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Abbreviations

AIDS	acquired immunodeficiency syndrome
AV	anchoring villus
BSA	bovine serum albumin
CT	cytotrophoblast
DAPI	4`,6-diamino-2-phenylindole
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
E	early
FcRc	neonatal Fc receptor
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FV	floating villus
gB	glycoprotein B
HBSS	Hanks` Balanced Salt Solution
HEPES	hydroxyethylpiperazine-N'-2-ethanesulfonic acid
hCG	human chorionic gonadotropin
HCMV	human cytomegalovirus
HIV	human immunodeficieny virus
IL	Interleukin
IE	immediate early
IgG	immunoglobulin
L	late
Mab	monoclonal antibody
M/DC	macrophage/dendritic cell
MOI	multiplicity of infection
NK	natural killer cell
ORFs	open reading frames
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PFU	plaque forming unit
PMN	polymorphonuclear leukocyte
ST	syncytiotrophoblast

Introduction

Human Cytomegalovirus

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 (Figure 1). It has been classified as a betaherpesvirus on the basis of several biochemical criteria such as the genome size, guanosine and cytosine contents, the slow replicative cycle, and the restricted *in vitro* and *in vivo* tropism [1]. 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 fibroblast. All known strains of HCMV are genetically homologous, but none seem to be genetically identical, unless they are obtained from epidemiologically related cases [2, 3]. 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 [4]. The massive HCMV genome is approximately 50% larger than the genome of herpes simplex.

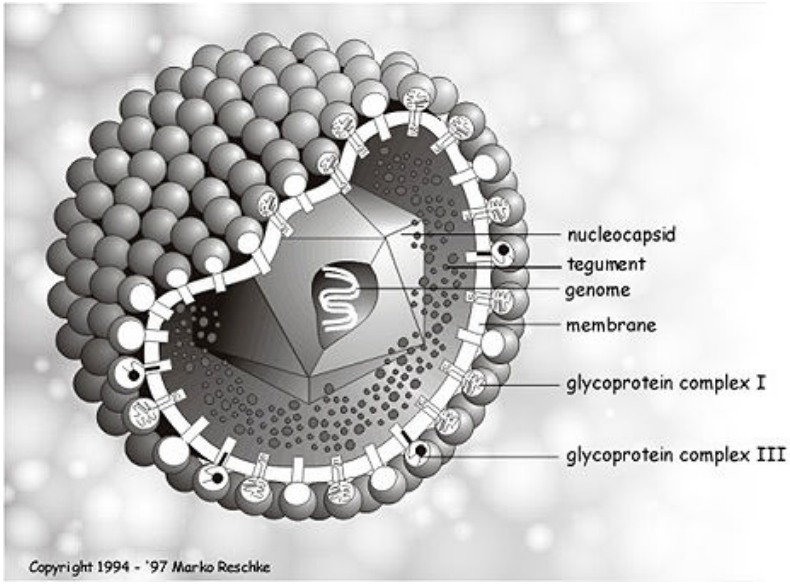

Figure 1.: Human Cytomegalovirus morphology. http://www.virology.net/Big_Virology/BVDNAherpes.html

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 (Figure 1). 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 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 [1]. The lipid-containing envelope is composed of virion glycoproteins and host-derived membrane lipids (Figure 1). The envelope of HCMV is thought to play an essential role in the initial steps of virus-host cell interactions and host immune responses against virion componenst are thought to be important in protective immunity. 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 [5]. 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 [6]. The complete DNA sequence of AD169 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 (Figure 2) [7]. 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 (ORFs), 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 [4, 8-10]. Most genes are highly conserved in sequence between HCMV strains, but some are characterized by a striking degree of variability, as revealed by examination of individual genes [4] and by whole genome comparisons [7, 11]. These variable genes are generally predicted to encode membrane-associated or secreted proteins [7]. 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 [12-14]. 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 (Figure 2). The UL146 gene encodes a chemokine, designated vCXC-1, that induces calcium mobilization, chemotaxis and 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 interleukin-8 (IL-8). As the first example of a virus-encoded a 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 [15]. The most variable gene in the vicinity of UL146 is UL139, which is predicted to encode a type I membrane glycoprotein. Variability is concentrated in a region of the ectodomain [7].

