Factors affecting the transplacental transmission of human cytomegalovirus

Ph.D. Thesis

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Introduction

Human cytomegalovirus (HCMV) the largest and structurally most complex member of the Herpesviridae family, is a ubiquitous virus that infects almost all humans at some time in their lives. It has been classified as a betaherpesvirus on the basis of several biochemical criteria, such as the genome size, the guanosine and cytosine contents, the slow replicative cycle, and the restricted *in vitro* and *in vivo* tropism. Although HCMV has been shown to infect a broad spectrum of cells *in vivo*, the only cells that are fully permissive for HCMV replication *in vitro* are human fibroblasts. All known strains of HCMV are genetically homologous, but none seem to be genetically identical, unless they are obtained from epidemiologically related cases. The genetic and immunologic variability and the differences in *in vitro* growth characteristics are well documented and strain differences may affect HCMV virulence. Comparative genetic analysis of HCMV strains is primarily limited by the size and complexity of the viral genome, which comprises 220–240 kB pairs of linear double-stranded DNA, depending upon the strain. The massive HCMV genome is approximately 50% larger than the genome of herpes simplex virus.

Many of the capsid proteins appear to share structural, functional and even antigenic similarities with capsid proteins from other human herpesviruses. Outside the capsid, but beneath the envelope, is tegument or matrix. The tegument of HCMV is the most complex and heterogeneous structure in the virion. Proteins within the tegument are characteristically phosphorylated and in many cases serve regulatory functions for virus replication. Some tegument proteins appear to have a primary role in the maintenance of the structural integrity of the virion. However the tegument contains the most immunogenic proteins of the virions, including the immunodominant targets of T lymphocyte responses and antibody responses. The lipid-containing envelope is composed of virion glycoproteins and host-derived membrane lipids. The envelope of HCMV plays an essential role in the initial steps of virus-host cell interactions. Since the envelope glycoprotein B (gB) of HCMV has been implicated in host cell entry, cell-to-cell viral transmission and fusion of infected cells in addition to being an important target for both antibody- and cell- mediated immune response, it is a candidate as a HCMV virulence factor. It is encoded by the gene UL55. Certain regions of the gB gene vary considerably between different virus strains. On the basis of the nucleotide sequence coding for the variable region, HCMV strains can be classified into 4 gB genotypes. The complete DNA sequence of AD169, a laboratory-adapted HCMV strain, has been determined, but an additional 22 viral genes have been found in low-passage clinical isolates that are missing in AD169. The wild-type HCMV genome has been predicted to encode approximately 165 genes. HCMV genes encode for structural proteins, which are incorporated in the architecture of virions (nucleocapsid, tegument or envelope). In addition to structural genes, the genome displays regulatory open reading frames, whose functions are associated with the onset and progression of HCMV infection and replication through the immediate early (IE), early (E) and late (L) phases. Most genes are highly conserved between strains, but some are characterized by a striking degree of variability, as revealed by the examination of individual genes and by whole genome comparisons. These variable genes are generally predicted to encode membrane-associated or secreted proteins. The hypervariation is probably due to immune system selection on the scale of human evolutionary history or longer, and the genotypes are maintained stably within and among infected individuals. Several hypervariable genes are located in the sequence at the right end of UL that is absent from laboratory-adapted strains AD169 and Towne. Two notable examples are UL146 and UL139, which are located 5.2 kbp apart and oriented leftward. The gene UL146 encodes a protein, designated vCXC-1, that induces calcium mobilization, chemotaxis and the degranulation of neutrophils. High-affinity vCXC-1 binding has been shown to be mediated via CXCR2, but not CXCR1. vCXC-1 exhibits a potency approaching that of human IL-8. As the first example of a virus-encoded chemokine, vCXC-1 may ensure the active recruitment of neutrophils during cytomegalovirus infection, thereby providing for efficient dissemination during acute infection and accounting for the prominence of this leukocyte subset in cytomegalovirus disease. The most variable gene in the vicinity of UL146 is UL139, which is predicted to encode a type I membrane glycoprotein. The variability is concentrated in a region of the ectodomain.

