and only 6% of term cells were infected at an MOI of 38. Thus, not only do trophoblasts require higher virus concentrations for productive interaction to an IE-positive stage than fibroblasts, but the greater majority are resistant to infection.

**Permissive infection of trophoblasts is not unique to CMV strain AD169.** To determine whether CMV strains infected trophoblasts with differing efficiencies, cultured cells were challenged with AD169, two other laboratory strains (Davis and Towne [32]), and a low-passage clinical isolate from a congenitally infected infant. Infection was determined by using the criteria of IE and pp65 antigen expression and production of infectious virus 12 days after virus challenge. All strains permissively infected trophoblasts, albeit to different degrees, and >99% of infectious progeny virus was cell associated (Table 1). The strain variability (AD169 ~ Towne > Davis ~ congenital isolate) was reproducible in three independent experiments using cells from different placentas.

**DISCUSSION**

The crucial location of placental villous trophoblasts separating maternal blood from fetal tissues suggests that it plays a role in preventing or disseminating CMV infection from mother to fetus during pregnancy. Previous studies have indicated the villous trophoblast is infected only under very specific conditions: in term placentas, trophoblasts rarely showed signs of permissive infection compared to fetal mesenchymal cells (18, 33, 37, 38, 43, 51), and permissive CMV infection in vitro occurred only after enhancement by coinfection with another virus, either HIV-1 (59) or HTLV-1 (58). Our results suggest an alternative view. We demonstrate that cultured term tro-
phoblasts are readily infected but require a high CMV inoculum, infection progresses more slowly than in fibroblasts, and progeny virus remains predominantly cell associated. Our results argue that permissive infection of villous ST or CT in late gestation by cell-free CMV can occur but is unlikely unless the virus titer in the maternal circulation is very high. Such levels could occur during primary infections because of the transient absence of neutralizing antibody and may partially explain why vertical transmission is much more frequent in primary than recurring infections (8, 66).

CMV crosses the placenta at all stages of gestation (54), and villous trophoblasts from first trimester placentas show frequent signs of permissive infection in vivo (18, 61). However, in vitro, Rosenthal et al. (46) found CMV-infected first trimester placental fibroblasts but not trophoblasts. We confirm that first trimester placental fibroblasts are readily infected but also find that first trimester trophoblasts are infected. The infection of first trimester trophoblasts is intermediate between placental fibroblasts and term trophoblasts in two aspects: the fraction of cells infected at near saturating virus titers and the kinetics of the infection. Twenty-four hours after challenge, all placental fibroblasts are IE antigen positive at an MOI of 3.5, 15% of first trimester trophoblasts are IE antigen positive at an MOI of 16, and only 6% of term trophoblasts are IE antigen positive at an MOI of 38 (Fig. 7). The kinetics of progression from the IE to early-late infection stage can be visualized by plotting the ratio of pp65-positive to IE-positive foci in cultures as a function of time (Fig. 8). After CMV challenge of placental fibroblasts at an MOI of 0.19, measurable pp65 antigen is observed within 24 h, and over half of infected cells have progressed to the early-late stage by 48 h. In contrast, even at a challenge MOI of 1, pp65 antigen does not appear in -EGF term trophoblasts until after day 4 and the pp65/IE ratio never exceeds 0.3 over a 21-day culture period. EGF-treated first trimester trophoblasts show intermediate progression kinetics. At an MOI challenge of 1, pp65 antigen expression appears within

FIG. 5. Appearance of IE-positive foci and release of infectious virus as a function of time. Panels A and C and panels B and D depict the same experiment carried out with cells isolated from two different placentas. Term trophoblasts were treated with (+EGF) or without (-EGF) epidermal growth factor and challenged with CMV strain AD169 at an MOI of 1.0 as described in Materials and Methods. (A and B) Number of IE-positive foci, determined immunohistochemically, as a function of culture time; (C and D) supernatant infectious virus titers, determined by HEL assay, as a function of time. The mean ± SD of three replicates are plotted as a function of postinfection time.
described in Materials and Methods. Cell number per microwell was calculated as the mean number of positive cells per microwell of four replicate wells from one of IE-positive cells was determined after 24 h. Percentages were calculated from culture plates as described in Materials and Methods. Cells were challenged with and first trimester placentas cultured with EGF were prepared in 96-well tissue cells as a function of virus concentration. HEL cells and trophoblasts from term (13, 31) even though first trimester trophoblasts are more rapidly infected. A placental barrier that retains infectious progeny virus is in accord with studies by Griffith et al. (22) in vivo (18, 61).