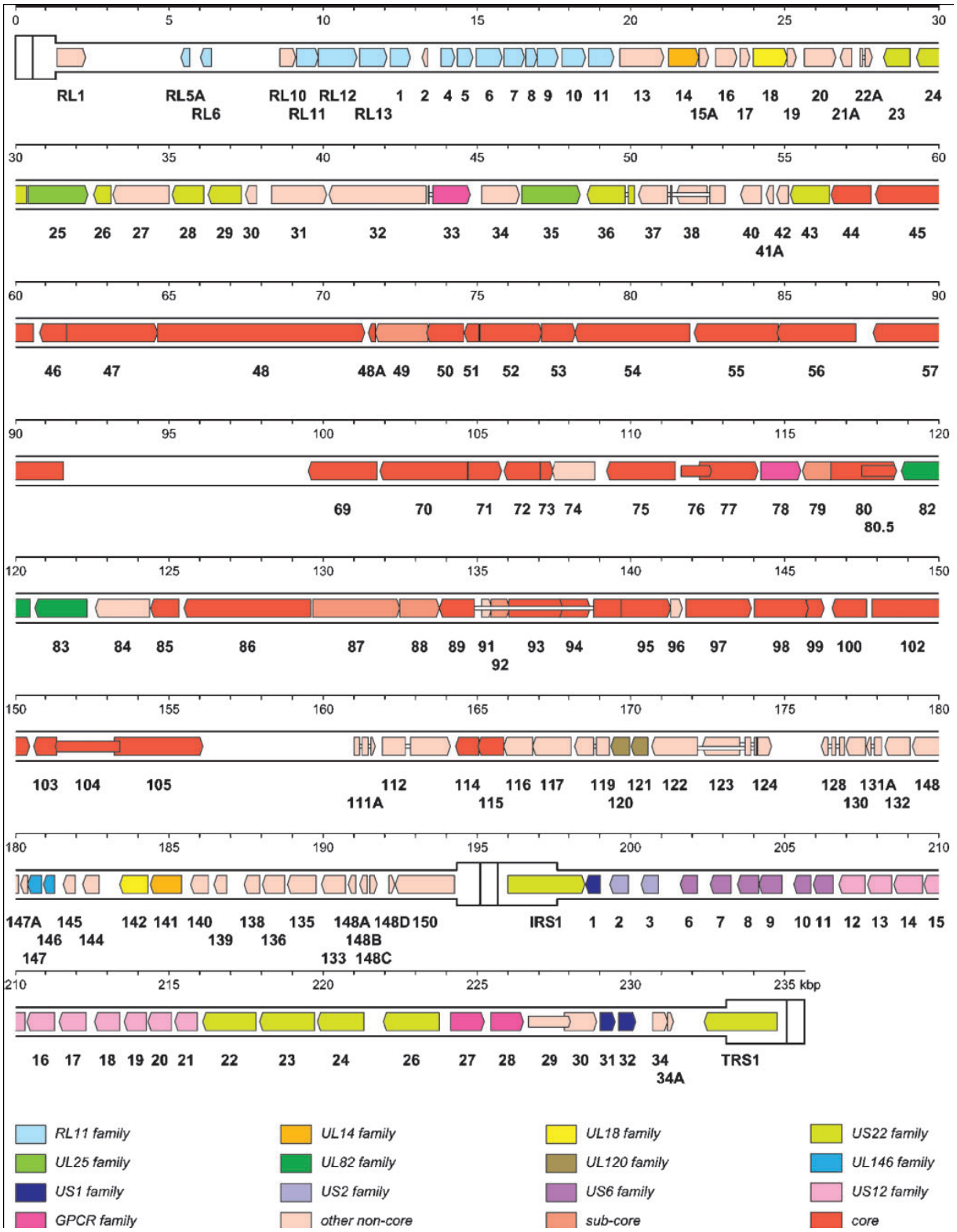

Figure 2.: Consensus genetic map of wild-type HCMV based on the Merlin genome [7].

Epidemiology of HCMV infection

Distribution and transmission

HCMV infections are endemic and has no seasonal variation. Studies on the age-related prevalence of infection with HCMV suggest that there may be three periods with particularly high rates of acquisition of the virus: early childhood, adolescence and the childbearing years. The prevalence of HCMV infection in the normal population varies widely, between 40% and 90%, depending on the race, the gender, the age, hygienic circumstances and the socioeconomic factors [16]. In general, HCMV is more prevalent among people in low socioeconomic brackets living in crowded conditions and in people living in undeveloped countries, with 80% of 3-year-old children and most adults in such groups being infected with HCMV [16-18]. In Hungary, the seroprevalence of HCMV at the age of 10 years is 72%, which increases to 96% by the age of 50 [19]. The level of immunity among women of childbearing age, which is an important factor in determining the incidence and significance of congenital and perinatal HCMV infections, varies widely among different populations. The prospective studies of pregnant women in the United States indicate that the rate of HCMV acquisition for childbearing-aged women of middle to higher socioeconomic background is approximately 2% per year, whereas it is 6 % per year among women of lower socioeconomic background [20-22].

Infection with HCMV can be classified as either primary or recurrent. Similar to other herpesvirus infections, primary HCMV infection is followed by persistent and/or recurrent infections, most often due to reactivation of a latent infection, residing in the host throughout life. It is reactivated periodically during episodes of mild immunosuppression caused by intercurrent infection, pregnancy or stress. Recurrent infections are fairly common [1]. Intermittent excretion of the virus can be anticipated in a significant proportion of seropositive adults. Reinfection by a new strain of HCMV has been documented in immunocompromised individuals, women attending a clinic for sexually transmitted diseases and healthy children attending a day-care center [23-26].

HCMV rarely causes symptoms in an immunocompetent host, and these can be nonspecific symptoms, such as malaise, fever, sweats, aching muscles, atypical lymphocytosis and mild hepatitis during the self-limiting primary infection [27]. However, it can give rise to serious disease in an immunodeficient person, such as those with acquired immunodeficiency syndrome (AIDS) [28] and those with organ and bone marrow transplants

[29], thereby causing severe morbidity and eventually mortality, or neonates, and especially premature babies [1]. Reactivation is asymptomatic, except in severely immunocompromised individuals [28, 29].

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 urine, oropharyngeal, cervical and vaginal secretions, breast milk, semen and tears, and can be shed intermittently for years. Spread of HCMV also occurs frequently in child care centers. Cohort studies indicate that children in day care centers frequently transmit virus to each other. It is reasonable to expect that approximately 50% of susceptible children between the ages of 1 and 3 years who attend group day care will aquire HCMV from their playmates and become an important potential source of infection for serosusceptible child-care personnel and parents, particulary women of childbearing age [20]. Fomites may also play role in transmission because HCMV has been shown to retain infectivity for hours on plastic surfaces and has been isolated from randomly selected toys and surfaces in day-care center [22].

HCMV can also be transmitted vertically, from mother to fetus by three routes: transplacental, intrapartum and human milk [21]. The materno-fetal transmission of the HCMV is the leading cause of congenital viral infection, affecting 0.3-2% of newborns in developed countries, and the transmission could well be higher in developing countries [30]. The prevalence displays a specific geographical pattern: it is 0.9% in Hungary [1, 31]. The individual course of the infection may vary between asymptomatic virus shedding, abortion or stillbirth, and a congenital syndrome. Between 10% and 15% of infants infected with congenital HCMV exhibit the clinically apparent or symptomatic form of the disease, characterized by petechiae, hepatomegaly, splenomegaly, jaundice, periventricular calcifications, microcephaly, hearing impairment and chorioretinitis. The remaining 85% to 90% of infected infants are asymptomatic at birth, but 15% of them will develop delayed sequelae, especially progressive hearing loss [32].

The natural history of HCMV during pregnancy is particulary complex and has not been fully explained.

Intrapartum transmission of HCMV is related to local shedding of virus. Approximately 10% of woman shed HCMV from the vagina or cervix; near the time of delivery, rates of 2% to 28% have been reported [21]. If the virus is present in the maternal genital tract at the time of delivery, the rate of transmission to newborn is around 50%.

The most common route for mother to infant transmission of HCMV is human milk [1]. The virus was isolated from human milk more than 30 years ago, but the roles of milk cells and cell-free virus in transmission have remained unclear. More recent studies using polimerase chain reaction (PCR) to detect HCMV DNA in milk from seropositive mothers reported a strong relationship between the presence of viral DNA in milk and transmission of HCMV to the infant. Breastfeeding significantly influences the epidemiology of HCMV infection [33].