The prevalence of HCMV infection in the normal population varies widely, between 40% and 90%, depending on the race, the gender, the age, the hygienic circumstances and the socioeconomic factors. HCMV is not very contagious: spreading of the infection appears to require close or intimate contact of either a nonsexual or a sexual nature with another person who is shedding the virus in the bodily secretions. The virus is present in the urine, oropharyngeal, cervical and vaginal secretions, breast milk, semen and tears, and can be shed intermittently for years. HCMV can also be transmitted vertically, from mother to fetus. The *in utero* transmission of HCMV can occur as a consequence of primary and recurrent infections, with equal frequency during all three trimesters. Primary infection with HCMV occurs in 0.7-4.1% of pregnancies, with a mean reported rate of transmission to the fetus of 40% (range 25-75%). In contrast with the high rate of transmission in primary infection, that during recurrent infection is much lower (1-1.2%). Primary infection occuring in the mother and as intrauterine transmission during the first 16 weeks of pregnancy has a much greater clinical impact on the fetus than nonprimary infections and infections occurring during the last trimester of pregnancy.

After primary maternal infection, the most likely sequence of events leading to congenital HCMV infection is maternal viremia, placental infection and hematogenous dissemination to the fetus. During the viremic phase, the virus circulates and disseminates, carried by leukocytes. HCMV is thought to be transmitted when infected leukocytes cross the placental barrier to reach the fetal circulation via the umbilical cord vessels. Other routes may also be accessible to viral transmission. Infected leukocytes may reach the fetal endothelium directly, through breaches of the syncytiotrophoblast (ST) layer of the placenta, particularly in the last stages of gestation. Virus coated by specific antibody may cross this layer by transcytosis and be released, still infectious, to the underlying cytotrophoblast (CT). Located at the boundary between the maternal circulation and the fetal mesenchyma, the ST is a central component of the placental barrier, imposing physical and possibly immunological constraints on the passage of microbial pathogens and/or maternal cells into the fetal compartment. An additional possibility is that the virus may ascend from the vagina via the ruptured membranes, to reach the decidua or amniotic cells. Consequently, infected amniotic cells may be ingested by the fetus, after which the virus may replicate in the oropharynx and invade the fetal circulation to reach the target organs.

Despite the morbidity and mortality associated with prenatal HCMV infection, little is known about how the virus infects the conceptus. Approximately 15% of women with primary infections during early pregnancy abort spontaneously. In this case, the placenta, but not the fetus, shows evidence of infection, which suggests that placental involvement is important in its own right and precedes virus transmission to the fetus. Later in pregnancy, HCMV infection causes premature delivery and (in 25% of the affected infants) intrauterine growth retardation, outcomes that are often associated with placental pathology. Numerous reports indicate that the placentas from these births also contain viral proteins, suggesting that placental infection and virus transmission to the infant are related causally.

Although the pathogenesis of HCMV transmission to the fetus during pregnancy is unknown, congenital HCMV infections are commonly associated with chronic villitis and infection of the placenta. Thus, passage probably occurs through the placenta, which may also act as a viral reservoir.

The human placenta is composed of villi that float in maternal blood and also villi within the uterine wall that anchor the placenta and attach the fetus to the mother. The individual chorionic villus has a connective core that contains fetal blood vessels and numerous macrophages (Hofbauer cells) that often lie under a thick basement membrane.

Three major trophoblast populations can be identified during placentation: CT stem cells and two differentiated derivative cell types: ST and extravillous CT. The undifferentiated trophoblastic stem cell of the placenta, the CT, is the first fetal cell type arising during embryogenesis. The CT stem cell of the placenta undergoes multistep differentiation and finally gives rise to villous (non-invasive) and extravillous (invasive) trophoblast cell populations.

In the human placenta, proliferating CT stem cells are attached to an extensive basement membrane that surrounds the stromal core of two types of chorionic villi. In floating villi, CTs differentiate by fusion to form an overlying layer of multinucleated ST. These cells are in immediate contact with

maternal arterial blood that bathes the floating villi, and their primary function is to perform nutrient, waste and gas exchange between the maternal and fetal circulations. In anchoring villi, beginning with the third post-ovulatory week, a subset of CTs at the distal tips of the villi proliferate and differentiate by leaving their basement membrane to form multilayered cell columns consisting of highly migratory, non-polarized, invasive CTs, covered by a thin layer of syncytium. The cell columns expand distally, and populate the decidualized endometrium and the first third of the myometrium, thereby anchoring the villous tip to the uterine wall. The penetrating cells spread over the maternal decidual cells, forming the cytotrophoblastic shell. Some of these deeply invasive cells penetrate the inner walls of the maternal spiral arteries up to their myometrial segments and, by replacing the endothelial lining and the smooth muscle cells, transform them into maximally dilated inert tubes.

The mechanisms by which human trophoblast cells influence the vertical transmission of HCMV have not been well studied. The cellular organization of the decidual-placental interface suggests potential routes by which HCMV reaches the placenta. The virus might disseminate from infected maternal blood cells to the decidua, interstitial and endovascular CTs in the uterine wall, CT columns of anchoring villi and/or floating villi.