We also find that although infectious progeny virus is rapidly released from placental fibroblasts, virus remains predominantly cell associated in both term and first trimester trophoblasts. Although basal release of infectious virus has yet to be demonstrated, such release from either first trimester or term trophoblasts would explain why vertical transmission does not appear to occur more frequently in the first than third trimester (13, 31) even though first trimester trophoblasts are more readily infected. A placental barrier that retains infectious progeny virus is in accord with studies by Griffith et al. (22) in guinea pig models showing that the placenta can accumulate CMV without transmission to the fetus.

The cell isolation procedures and culture models used in this study were essential for a complete characterization of trophoblast infection by CMV. Infectious challenge of cultures that contained very low frequencies of placental fibroblasts and direct demonstration of IE and pp65-positive cells that were vimentin negative (and thus trophoblasts) eliminated the possibility that the 5 to 15% CMV infection frequencies observed were due to preferential infection of placental fibroblasts. Crucial to the demonstrations of productive infection and the slow progression of infection in trophoblasts was the ability to maintain viable cultures for greater than 3 weeks without overgrowth by proliferating placental fibroblasts.

Interestingly, most of the very few vimentin-positive cells (fibroblasts) in these cultures were uninfected. Possible reasons include the following: (i) there is a disadvantageous target ratio (there are ≥4,000-fold more trophoblasts); (ii) infected vimentin-positive cells may lyse and not be detected, although lack of high titers of infectious virus in supernatants during the first week of culture argues against this; (iii) fibroblasts, which strongly adhere to tissue culture plastic, may lie beneath the

![Image](http://jvi.asm.org/)

**FIG. 6.** Infection of first trimester placental trophoblasts with CMV strain AD169 as a function of time. Villous trophoblasts from first trimester placentas were cultured 3 days with EGF as described in Materials and Methods. (A) The cells were challenged with CMV strain AD169 at an MOI of 1.0, cultured for the indicated periods of time (horizontal axis), and immunohistochemically stained for CMV IE and pp65 antigens, and the percent infected cells was determined as described in the legend to Fig. 2. (B) At the indicated times (horizontal axis), released and cell-associated infectious virus titer was assessed as IF/milliliter as described in the legend to Fig. 4. The results are depicted as the mean ± SD of three replicate cultures and are representative of two independent experiments with the same results.

<table>
<thead>
<tr>
<th>CMV strain</th>
<th>No. of nuclei positive for IE</th>
<th>IF/m of equal vol of Lysate</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD169&lt;sup&gt;a&lt;/sup&gt;</td>
<td>540 ± 130</td>
<td>16 ± 7.6</td>
</tr>
<tr>
<td>Towne&lt;sup&gt;a&lt;/sup&gt;</td>
<td>290 ± 63</td>
<td>19 ± 10</td>
</tr>
<tr>
<td>Davis&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25 ± 19</td>
<td>2.8 ± 0.96</td>
</tr>
<tr>
<td>Congenital isolate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54 ± 21</td>
<td>2.3 ± 2.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Term trophoblasts cultured with EGF as described in Materials and Methods and challenged with the laboratory CMV strains AD169, Towne, and Davis at an MOI of 1.0.
<sup>b</sup> Term trophoblasts cultured without EGF challenged at an MOI of 1.0.
<sup>c</sup> Mean ± SD of four microwells.
<sup>d</sup> Determined as described in Materials and Methods.
<sup>e</sup> Mean ± SD of six microwells.
<sup>f</sup> Mean ± SD of five microwells.
<sup>g</sup> Mean ± SD of three microwells.