HCMV infection in pregnancy

The *in utero* transmission of HCMV can occur as a consequence of primary and recurrent infections, with equal frequency during all three trimesters [34]. 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 to the high rate of transmission in primary infection, that during recurrent infection is much lower (1-1.2%) [35-40]. Primary infection occurring 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. Anatomical considerations argue that *in utero* infection involves the passage of virus or virus-infected cells across the placenta into the fetal compartment [41-43].

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 [41, 42]. 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) [42]. 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 to the passage of microbial pathogens and/or maternal cells into the fetal compartment [41]. An additional possibility is that the virus may ascend from the

vagina via the ruptured membranes, to reach the decidua or amniotic cells [44]. 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 [35]. 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 [45-47]. Later in pregnancy, HCMV infection causes premature delivery and (in 25% of affected infants) intrauterine growth retardation, outcomes that are often associated with placental pathology [48]. 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 [47, 49].

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 [50].

Placenta

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 (Figure 3). 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 [51].

Three major trophoblast populations can be identified during placentation: CT stem cells and two differentiated derivate cell types: ST and extravillous CT [52]. The undifferentiated trophoblastic stem cell of the placenta, the CT, is the first fetal cell type arising during embryogenesis. It derives from external trophectodermal cell layer of the blastocyst, thus it is extraembryonic in origin. It represents the earliest epithelium and forms a variety of different structures e.g., placental villi and fetal membranes [53]. Just as undifferentiated basal layer of the skin gives rise to differentiated keratinocytes, the CT stem cell of the placenta undergoes multistep differentiation and finally gives rise to villous (non-invasive) and extravillous (invasive) trophoblast cell populations [54, 55].

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 (Figure 3). 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 [56, 57]. 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 in maximally dilated inert tubes. This process is termed endovascular invasion [53, 58-62]. This unusual invasive behavior has two important roles: (1) CT invasion phisically anchors the placenta to the uterus, and (2) by replacing the endothelial lining of the spiral arteries invasive extravillous CTs create the large-diameter, low-resistance vessels that carry blood to the floating villi at the material-fetal interface.

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 [63]. The virus might disseminate from infected maternal blood cells to the decidua (Figure 3, site 1), interstitial and endovascular CTs in the uterine wall (site 2), CT columns of anchoring villi (site 3), and/or floating villi (site 4). An endothelial cell-tropic HCMV strain replicates in uterine microvascular endothelial cells and spreads to invasive CTs *in vitro* [64], suggesting that hematogenous transmission occurs *in utero*.

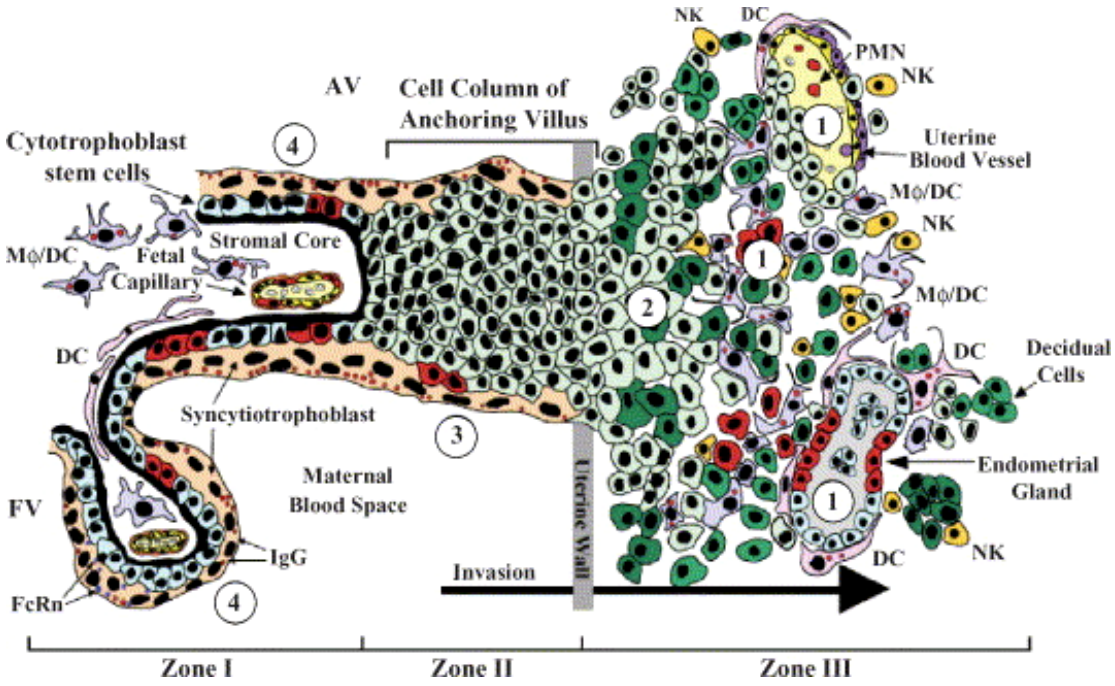

Figure 3: Diagram of the placental (fetal)-decidual (uterine) interface near the end of the first trimester of human pregnancy (10 weeks of gestational age). Sites proposed as routes of HCMV infection at the maternal–fetal interface are numbered 1–4; FcRn, neonatal Fc receptor; AV, anchoring villus; FV, floating villus; DC, dendritic cell; PMN, polymorphonuclear leukocyte; NK, natural killer cell; M/DC, macrophage/dendritic cell [51].

Numerous investigations have indicated that placental cells can be productively infected by HCMV [42, 64-67]. 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 [68]. Placental macrophages and trophoblastic cells are known to constitutively secrete a variety of cytokines and prostaglandins, which play crucial roles in normal reproductive processes [69]. 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 [70-74]. Intrauterine infections are associated with the expression of placental cytokines such as IL-1 and IL-8 [71]. IL-8 has been demonstrated to upregulate the replication of laboratory-adapted HCMV strains in various cells, including the ST [75-77]. 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, and by this mechanism latent virus in the placenta develops into a fulminating infection.

Griesinger et al. evaluated the influence of bacteria that have been identified in uterine infections on cytokine expression of highly purified primary trophoblast cultures [78]. Their results indicated that the potential of various bacteria to stimulate cytokine production in these cells varies. *E. coli* and *B. fragilis* exhibited the highest potencies to stimulate IL-8 expression.