Numerous investigations have indicated that placental cells can be productively infected by HCMV. Experiments have been performed *in vitro* by using laboratory-adapted strains and a high multiplicity of infection (MOI), which may not be entirely representative of the behavior of wild strains. Placental trophoblastic cells closely resemble macrophages; both macrophages and trophoblasts are invasive, form syncytia, and express CD14, Fc receptors, multiple cytokines and receptors for many cytokines. Placental macrophages and trophoblastic cells are known to constitutively secrete a variety of cytokines and prostaglandins, which play crucial roles in normal reproductive processes. In the placenta, proinflammatory cytokines, including IL-1, IL-6, IL-8 and TNF-α, and anti-inflammatory cytokines such as IL-4 and IL-10, are produced by the trophoblast. Intrauterine infections are associated with the expression of placental cytokines such as IL-1 and IL-8. IL-8 has been demonstrated to upregulate the replication of laboratory-adapted HCMV strains in various cells, including the ST. Furthermore, IL-8 could attenuate the antiviral activities of interferon, particularly, type I interferon. These observations would imply that endogeneously produced IL-8 in the placental microenvironment may play a regulatory role in the expression of HCMV.

Aims

The present study was designed to address the following aims:

Aim 1: To investigate the production of proinflammatory cytokines of ST cultures infected with clinical HCMV isolates.

Aim 2: To examine the interrelationship between the level of IL-8 secreted and the replication of HCMV in epithelial cells.

Aim 3: To determine the distribution of the genotypes of the HCMV genes UL55, UL146 and UL139 among HCMV strains, and to investigate the association between polymorphisms within the genes UL55, UL146, and UL139, and the ability to induce IL-8 production.

Materials and Methods

Cytotrophoblasts

A pure population of villous CTs was separated from individual first-trimester (6 to 12 weeks) human placenta villi by sequential trypsinization. As a first step, a cell preparation highly enriched in CT was obtained by using a discontinuous percoll gradient as described by Kliman et al. The purity of the isolated CTs was tested by immunochemical staining with monoclonal mouse antibodies to human cytokeratins (clone: MNF 116; Dako A/S, Glostrup, Denmark), which stain only the trophoblasts within the placental villi, and with monoclonal mouse antibodies to vimentin (clone: V9; Dako A/S, Glostrup, Denmark). The viability of the percoll-enriched cells was estimated by trypan-blue exclusion. The viability was constantly >95%.

At this point the isolated cells were frozen (in cold 10% dimethylsulfoxide in fetal calf serum (FCS) to give 5-10x10^6 cells/ml) and stored in liquid N_2 until needed. When required, the cells were

thawed and subjected to an immunomagnetic separation procedure to remove the remaining contaminating cells. Cells were tested routinely for bacterial infection, including mycoplasma, chlamydia and infection with fungi or HCMV. The cells were treated with mouse monoclonal antibodies against human HLA-ABC antigen, (clone W6/32) and human HLA-DP, DQ, DR antigen (clone CR3/43) (Dako A/S, Glostrup, Denmark), followed by magnetic microspheres coated with goat anti-mouse immunoglobulin (IgG) (Dynal AS, Oslo, Norway). The purity of the CT population was tested by cytokeratin and vimentin staining. The CT preparations from first-trimester placentas used in this study were highly pure (>99.99%).

For the *in vitro* differentiation of CTs (10^6 cells/ml), keratinocyte growth medium (Gibco BRL, Karlruhe, Germany) supplemented with 15% FCS was used. CTs were seeded in 24-well plates containing $5x10^5$ cells/well. Five-day-old ST cultures were used in all experiments.

A549 cell

Cells of the human A549 lung carcinoma cell line were cultivated in Eagle's minimum essential medium (MEM) supplemented with 10% FCS and antibiotics. The cells were grown on glass coverslips in 24-well plates containing $2x10^5$cells/well for cytokine production, immunofluorescence and virus replication studies.

Viruses

Thirty-six HCMV clinical isolates of different origins and 6 laboratory strains were used. The stock of HCMV laboratory strains and clinical isolates were propagated in confluent MRC-5 cells grown in RPMI medium supplemented with 10% FCS and antibiotics. The clinical isolates were passaged <5 times. The infectivity titers were determined by plaque assay, with the inoculation of confluent MRC-5 cultures in 24-well plates.

The clinical samples were obtained in accordance with local ethical guidelines from congenitally infected infants and from immunocompromised and immunocompetent individuals.