![Image](http://jvi.asm.org/)

**FIG. 7.** Infection of term and first trimester placental trophoblasts and HEL cells as a function of virus concentration. HEL cells and trophoblasts from term and first trimester placentas cultured with EGF were prepared in 96-well tissue culture plates as described in Materials and Methods. Cells were challenged with CMV strain AD169 at the MOI indicated on the horizontal axis, and the number of IE-positive cells was determined after 24 h. Percentages were calculated from the mean number of positive cells per microwell of four replicate wells from one of two independent experiments. Cell number per microwell was calculated as described in Materials and Methods.
The data are expressed as the ratio of pp65- to IE-positive foci from the means histochemically stained for foci of CMV IE and pp65 antigens (in separate wells). The trophoblasts and fibroblasts were challenged with CMV strain AD169 at MOIs of 1.0 and 0.19, respectively, cultured for the indicated periods of time (horizontal axis), and immunoblots were challenged with EGF as described in Materials and Methods. The trophoblasts and fibroblasts from term and first trimester placentas were cultured as a function of time. Villous trophoblasts from term and first trimester placentas were cultured until the number of infected nuclei increases (data not shown) but the number of foci does not. An increase in the number of foci coincides with release of progeny virus into culture supernatants, suggesting that free virus dissemination also exists. In the absence of trophoblast proliferation in culture, cell loss due to death or shedding leads to a decrease in DNA content over time. CMV infection did not increase this loss of DNA content; thus, infected cells are not preferentially lost, a conclusion supported by the observation that the number of infected cells always increased and never peaked or decreased. These observations indicate that CMV infection, at least up to 3 weeks after virus challenge, does not damage ST, possibly because of slow virus accumulation in infected syncytiotrophoblasts and be protected from virus challenge; and (iv) EGF down modulates CMV production from infected placental fibroblasts as it does with other human fibroblasts (30).

Our results differ from those of Toth et al. (59), who found that CMV infection of syncytialized term trophoblasts was abortive and became fully permissive only if the cells were preinfected with HIV-1. The reasons for the different results are not clear, but it is possible that different CT subpopulations were isolated by the slightly different negative selection methods used in the two laboratories: elimination of MHC class I, MHC class II, and CD9-expressing cells in our laboratory (28) and elimination of MHC class I and II cells in their laboratory (59). Alternatively, the stocks of the laboratory strain of CMV, AD169, used in both studies may be substantially different since, according to Cha et al. (12), long-term passage can result in loss of genetic information, explaining differences in tissue tropism and virulence. To confirm that the permissive infection that we observed was not a property of our laboratory AD169 strain, we tested two other well-known laboratory-adapted strains, Towne and Davis, and a low-passage congenital isolate. Although there was considerable variation in infection efficiency (AD169 and Towne infected much more efficiently), all strains were able to permissively infect term trophoblasts.

The ST is a rather unique tissue in that it is a continuous, multinucleated cell layer that, theoretically, covers entire villous branches. The EGF-treated cultures in this study, although not continuously syncytialized, nonetheless offer a useful model of the ST. Approximately 90% of nuclei are in cells containing >2 nuclei, with approximately 20% being in cells with as many as 50 nuclei (28). It was consistently observed that either all or no nuclei in CMV-challenged syncytialized cells were positive for CMV antigens; thus, all nuclei in an infected ST participate in infection. Since ST, both in culture and in vivo, does not proliferate (4, 19), any increases in the number of infected nuclei must come from free virus infection, fusion of infected with uninfected cells, or cell-to-cell transmission (focal spread [39]). We find that the spread of virus is initially focal since the number of infected nuclei increases (data not shown) but the number of foci does not.

ACKNOWLEDGMENTS

This work was supported by grants from the Hospital for Sick Children Foundation and the National Health Research Development of Canada to L.J.G. and J.G.N. was supported by studentship grants from the Alberta Heritage Foundation for Medical Research, and D.G.H. was supported by a grant from University of Alberta Perinatal Research Centre.

We thank Bonnie Lowen for expert technical assistance and the University of Alberta Perinatal Research Center laboratory staff and the OB/GYN nursing staff, both at the Royal Alexandra Hospital in Edmonton, for placental cell preparations.

REFERENCES

3. Amirhessami-Aghili, N., P. Manalo, M. R. Hall, F. D. Tibbits, C. A. Ort, and...