Aims

The present study was designated 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

Isolation of villous cytotrophoblasts

A pure population of villous CTs was separated from individual first-trimester (6 to 12 weeks) human placenta villi and differentiated as described previously [79]. Briefly, trophoblastic tissue was obtained from legal terminations of pregnancies performed in accordance with the Hungarian Abortion Law between 6 and 12 weeks of gestation. Ethical approval for this study was granted by the Human Investigation Review Board of the University of Szeged. Villous tissue from individual placentas was dissected manually rinsed and minced in Hanks' Balanced Salt Solution (HBSS) (pH=7.4) containing 200 U/ml penicillin and 200 μg/ml streptomycin (both from Sigma, Budapest, Hungary). The minced tissue was then incubated at 37°C four times for 20 minutes in HBSS containing 0.25% trypsin (Sigma, Budapest, Hungary), 50 U/ml DNase I (Sigma), 4.2 mM magnesium sulfate (Sigma), 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (Sigma) and antibiotics (200 U/ml penicillin and 200 μg/ml streptomycin). The supernatant containing the dissociated mixed placental cells was collected, and the trypsin activity was neutralized by addition of 10% Foetal Calf Serum (FCS) (Gibco, Life Technologies, Vienna, Austria). The neutralized supernatant was centrifuged at 800×g for 10 minutes and the resulting cell pellet was resuspended in Dulbecco's Modified Eagle Medium (DMEM) containing 25 mM HEPES, 200 μg/ml streptomycin. The cell suspension obtained was placed immediately into an incubator and was maintained at 37°C until the end of the entire dissociation procedure. Concomitantly, the remaining villous tissue was subjected to another 20 min trypsinization step. At the end of the dissociation procedure the remaining villous fragments were discarded. The four fractions of cell suspensions were pooled, filtered over a 100 μm nylon mesh to remove remaining villous fragments, centrifuged at 800×g for 10 minutes, and resuspended in 2-3 ml of the same medium without FCS. This cell suspension was layered over a 5% to 70% preformed discontinuous percoll gradient, according to method of Kliman et al [80]. The fraction containing the CTs was washed and resuspended in DMEM. 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 [81] 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 (5-10x10^6 cells/ml) and stored in liquid N_2 until needed. The percoll-purified cells were tested routinely for bacterial infection, including mycoplasma, chlamydia and infection with fungi or HCMV. When required, the cells were thawed and subjected to an immunomagnetic separation procedure to remove the remaining contaminating cells [82].

Storage of cells in liquid nitrogen

The percoll-purified cells were suspended in cold FCS containing 10% dimetyl sulfoxide (DMSO) (Sigma) to give 5-10×10^6 cells/ml. One ml aliquots of this cell suspension in 1.8 ml criogenic vials (Corning Inc., N.Y., USA) were placed in insulated cardboard containers and kept at -70°C overnight. Next day the tubes were rapidly transferred to liquid nitrogen for long term storage. When required, a tube was removed from liquid nitrogen and thawed in a water bath at 37°C. The thawed cells were diluted 1/10 in DMEM containing 20% FCS and centrifuged at 400×g for 5 minutes. The cell pellet was resuspended in fresh culture medium to required cell density.

Purification of cytotrophoblasts

Cells were incubated for 30 minutes at 4°C with 50 µl of a monoclonal anti-HLA-ABC (clone W6/32) and 50 µl of monoclonal anti- HLA- DP, DQ, DR (clone CR3/43; both from Dako A/S). All the primery antibodies were raised in mice and added undiluted to the cell suspension. After incubation, the cells were washed with phosphate buffered saline (PBS) (pH 7.4) containing 0.1% bovie serum albumin (BSA) (radioimmunoassay grade, Sigma). Prior to separation, 100 µl of magnetic particles coated with goat anti-mouse immunoglobulin (IgG) (Dynal AS, Oslo, Norway) were washed twice with 5 ml PBS containing 0.1% BSA and stored at 4°C until used. The cell suspension was then incubated with the prewashed magnetic particles at 4°C for 30 minutes with occasional gentle shaking. At the end of the incubation the tube was clamped to a magnetic concentrator (Dynal Laboratories, Oslo, Norway) for 5 minutes. The supernatant containing the purified CTs was removed from the tube and centrifuged at 800×g for 10 minutes at room temperature. The pelleted cells were resuspended in culture medium and plated as required.

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).

Differentiation of cytotrophoblasts

For the *in vitro* differentiation of CTs, Keratinocyte-SFM (Gibco BRL, Karlruhe, Germany) supplemented with 15% FCS was used. CTs were seeded in 24-well plates containing $5x10^5$ cells/well. The epidermal growth factor present in the Keratinocyte-SFM promotes the differentiation of villous CTs from first-trimester chorionic villi to form STs [81]. The media were changed every 48 h. All experiments were carried out on 5-day-old ST cultures.

A549 cells

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

HCMV clinical isolates of different origins and laboratory strains Davis (ATCC #VR-807), Toledo (ATCC), Towne (ATCC #VR-977), Merlin (ATCC #VR-1590), TB40/E (ATCC) and AD169 (ATCC #VR-538) (American Type Culture Collection) were used. (Table I.). Most strains were isolated in our laboratory from the urine of congenitally infected neonates and mothers, and strains 5DV and E5 from the urine of adults. Strains S and B were isolated by Dr. J. Schirm (Regional Health Laboratory, Groningen, The Netherlands) from the urine of neonates. Strains 20 and 74 originated from the blood of renal transplant recipients, strains 83 and 89 from congenital infection and were provided by Professor C. Bruggeman (University Hospital Maastrich, The Netherlands). The clinical isolates were passaged <5 times. The stock of HCMV laboratory strain Towne and clinical isolates were propagated in confluent MRC-5 cells grown in RPMI medium supplemented with 10% FCS and antibiotics. 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.