Hormone assay

The release of human chorionic gonadotropin (hCG) from the trophoblasts was measured in the culture supernatant daily after seeding in culture. The microparticle enzyme immunoassay kit used for hCG detection (WHO Matched Assay Reagents for Immunoassay of Hormones). The sensitivity of the assay was 2 mIU/ml.

Determination of cytokine production

Five-day-old ST cultures were infected with the laboratory-adapted strain or isolates of HCMV at a MOI of 0.1. The infected ST cultures were centrifuged at 300 x g for 60 min at room temperature and then incubated for 2 h at 37 ºC. The STs were washed 5 times with serum-free medium. After washing, fresh medium was added to the ST cultures.

Twenty-four-hour A549 cell cultures were infected with one or other of the laboratory-adapted strains Towne or AD169 or each isolate of HCMV at an MOI of 0.1. The infected cultures were centrifuged at 750xg for 60 min at room temperature and then incubated for 2 h at 37 ºC. The unabsorbed virus was removed and the cells were washed 5 times with serum-free medium.

Supernatants of virus-infected and mock-infected cultures were collected at different time intervals after infection and assayed for TNF-α, IL-1β, IL-6 and IL-8. Cytokine concentrations were measured in each supernatant by using ELISA kits according to the manufacturer's technical guidelines. All ELISA kits were purchased from Biosource Europe S.A., Nivelles, Belgium. Sensitivity of cytokine assay: the minimum detectable doses of hTNF-α, hIL-1β, hIL-6 and hIL-8 were 1.7, 1, <2 and <5 pg/ml, respectively.

Immunofluorescence assay

After a 48-h incubation, the HCMV-infected and mock-infected cultures were washed twice with cold PBS and fixed with cold acetone:ethanol (1:1) for 20 min at –20 ºC. The fixed cells were

stored at –20 ºC until immunofluorescence assay was performed. The nuclei of the cells were stained with 4',6-diamidino-2-phenylindole (DAPI), and the HCMV IE antigen was detected in the nuclei of cells by immunostaining, using monoclonal antibody (MAB810) (Chemicon International Inc., Temecula, CA, USA) and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Sigma, Budapest, Hungary).

Assay for virus replication in A549 cells

For analysis of the growth kinetics of HCMV strains of different IL-8-inducing abilities in A549 cultures, cells were infected at an MOI of 0.1 with strain 128V, E5 or Towne. During the adsorption period, the plates were first subjected to centrifugation at room temperature for 60 min at 750xg and then incubated at 37 ºC for 2 h.

The inoculum was removed and the cells were washed 5 times with serum-free medium to remove residual input infectivity completely. After washing, the cells were overlayed with MEM with 1% FCS and antibiotics, and were harvested at zero time point. After infection, culture supernatants were collected daily, and made cell-free by centrifugation. Cell-associated virus was collected by 3 freeze-thaw cycles of infected cells in medium. The cell-free and cell-associated virus-containing samples collected at the same time were pooled. The virus titres were determined by plaque assay, with inoculation of confluent MRC-5 cultures in 24-well plates.

Determination of the effect of exogenous IL-8 on IE antigen expression

A549 cells were incubated with various concentrations of recombinant human IL-8 (rhIL-8) (R&D Systems Europe Ltd.) before and after infection with strains of HCMV at an MOI of 0.1. At 48 h after infection, immunofluorescence assays were performed for the detection of IE antigen-producing cells.

DNA preparation

HCMV DNA was extracted by standard methods from cultured cells and urine. Two nucleic acid extraction kits were used for purification of viral DNA from urine. In experiments relating to the determination of the HCMV gB genotype the High Pure Viral Nucleic Acid Kit was used (Boehringer Mannheim GmbH, Indianapolis, IN, USA) according to the manufacturer`s instructions, and the viral DNA was resuspended in nuclease-free distilled water. In experiments involving the genotypic analysis of the HCMV genes UL139 and UL146, the NucleoSpin Tissue Kit (Macherey-Nagel GmbH & Co, Germany) was used according to the manufacturer`s instructions, and the viral DNA was resuspended in Elution Buffer BE (5 mM Tris/Cl, pH 8.5). In addition, DNA was isolated from cultured cells by using the QIAGEN FlexiGene DNA Kit (QIAGEN ltd., UK) following to manufacturer`s instructions, and the DNA was dissolved in hydration buffer (10 mM Tris.Cl, pH 8.5).