Table I. HCMV isolates of different origins and laboratory-adapted strains

Strain[a]	Clinical source[b]	Age/sex[c]	Clinical details[d]
16/5	U/P	Infant	C
128V	U/P	Infant	C
E24	U/P	Infant	C
16/3	U/P	Infant	C
E34AV	U/P	Mother	C+
5DV	U/P	Adult	CH
E5	U/P	Adult	C+
E33AV	U	Mother	C+
E33BV	U/P	Infant	C
E36BV2	U	Infant	C
E36AV1	U	Mother	C+
E41BV3	U	Infant	C
E41AV1	U	Mother	C+
E42BV1	U	Infant	C
E42AV1	U	Mother	C+
E43BV1	U	Infant	C
E43AV1	U	Mother	C
E62BV1	U	Infant	C
E62AV1	U	Mother	C+
E45BV1	U	Infant	C
E59BV1	U	Infant	C
E28BV1	U	Infant	C
16/2	U	Infant	C
E34B/BV	U	5m/M	C+
E60BV1	U	Infant	C+
E55BV1	U	Infant	C+
E63BV1	U	10 w/F	C+
E63AV1	U	Mother	C+
E49AV1	U	Mother	C+
E52BV1	U	14 m/M	C+
20	B/P	Adult	R
74	B/P	Adult	R
S	U/P	Infant	C
B	U/P	Infant	C
83	U	Infant	C
89	U	Infant	C
Davis	BP/P	Infant	C
Toledo	U/P	Infant	C
Towne	U/P	Infant	C
Merlin	U/P	Infant	C
TB40/E	TS/P	Adult	B
AD169	BP/P	Infant	C+

[a] Virus strains; [b] U, urine; BP, biopsy; B, blood; TS, throat swab; P, passaged in cell culture; [C] Ages are in weeks (w) or months (m). Sexes: M, male; F, female; [d] C+, HCMV positive by PCR or IgM in serum; C, congenital; CH, chronic hemodialysis patient; R, renal transplant recipient; B bone marrow transplant recipient;

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 Towne strain or isolates of HCMV (16/3, 128V and 20) at a MOI of 0.1. The MOI was calculated from the number of CTs seeded for syncytium formation. The infected ST cultures were centrifuged at 300xg for 60 min at room temperature and then incubated for 2 h at 37 ºC. The STs were washed five 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 in a Heraeus Megafuge 1.0 (Osterode, Germany) at room temperature and then incubated for 2 h at 37 ºC. The unabsorbed virus was removed and the cells were washed five 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 –20ºC. The fixed cells were stored at –20 ºC until the 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). Nuclei positive for IE antigen were counted under Leitz UV microscope.

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 in a Heraeus Megafuge 1.0 (Osterode, Germany) 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 titers were determined by plaque assay, with inoculation of confluent MRC-5 cultures in 24-well plates. All assays were performed in triplicate.

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

A549 cells were incubated with different concentrations of recombinant human IL-8 (rhIL-8) (R&D Systems Europe Ltd.) for 1 h at 37 ºC. After the removal of the medium, the cells were infected with various strains of HCMV at an MOI of 0.1. During the adsorption period, the plates were first subjected to centrifugation at room temperature for 60 min at 750xg in a Heraeus Megafuge 1.0 (Osterode, Germany) and then incubated at 37 ºC for 2 h. The inoculum was removed, the cells were washed five times with serum-free medium to remove residual input infectivity completely, and were overlayed with MEM with 1% FCS and antibiotics in the presence or absence of the various concentrations of IL-8. After 48 h incubation, the cultures were washed twice with cold PBS and fixed with cold acetone:ethanol (1:1) for 20 min at –20ºC. The fixed cells were stored at –20 ºC until 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 UL55 gene 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 as previously described [83]. Primers for amplification were selected from the published UL55 sequences of HCMV strain AD169 [6]. Amplification products (293-296 bp, the size varying with the strain) were analyzed by electrophoresis in a 2% agarose gel (BRL, Gaithersburg, Germany) stained with 5µl of 10 mg/ml ethidium bromide (Serva Feinbiochemica, Heidelberg, Germany) and subjected to restriction analysis by using Hinf I and Rsa I (Promega, Madison, WI, USA) as previously described [6]. Digested DNA was analyzed in a 3% agarose gel (Nu Sieve 3:1, FMC Bio Products, Rockland, ME, USA). Four distinct gB genotypes can be identified by the different lengths of restriction fragments (36-239 bp) [6].

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 (Table II). 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 using nested PCR (approximately 40% of the total) lacked 29 amino acid-encoding codons from the highly conserved C terminus. For the single (and first) round, 1 μl of DNA was added to the PCR reaction mixture, which consisted of 40 μl of water, 5 μl of buffer, 1 μl of 10 μM dNTPs, 1 μl of each the two primers (10 μM) and 1 μl (1 U) of DNA polymerase (Advantage 2, BD Clontech, Basingstoke, UK). The conditions for amplification were 95°C for 2 min followed by 35 cycles of 95°C for 2 minutes, 60°C for 30 sec and 68°C for 1 min. Second round PCR utilized 1μl of first round PCR products as template amplified under the same conditions. PCR reactions were set up in a dedicated, PCR product-free room. 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, including those where direct sequencing indicated the presence of more than one genotype of UL146 or UL139, fragments were cloned using a pGEM-T 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 using 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 using Pregap4 and Gap4 [84]. Nucleotide and imputed amino acid sequences were aligned using CLUSTAL W [85] and MAFFT [86]. 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 [87] was used for the generation of phylogenetic trees.

Table II. Primers Used for PCR and Sequencing of UL139 and UL146 genes

Gene	Primer	Sequence (5'-3')	Genome location[a]
UL146	AB4	TAGACACTACGTCGTAAATG	180494-180513
UL146	A162	TGTAGAATTAGTCTAGATTCCTGA	181524-181501
UL146	UL146-4A	GCTTGCGCGTTAGGATTGAGACAC	180571-180594
UL146	UL146-3A	ATACCGGATATTACGAATT	181341-181323
UL139	AB1	GTCATTGTGAAAGTGACGTCTCAG	186389-186412
UL139	AB2	ATCTACTGTAAACCCTCTGCTCTG	187148-187125
UL139	UL140-11A	GCGGCATTGGTGTACGCGTG	186553-186572
UL139	UL140-3A	GTGGAAATTTTTACGTCATT	187077-187058

[a] With reference to RefSeq accession NC_006273 (HCMV strain Merlin).

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 3 independent 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 [Figure 4A]. 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 (Figure 4A). 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 (Figure 4B). 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 (Table III).

Table III. Interrelationship between production of IL-8 and IE antigen in rensponse to infection with different HCMV strains in ST cells

HCMV strain	IL-8 (pg/ml)[a]	No. of nuclei positive for IE antigen±SD[b]
-	3.1±1.5	-
Towne	28.2±7.6	9.0±3.0
16/3	21.5±8.1	12.0±2.0
128V	150.4±15.5	323±45.0
20	59.3±2.1	46.0±8.0

[a] Values are means ± SD of the results of three independent experiments.
[b] Mean number ± SD of nuclei positive for IE (intermediate-early) antigen per microwell 48 h after infection of HCMV strains at an MOI of 0.1 from three independent experiments.