Polymerase chain reaction amplification of the gene UL55 and restriction analysis

A region of high peptide variability in the gene UL55 was amplified. To improve the sensitivity and to overcome sequence variation between strains, a nested PCR was used. Primers for amplification were selected from the published UL55 sequences of HCMV strain AD169. Amplification products (293-296 bp, the size varying with the strain) were digested with *Hin*f I and *Rsa* I (Promega, Madison, WI, USA). Digested DNA was analyzed in a 3% agarose gel (Nu Sieve 3:1, FMC Bio Products, Rockland, ME, USA), and visualized with ethidium bromide. Four distinct gB genotypes could be identified via the different lengths of restriction fragments (36-239 bp).

Polymerase chain reaction amplification of UL146 and UL139 genes

UL146 and UL139 were amplified separately by single round or nested PCR, using primers in conserved regions. Single (and first) round PCR of UL146 using AB4 and A162 generated a product of approximately 1 kbp, and second round PCR using UL146-4A and UL146-3A yielded an 800 bp product. Single (and first) round PCR of UL139 using AB1 and AB2 generated an 800 bp product, and nested PCR using UL140-3A and UL140-11A yielded a 500 bp product. UL140-11A is located within

the UL139 coding region, and as a consequence the sequences obtained by using nested PCR (approximately 40% of the total) lacked 29 amino acid-encoding codons from the highly conserved C terminus. Approximately one-third of the samples were tested on three separate occasions to assess reproducibility.

Purification, cloning and sequencing of PCR products

PCR products were separated by agarose gel electrophoresis. Appropriate DNA fragments were excised, purified using a Geneclean turbo kit (Q Biogene, Cambridge, UK), and eluted with 100 μl of nuclease-free water. The single round or second round primers were used for direct sequencing.

In some cases, fragments were cloned by means of a pGEM-T Easy kit (Promega, Southampton, UK). Following ligation and transformation into chemically competent *E. coli* TOP 10 cells, 5 recombinant colonies were picked and grown overnight at 37 ºC in 2YT-broth containing 100 μg/ml ampicillin. Plasmid DNA was purified with a QIAprep Spin miniprep kit (Qiagen, UK). Plasmid inserts were sequenced via universal forward and reverse primers. Sequencing was carried out on both DNA strands, using a BigDye terminator kit (Applied Biosystems, Warrington, UK) in an ABI 3730 instrument.

Sequence analysis

Sequence chromatograms were viewed via Editview (Applied Biosystems, Warrington, UK) and analyzed by using Pregap4 and Gap4. Nucleotide and imputed amino acid sequences were aligned through CLUSTAL W and MAFFT. Full-length sequences were used for the UL146 data and a subset of the UL139 data, and another subset of the UL139 data was analyzed by using sequences lacking the conserved C terminus. MEGA4.0 was used for the generation of phylogenetic trees.

Statistical analysis

The results on the variability in proinflammatory cytokine secretion among ST cultures from three independent experiments are presented as means ± standard deviation (SD). Data on ST cultures infected with HCMV strains were compared with those on mock-infected ST cultures; significant differences were determined by two-way repeated measures analysis of variance (ANOVA) followed by the Bonferroni post test (GraphPad Prism_Home, San Diego, CA, USA). A p value <0.05 was considered significant. The mean number ± SD of nuclei positive for IE antigen per microwell 48 h after infection was calculated from 3independent experiments.

The data on A549 cell cultures infected with HCMV strains were expressed as the mean percentage ± SD of IE antigen-positive cells per 30 microscopic fields. Total cell numbers were determined via the numbers of DAPI-stained nuclei.

Results

1. *Production of proinflammatory cytokines of ST cultures infected with clinical HCMV isolates*

In order to determine the production of proinflammatory cytokines by HCMV-infected STs, STs were differentiated from first-trimester placenta-derived CTs. Three independent experiments were carried out on 3 different placental trophoblast preparations. The ST cultures were infected with HCMV strains or the laboratory-adapted strain Towne. To resemble a natural infection, clinical HCMV isolates and a low MOI were used. There was no difference in hCG release between virus- and mock-infected STs in any of the experiments. Up to 72 h post-infection, TNF-α and IL-1β activities could not be detected in any of the ST culture supernatants, regardless of whether these were mock-infected or infected with any of the HCMV strains. The amount of IL-6 measured in the mock-infected ST cultures was observed to increase in a time-dependent manner. Similar or slightly increased amounts of IL-6 were found in most HCMV-infected ST cultures. One strain (128V) induced the production of a significantly ($p < 0.05$) higher amount of IL-6 as compared with that in the mock-infected cultures. Analyses of the IL-8 levels in the supernatants of the ST cultures revealed that, in contrast with the other strains, strain 128V was a potent IL-8 inducer. The second most potent IL-8-

inducing strain was strain 20. The number of IE antigen-containing cells in ST cultures infected with different HCMV strains seemed to be dependent on the amount of IL-8 produced.