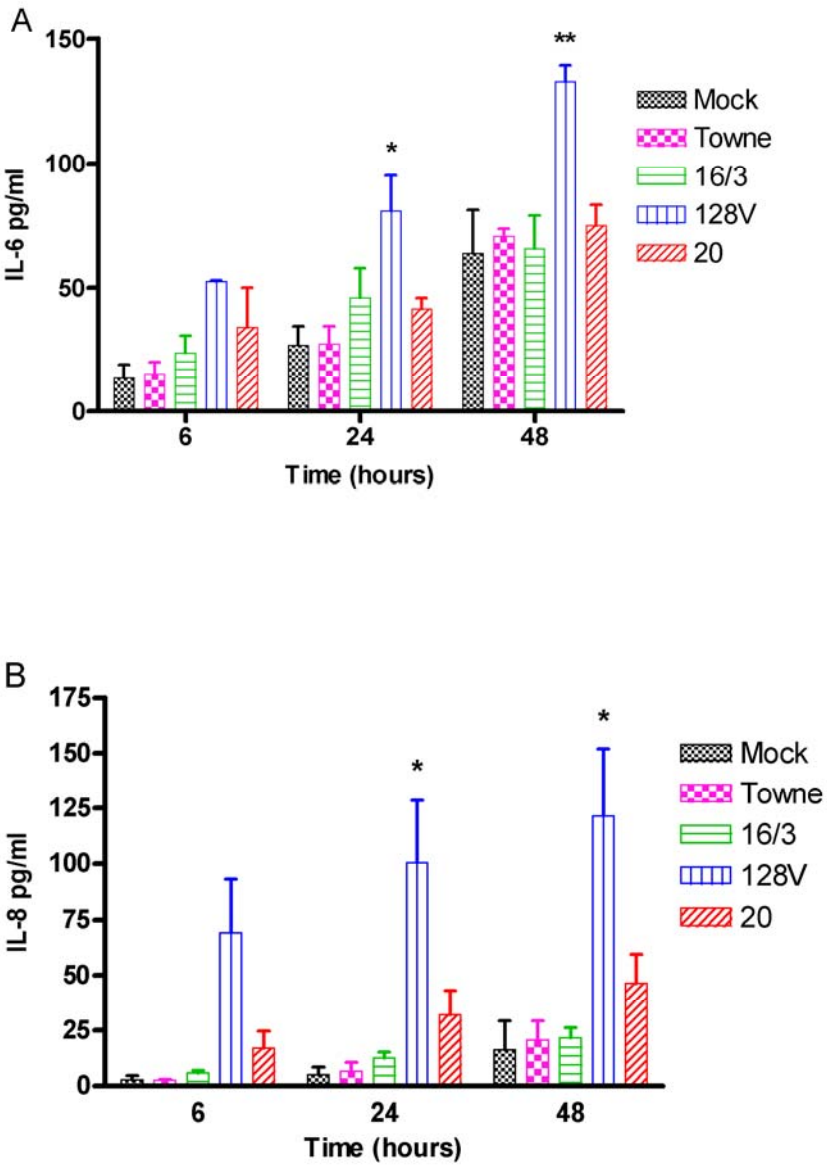

Figure 4. IL-6 (A) and IL-8 (B) levels in the medium of ST cultures at various time points after exposure to different HCMV strains. Values are means of the results of three independent experiments carried out on three different placental villous trophoblast preparations and error bars indicate standard deviation. Statistical analysis shows significant differences (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$) between mock- and 128V-infected cultures.

2. The interrelationship between the level of IL-8 secreted and the replication of the virus 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 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. Analyses 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 (Table IV).

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 (Table IV). A very low incidence of infection was seen for the two laboratory-adapted strains (Towne and AD169).

Three HCMV strains with different IL-8-inducing abilities 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 at an MOI of 0.1 in A549 cells demonstrated the production of infectious virus. There was a 4 log difference between the highest titres of strains 128V and E5 (Figure 5). In the cell culture infected with the laboratory-adapted Towne strain, no infectious virus was detected. 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 investigated. Results of a typical experiment are shown in Figure 6. Exogenous IL-8 increased 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 (Figure 6).

To explore the specificity of the enhancing effect of IL-8, the effect of neutralizing anti-IL-8 Mab on IL-8 enhanced IE gene expression was examined. The anti-IL-8 Mab reduced the number of IE antigen-expressing cells nearly to the unstimulated levels (unpublished data).

Table IV. Interrelationship between production of IL-8 and IE antigen in response to infection with different HCMV strains in A549 cells [1]

HCMV strain[2]	IL-8 (pg/ml)	Nuclei positive for IE[3] antigen (%)
-	108	-
128V	5009	3.5 ± 0.7
E33	385	1.4 ± 0.6
E5	764	1.8 ± 0.7
20	687	NT[4]
S	466	NT
74	508	NT
Towne	156	0.5 ± 0.1
AD169	329	0.7 ± 0.3

[1] 48 h after infection; [2] at an MOI of 0.1; [3] immediate-early antigen; [4] not tested

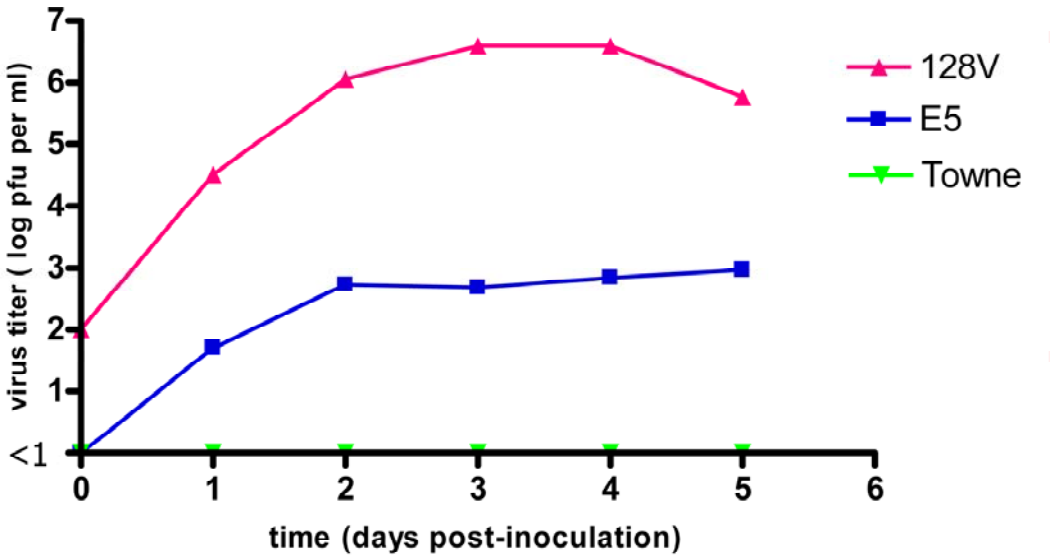

Figure 5. Replication of HCMV strains in A549 cells. For analysis of virus growth kinetics of HCMV strains of different IL-8 inducing ability in A549 cultures, cells were infected at a MOI of 0.1. A549 culture lysates and supernatants were pooled at varies times after infection and assayed for infectious virus. The virus titers were determined by plaque assay. Results are representative of three experiments.

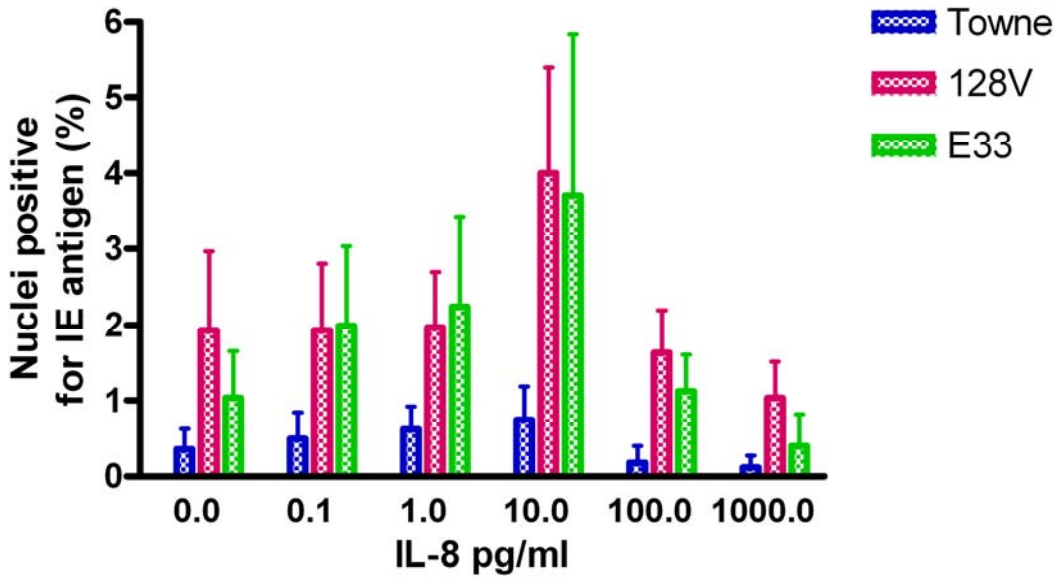

Figure 6. Enhancement of IE gene expression of HCMV in A549 cells by IL-8. A549 cells were incubated with the indicated concentrations of IL-8 before and after infection with HCMV. Cells were infected at a MOI of 0.1. At 48 h after infection the IE antigen-positive cells were detected by using immunfluorescence assay. Data are expressed as mean percentage ± of IE antigen-psitive cells per 30 microscope fields calculated.

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 though the different lengths of restriction fragments (36-239 bp).

One sample (E24) 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. In the isolates from 15 congenitally infected infants 11 gB1, 3 gB2 and 2 gB3 genotypes were detected (Table V).

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. A total of 9 samples failed to yield products from either gene (Table V).

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 [7, 13]. The genotye system of Dolan et al. [7] was used for UL146 and for UL139.

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

UL146 genotypes

The UL146 coding sequences range in length in the interval 342 to 378 bp (114-126 codons). All fall into 14 genotypes defined previously [7] and are designated G1-G14. UL146 is hypervariable throughout its length and only a few amino acid residues are completely conserved in all sequences.

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 (Table V).

UL139 genotypes

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 [88]. The protein encoded by each HCMV UL139 genotype contains a putative signal peptide sequence and a transmembrane region. Variation is concentrated in the N-terminal portion of the protein. Variation within genotypes tends to be higher in UL139 than in UL146, but that among genotypes is lower.

Seven UL139 genotypes (G1-7) were found in congenitally infected infants (Table V). Isolate E24 is assigned to 2 genotypes (G4 and G7). After resequencing and analysis of 5 clones, these assigments still held.

The gB genotype (gB1) and 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 (Table V).

Polymorphisms within UL55, UL146 and UL139 genes and ability to induce IL-8 production

The number of strains whose ability to induce IL-8 production has been examined is limited, and only one strain (128V) induces high levels (Table VI). However, there appears to be no obvious correlation between the IL-8-inducing ability with 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, since no other strain has the same genotype (G9) as strain 128V.

Table V. Genetic variations in the UL55, UL146 and UL139 genes of HCMV strains.

Strain[a]	Clinical source[b]	Age/sex[c]	Clinical details[d]	gB genotype[e]	UL146 genotype[f]	UL139 genotype[f]
16/5	U/P	Infant	C	1	G11	G2
128V	U/P	Infant	C	3	G9	G4
E24	U/P	Infant	C	1,2	G9	G4, G7
16/3	U/P	Infant	C	1	G11	G2
E34AV	U/P	Mother	C+	1	G13	G2
5DV	U/P	Adult	CH	1	G10	G4
E5	U/P	Adult	C+	3	G1	G4
E33AV	U	Mother	C+	1	G7	G2
E33BV	U/P	Infant	C	1	G7	G2
E36BV2	U	Infant	C	ND	G7	G4
E36AV1	U	Mother	C+	ND	ND	ND
E41BV3	U	Infant	C	1	G12	G5
E41AV1	U	Mother	C+	1	ND	ND
E42BV1	U	Infant	C	1	G8	G7
E42AV1	U	Mother	C+	1	ND	G1
E43BV1	U	Infant	C	2	G11	G4
E43AV1	U	Mother	C	ND	ND	G1
E62BV1	U	Infant	C	ND	G13	G4
E62AV1	U	Mother	C+	ND	ND	G4
E45BV1	U	Infant	C	1	ND	ND
E59BV1	U	Infant	C	ND	ND	ND
E28BV1	U	Infant	C	ND	ND	G2
16/2	U	Infant	C	1	ND	ND
E34B/BV	U	5m/M	C+	1	ND	ND
E60BV1	U	Infant	C+	1	ND	ND
E55BV1	U	Infant	C+	ND	ND	ND
E63BV1	U	10 w/F	C+	ND	ND	G1
E63AV1	U	Mother	C+	ND	ND	ND
E49AV1	U	Mother	C+	1	G2	ND
E52BV1	U	14 m/M	C+	2	G10	G2
20	B/P	Adult	R	4	G1	G4
74	B/P	Adult	R	3	G12	G3
S	U	Infant	C	1	G7	G2
B	U	Infant	C	3	G7	G6
83	U	Infant	C	1	ND	G2
89	U	Infant	C	1	ND	G2
Davis	BP/P	Infant	C	1	G5	G4
Toledo	U/P	Infant	C	2	G1	G4
Towne	U/P	Infant	C	1	G7	G5
Merlin	U/P	Infant	C	ND	G2	G1
TB40/E	TS/P	Adult	B	ND	G8	G4
AD169	BP/P	Infant	C+	2	ND	ND