2. *Interrelationship between the level of IL-8 secreted and the replication of HCMV in epithelial cells*

In order to investigate the IL-8-inducing capacities of clinical isolates of HCMV by comparison with the permissiveness in another epithelial cell, A549 cell cultures were infected with HCMV strains of different origins or laboratory-adapted strains at an MOI of 0.1. The interrelationships between the amount of IL-8, the percentage of nuclei positive for HCMV IE antigen and the replication of the virus were examined. The effect of exogenous IL-8 on IE antigen expression was determined.

The amount of IL-8 measured in the mock and virus-infected A549 cultures initially increased in a time-dependent manner, but did not rise further after 48 h. Analysis of the IL-8 levels in the supernatants of the A549 cultures at 48 h after infection revealed that strain 128V was the most potent IL-8 inducer, followed in sequence by strains E5, 20, 74, S and E33.

The percentages of cells positive for IE antigen in the A549 cultures infected with the different HCMV strains seemed to be dependent on the amount of IL-8 produced. A low incidence of infection was seen for the two laboratory-adapted strains (Towne and AD169).

Three HCMV strains with different IL-8-inducing capacities (128V, E5 and Towne) were chosen to investigate the interrelationship between the level of IL-8 secreted and the replication of the virus in A549 cells. The single-step growth curves of the HCMV isolates in A549 cells demonstrated the production of infectious virus. The greater the amount of IL-8 produced in the HCMV-infected A549 culture, the higher the yield of infectious virus.

The effects of exogenous IL-8 on the IE antigen expression of HCMV strains with different IL-8–inducing abilities (128V, E33, and Towne) were examined. Exogenous IL-8 enhanced the number of IE antigen-producing cells at concentrations of from 0.1 to 10 pg of IL-8 per ml, in a dose-dependent manner.

The anti-IL-8 Mab reduced the number of IE antigen-expressing cells nearly to the unstimulated levels (unpublished data).

3. *Distribution of the genotypes of the genes UL55, UL146 and UL139 among HCMV strains, and the association between polymorphisms within the genes UL55, UL146 and UL139 and the ability to induce IL-8 production*

UL55 genotypes

gB genotyping was carried out on 26 samples. A region of high peptide variability in the gene UL55 was amplified by nested PCR. Amplification products were analyzed by electrophoresis and subjected to restriction analysis by using *Hin*f I and *Rsa* I. Four distinct gB genotypes (gB1-gB4) could be identified via the different lengths of restriction fragments (36-239 bp).
One sample contained 2 genotypes (gB1 and gB2). The overall distribution of the 27 genotypes was as follows: 19 gB1, 3 gB2, 4 gB3, and 1 gB4.

UL146 and UL139 sequences

The UL146 and UL139 genotypes in 36 samples were investigated by PCR and sequencing using primers in conserved regions. UL146 was amplified from 36 samples and sequences were determined from 20, and UL139 was amplified from 36 samples and sequences determined from 26.

The relevant genes were amplified by PCR, using validated primer sets. In some cases, nested PCR was used. Most products were sequenced directly, and some were cloned into plasmids and then sequenced. For reference sequences, we included previously analyzed strains: Toledo, Towne, TB40/E, Davis and Merlin. The genotye system of Dolan et al. (2004) was used for UL146 and for UL139. The UL146 coding sequences range in length from 342 to 378 bp (114-126 codons). All fall into 14 genotypes and are designated G1-G14. The UL139 coding sequences range in length from 372 to 444 bp (124-148 codons). All fall into 8 genotypes and are designated G1-G8.

The total number of strains for which genotype data were obtained was 31, including 7 mother-baby pairs.

UL146 genotypes

Nine UL146 genotypes (G1-2, and G7-13) were apparent in our samples. Seven different UL146 genotypes (G7-13) were detected in strains from congenital infections.

UL139 genotypes

Seven UL139 genotypes (G1-7) were found in congenitally infected infants.
Isolate E24 is assigned to 2 genotypes (G4 and G7). The DNA sequence of genotypes UL146 (G11) and UL139 (G2) in an infant did not change in the 17 months during which samples (16/3 and 16/5) were obtained.

The analysis to determine whether an association exists between the UL55, UL146 and UL139 genotypes and the ability to induce IL-8 production in STs and A549 cells was underpowered, because of the unexpectedly high-frequency variation in the genes UL146 and UL139.