[a] Virus strains; [b] U, urine; BP, biopsy; B, blood; TS, throat swab; P, passaged in cell culture; [C] Ages are in weeks (w) or months (m). Sexes: M, male; F, female; [d] C+, HCMV positive by PCR or IgM in serum; C, congenital; CH, chronic hemodialysis patient; R, renal transplant recipient; B bone marrow transplant recipient [e] Genotypes are denoted gB1-gB4 for UL55 [f] Genotypes are denoted G1-G14 for UL146 and G1-G8 for UL139. Multiple genotypes are separated by commas. ND, not determined.

Table VI. Relationship between genotypes and production of IL-8 in STs and in A549 cells

Strain[a]	**Clinical Source**[b]	**Genotype**			**IL-8 pg/ml**	
		UL55[c]	**UL146**[d]	**UL139**[e]	**in STs**	**in A549 cells**
E33	U	1	7	2	ND	385
S	U	1	7	2	ND	466
16/3	U	1	11	2	21.5	ND
128V	U	3	9	4	150.4	5009
20	B	4	1	4	59.3	687
74	B	3	12	3	ND	508
E5	U	3	1	4	ND	764
Towne	U	1	7	5	28.5	156

[a]virus strain; [b]U, urine; B, blood; [c]Genotypes are denoted gB1-gB4 for UL55; [d]Genotypes are denoted G1-G14 for UL146; [e]Genotypes are denoted G1-G8 for UL139; ND, not determined

Discussion

Congenital HCMV transmission results in substantial long-term neurodevelopmental morbidity in newborns, including mental retardation and sensorineural hearing loss (SNHL) [89]. While antiviral therapies may limit the severity of SNHL, therapeutic interventions have only a limited ability to reverse other neurologic injuries [90]. The most effective strategies for the control of congenital HCMV may require the prevention of fetal infection by preconceptual vaccination or anti-HCMV immunoglobulins [91, 92]. However, vaccines and immunoglobulins are not yet licensed for prevention of congenital HCMV infection. Continued study of the molecular pathophysiology of HCMV infection in cell types relevant to congenital transmission is therefore warranted, in order to facilitate the development of novel disease prevention strategies.

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

Numerous investigations have indicated that placental cells can be productively infected by HCMV [42, 64-67]. 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-1, IL-6, IL-8 and TNF-α, and anti-inflammatory cytokines such as IL-4 and IL-10, are produced by the trophoblasts [70-74]. Intrauterine infections are associated with the expression of placental cytokines such as IL-1 and IL-8 [71]. IL-8 has been demonstrated to upregulate the replication of laboratory-adapted HCMV strains in various cells, including the ST [75-77]. 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 foetus.

We have found that HCMV strains induce the production of different amounts of IL-8 in STs differentiated from first-trimester CTs, and the IE gene expression of the virus increases with the amount of IL-8 produced. This observation indicates that IL-8 may be involved in the materno-foetal transmission of HCMV. Our findings suggest that certain HCMV strains induce a high level of IL-8 in the 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 [78].

The fact that the IL-6-mediated stimulation of trophoblast cells did not result in an enhancement of IL-8 production [93] 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 [4, 7]. 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 [15].

2. The interrelationship between the level of IL-8 secreted and the replication of the virus in epithelial cells

HCMV displays a broad host cell range and infects many different cell types [94]. 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 [95]. 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 [96]. 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 [75-77]. Our results indicate that exogenous IL-8 increases 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. 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 [95]. Other strains can replicate in epithelial cells if IL-8 is provided by coinfecting agents, i.e. viruses or bacteria. The potential of various bacteria to stimulate IL-8 production in epithelial cells varies [78, 97].

Our results showed that HCMV replication in A549 cells is strain-dependent, in support of the view 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. 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 structures are 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.

The gB1, genotype is the most prevalent in congenital infections followed by genotypes gB3, and gB2, as it observed in erlier studies in our laboratory [83, 98]. The demonstrated predominance of gB genotype 1 could reflect the infection type of the study population.

The gB genotype (gB1) and the DNA sequences of genotypes UL146 and UL139 did not change in an infant in the period during which samples were obtained (16/3 and 16/5) as observed in persistently infected renal transplant recipients [14]. Since the gene UL 146 is variable, many studies have addressed the stability of UL146 upon isolation over many years [7, 12-14, 99].

As found in previous studies, mixed infections with different HCMV strains were common [100]. In one sample (E24), a single UL146 genotype and multiple UL 55 (gB1 and gB2) and UL139 genotypes (G4, G7) were detected, which indicates that this infant was

infected with two different HCMV isolates as seen in both congenital infections and immune compromised patients[7, 8, 13].

This could be due to different strains happening to contain the same genotype at one locus but not at the other, or to the limitations of amplifying sequences present as mixtures in unequal proportions.

Evidence for a genetic linkage between the three loci was unconvincing, consistent with a role for interstrain recombination during HCMV evolution [4]. The fact that the genotyping of one gene therefore does not shed light on the genotypes of other genes justifies the need to determine genotypes separately for each target locus.

The analysis to determine whether an association exists between the UL55, UL146 and UL139 genotypes and ability to induce IL-8 production in STs and A549 cells was underpowered, because of the unexpectedly high frequence variation in the genes UL146 and UL139. The small sample size we does not allow to draw a direct link between the genotypes and the ability to induce IL-8 production. However, a correlation with UL146 remains possible.

The analysis of further strains is required, especially those able to induce high levels of IL-8 or those known to have a UL146 genotype G9. Additionally data for other loci (for which data collection is ongoing) will broaden the analysis.

Continuation of the investigation will help us determine whether particular genotypes of UL146 (and other genes) are associated with IL-8 induction in physiologically relevant STs or other epithelial cells.

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 cells.

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 another 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 apparent 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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