Discussion

1. *Production of proinflammatory cytokines of ST cultures infected with clinical HCMV isolates*

Numerous investigations have indicated that placental cells, including STs, can be productively infected by HCMV. Experiments have been performed *in vitro* by using laboratory-adapted strains and a high MOI (10 or 1), which may not be entirely representative of the behavior of wild strains. In the placenta, proinflammatory cytokines, including IL-1β, IL-6, IL-8 and TNF-α, and anti-inflammatory cytokines such as IL-4 and IL-10, are produced by the trophoblasts. Intrauterine infections are associated with the expression of placental cytokines such as IL-1β and IL-8. IL-8 has been demonstrated to upregulate the replication of laboratory-adapted HCMV strains in various cells, including the ST. The local cytokine environment may modulate the transplacental transmission of HCMV.

To resemble a natural infection of STs, in our experiments clinical HCMV isolates and a low MOI were used. STs were differentiated from first-trimester placenta-derived CTs. We found that the IL-8-inducing capacities of the HCMV strains differed in ST cultures, the IE gene expression of the virus increasing with the amount of IL-8 produced. This observation indicates that IL-8 may be involved in the materno-fetal transmission of HCMV. The presence of this cytokine in the placental microenvironment may play a regulatory role, potentially promoting the progression of latent HCMV infection in the placenta into a fulminating infection that can be transmitted to the fetus.
Our findings suggest that certain HCMV strains induce a high level of IL-8 in STs, which in turn enhances productive HCMV expression in the placenta, while others replicate if the IL-8 is provided by co-infection agents, i.e. human immunodeficiency virus (HIV), human herpesvirus-6 or bacteria. The potential of various bacteria to stimulate cytokine production in highly purified primary trophoblast cultures varies. *E. coli* and *B. fragilis* exhibited the highest potencies to stimulate IL-8 expression.

The fact that the IL-6-mediated stimulation of trophoblast cells did not result in an enhancement of IL-8 production suggests that particular genotypes of the target HCMV genes may be associated with IL-8 induction in physiologically relevant STs. In recent years, it has become clear that many HCMV genes are strikingly variable in sequence between strains, with at least 25 of the 165 genes present in wild-type virus strains being hypervariable. The anticipated association between pathogenic properties and genotypes is a subject of importance, but has yet to find conclusive experimental support. We intend to focus on several hypervariable genes whose genotypic structures are well characterized and which encode products with potentially relevant functions. The key target is UL146, which encodes a potent CXC chemokine similar to IL-8.

2. *Interrelationship between the level of IL-8 secreted and the replication of HCMV in epithelial cells*

HCMV displays a broad host cell range and infects different cell types *in vivo*. Epithelial cells are major targets of permissive infection by HCMV and play a role in the spread of the virus in infected tissues during acute infection. Little is known about the viral factors that determine HCMV epithelial cell tropism. Most analyses of HCMV epithelial cell tropism have been performed with the use of laboratory-adapted strains and a high MOI. Potential differences between HCMV strains were not taken into consideration.

A549 epithelial cells were infected with HCMV isolates of different origins with a view to acquiring support for our earlier suggestion that interstrain differences in HCMV epithelial cell tropism depend on the IL-8-inducing capacity and determine the outcome of HCMV infection. The data revealed that the IL-8-inducing capacities of the various HCMV isolates differed in A549 cells too. The IE gene expression and the yield of infectious virus were dependent on the amount of IL-8 produced in these cells.

IL-8 has been demonstrated to upregulate the replication of laboratory-adapted HCMV strains in various cells. According to our results, exogenous IL-8 increases the number of IE antigen-producing cells, in a dose-dependent manner in A549 cells infected with HCMV strains of different IL-8-inducing abilities. The presence of this cytokine in the microenvironment may promote the progression of HCMV infection.

Our findings support our earlier suggestion that certain HCMV strains induce a high level of IL-8 in epithelial cells, which in turn enhances productive HCMV infection in these cells. Other strains can replicate in epithelial cells if IL-8 is provided by coinfecting agents.

3. Distribution of the genotypes of the genes UL55, UL146 and UL139 among HCMV strains, and the association between polymorphisms within the genes UL55, UL146 and UL139 and the ability to induce IL-8 production

We focused on hypervariable genes whose genotypic structure has been well characterized and which encode products with potentially relevant functions. The primary targets were UL146, which encodes a potent CXC chemokine similar to IL-8, and UL139, which maps about 5 kbp distant and potentially encodes a highly O-glycosylated membrane protein. Genetic variability in the gB gene was also investigated. gB is an important target for neutralizing antibodies and participates in cell-receptor interactions. With the same approach, the genotypes of several paired HCMV samples from mothers and babies were also determined.

Evidence for a genetic linkage between the three loci was unconvincing, consistent with a role for interstrain recombination during HCMV evolution. The fact that the genotyping of one gene does not shed light on the genotypes of other genes justifies the need to determine the genotypes separately for each target locus. The DNA sequences of genotypes in an infant did not change in the period during which samples were obtained (16/3 and 16/5), as was observed in persistently infected renal transplant recipients.

The number of strains whose ability to induce IL-8 production in STs and A549 cells has been examined is limited, and only one strain (128V) induces high levels. However, there appears to be no obvious correlation between the IL-8-inducing ability and the genotype of UL55 (compare strain 128V with strains 74 and E5) or UL139 (compare strain 128V with strains 20 and E5). A correlation with UL146 remains possible. The analysis of additional strains is required, especially those able to induce high levels of IL-8 or those known to have a genotype G9 gene UL146. Data for other loci (for which data collection is ongoing) will broaden the analysis.

Our results confirmed that HCMV replication in epithelial cells is strain-dependent, suggesting that specific viral gene(s), including UL146, which encodes a potent CXC chemokine similar to IL-8, are required for efficient replication in this cell type.

A better understanding of the molecular basis of the interactions between HCMV isolates and epithelial cells, including trophoblasts, may yield novel strategies with which to prevent the progression of the virus. IL-8 may be an important new target for the control of HCMV replication.

Summary

Forty per cent of women with primary HCMV infection during gestation transmit the infection to their fetuses, which may result in abnormalities for the newborn, varying in degree from mild to severe. Since placental HCMV infection has been detected both in the presence and in the absence of fetal infection, the placenta should be considered the most important site of either protection of the fetus from HCMV infection or transmission, by acting as a viral reservoir and allowing the infection to reach the fetal compartment.

The factors whereby HCMV in the placenta develops into a fulminating infection and spreads to the fetus are not known.

This study concerned three main features:

1. The production of proinflammatory cytokines was investigated in ST cultures infected with HCMV strains. The interrelationships between the cytokines produced in the ST cultures and the number of nuclei of ST expressing the HCMV IE gene were examined. To resemble a natural infection, clinical HCMV isolates and a low MOI were used.

TNF-α and IL-1β were not detected in the supernatants of any ST cultures. Similar or increased amounts of IL-6 were found in the HCMV-infected cultures. The IL-8-inducing capacities of the HCMV strains differed in the ST cultures. The IE gene expression of the virus provided was dependent on the amount of IL-8 produced in the STs.

Our observations indicate that IL-8 in the placental microenvironment may play a regulatory role, potentially promoting the progression of HCMV infection in the placenta into a fulminating infection that can be transmitted to the fetus. Certain HCMV strains induce high amounts of IL-8, which in turn enhances HCMV replication in the placenta, while others can replicate if the IL-8 is provided by a co-infecting agent.

2. The interrelationship between the level of IL-8 secreted and the replication of HCMV was examined in another epithelial cell type, A459 cell.

The data revealed that the IL-8-inducing ability of the various HCMV isolates differed in A549 cells too. The IE gene expression and the yield of infectious virus were dependent on the amount of IL-8 produced in these cells. Exogenous IL-8 increased the number of IE antigen-producing cells, in a dose-dependent manner, in A549 cells infected with HCMV strains with different IL-8-inducing abilities.

Our results showed, that HCMV replication in epithelial cells is strain-dependent, suggesting that specific viral gene(s), including UL146, which encodes a potent CXC chemokine similar to IL-8, are required for efficient replication in this cell type.

3. Genotyping was carried out to elucidate whether there is any link between the ability of HCMV isolates to induce IL-8 production in STs and in other epithelial cell type, A549 cell. We focused on hypervariable genes (UL55, UL146 and UL139), whose genotype structure has been well characterized, and which encode products with potentially relevant functions.

The total number of strains for which genotype data were obtained was 31, including 7 mother-baby pairs. Four gB genotypes (gB1-gB4), 9 UL146 genotypes (G1-2 and G7-13), and 7 UL139 genotypes (G1-7) were to be found in our samples.

The number of strains whose ability to induce IL-8 production in STs and A549 cells has been examined is limited. However, there appears to be no obvious correlation between the IL-8-inducing ability and the genotype of UL55 or UL139. A correlation with UL146 remains possible. The analysis of additional strains is required, especially those able to induce high levels of IL-8 or those known to have the genotype G9 gene UL146.

A better understanding of the molecular basis of the interactions between HCMV isolates and trophoblasts may yield novel strategies with which to prevent the vertical transmission of the virus. IL-8 may be an important new target for the control of HCMV replication.

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