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*J Immunol* 2000; 164:805-811; doi: 10.4049/jimmunol.164.2.805

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Human Cytomegalovirus Gene Products US3 and US6 Down-Regulate Trophoblast Class I MHC Molecules

Youngsoo Jun,* Eunok Kim,* Mirim Jin,* Ha Chin Sung,* Hoon Han,† Daniel E. Geraghty,‡ and Kwangseog Ahn*‡

The epidemiological correlation between human CMV (HCMV) infection and spontaneous fetal loss has been suggested, but the underlying mechanism is not well understood. Fetal cytotrophoblasts, which are in direct contact with the maternal immune system in the uterus during pregnancy, do not express HLA-A and HLA-B, but express the nonclassical class I HLA-G and HLA-C. It has been shown that both HLA-G and HLA-C are capable of inhibiting NK-mediated cell lysis. In our present study, using human trophoblast cell lines as well as other cell lines stably transfected with the human class I genes, we have demonstrated that HCMV US3 and US6 down-regulate the cell-surface expression of both HLA-G and HLA-C by two different mechanisms. HCMV US3 physically associates with both trophoblast class I MHC species, retaining them in the endoplasmic reticulum. In contrast, HCMV US6 inhibits peptide transport by TAP and thus specifically the intracellular trafficking of class I molecules. Therefore, these findings suggest for the first time a possible molecular mechanism underlying HCMV-related spontaneous pregnancy loss. The Journal of Immunology, 2000, 164: 805–811.

The absence of a harmful maternal immune response against the fetal allograft of a pregnancy remains a major enigma in current biology. Among the different mechanisms proposed is the suggestion that maternal tolerance to the fetus may be primarily achieved by the down-regulation of classical HLA class I MHC Ag expression on the fetal cytotrophoblasts, which are in direct contact with the maternal immune system in the uterus during pregnancy (1), thus preventing T lymphocyte activation against the semiallogenic fetus. However, a complete lack of class I MHC molecules on the cytotrophoblast would render these cells susceptible to attack by maternal NK cells. It has now become evident that trophoblast cells actually do express both the nonclassical class I MHC molecules HLA-G (2, 3) and the classical class I molecule HLA-C (4).

Although reduced expression of HLA-G is associated with some abnormalities during pregnancy, its function at the maternal-fetal interface still needs to be investigated. One recognized role of NK cells is to eliminate cells that have lost class I MHC surface expression because of events like malignant transformation or viral infection. There is mounting evidence that HLA-G protects trophoblast cells from being recognized by NK cells (5, 6). It has been shown with a class I MHC-negative B lymphoblastoid cell line 721.221 that expression of HLA-G protected these otherwise susceptible target cells from NK-mediated cytotoxicity (7). Several studies have revealed that HLA-C molecules are the dominant ligands that prevent the killing of a target by NK cells (8, 9). But it is not yet known to what extent HLA-G or HLA-C are involved in the protection of trophoblast from NK cell attack in vivo. The HLA-C molecules may have a complementary purpose or be a redundant counterpart of HLA-G in the mechanism that lets trophoblast cells evade NK-mediated lysis.

Human CMV (HCMV) (10), a β-herpesvirus, exists ubiquitously in human populations worldwide, with a primary infection being followed by lifelong persistence of the virus in the host. HCMV contributes in particular to the high morbidity and mortality of immunocompromised patients, most notably organ transplant recipients and AIDS patients, as well as infants infected in utero where it infects the trophoblast (11). Thus, an epidemiological correlation between HCMV infection and spontaneous fetal loss has been reported (12). However, to date, a mechanism that would explain this linkage remained to be discovered.

HCMV has evolved multiple strategies in causing down-regulation of HLA-A and -B expression on the surface of cells after they have become infected with the virus (13). At present, four different gene products, US2, US3, US6, and US11, have been identified that can independently reduce class I H chain expression on the cell surface. The US2 and US11 gene products induce rapid export of the class I H chains out of the endoplasmic reticulum (ER) into the cytosol, where they are degraded by proteasomes (14, 15). US3 expression causes MHC class I molecules to accumulate in the ER by preventing the transport of the assembled class I Ags to the cell surface (16, 17). Furthermore, US6 prevents peptide loading of the class I MHC molecules by inhibiting TAP-mediated peptide translocation into the ER. The US6 gene product encodes a type I transmembrane glycoprotein that binds directly to TAP in the ER lumen, consequently inhibiting peptide translocation (18–20).

The sequence similarities between HLA-G and -C and HLA-A and -B (21) along with the molecules’ ability to form trimeric complexes with β2-microglobulin (β2-m) and peptides (22)

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Received for publication September 8, 1999. Accepted for publication October 26, 1999.

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1 This work was supported by a grant for Molecular Medicine Research Group from the Ministry of Science and Technology, Korea.
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3 Abbreviations used in this paper: HCMV, human CMV; ER, endoplasmic reticulum; β2-m, β2-microglobulin; LCL, lymphoblastoid cell line; Endo H, endoglycosidase H; US, unique sequence; vv, vaccinia virus; WT, wild type.
suggests that the US proteins may act on the placenta-specific class I molecules HLA-G and -C in a manner that is analogous to their action on their classical counterparts. The down-regulation of trophoblast class I Ag presentation on the cell surface upon HCMV infection may expose the cells to NK-mediated lysis, which in turn may cause the spontaneous fetal abortion. However, recent findings by Schust et al. (23) argue against such a scenario. These researchers showed that, unlike the classical class I molecules, both HLA-G and HLA-C are fully resistant to rapid degradation associated with US2 and US11 HCMV gene expression. However, these data do not support the conclusion that there is a general resistance of trophoblast cells to class I down-regulation upon HCMV infection. The possibility remained that US3 or US6 or other unidentified molecules could be the cause of the down-regulation of class I MHC surface expression on the trophoblast.

We found in our study described here that both US3 and US6 can independently down-regulate HLA-G and HLA-C expressions on the cell surface. In a manner similar to their classical counterparts, both trophoblast class I molecules are bound by US3 and sequestered inside the ER, while the US6 gene product inhibits peptide transport and thus also down-regulates surface HLA-G and HLA-C expression. In so far, our data suggest that down-regulation of HLA-G and HLA-C may be one of the mechanisms involved in spontaneous abortions in HCMV infection.

Materials and Methods

Cell lines and culture

The trophoblast-derived choriocarcinoma cell line JEG-3 and mouse NIH3T3 fibroblasts were obtained from the American Type Culture Collection (Manassas, VA) and cultured in MEM (Life Technologies, Rockville, MD) supplemented with 10% FBS (HyClone, Logan, UT), penicillin (50 U/ml), and streptomycin (50 μg/ml). The lymphoblastoid cell line (LCL) 721.221 transfectants stably expressing HLA-G1 molecules were generated as described previously (22) and maintained in RPMI 1640 medium (Life Technologies) containing 10% FBS, 2 mM glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml).

Constructs and transfection

The cloning of cDNAs for US2, US3, US6, US11, and ICP47 have been described (16, 18, 24). The cDNA coding for human CD4 was kindly provided by Dr. Y. Yang (R. W. Johnson Pharmaceutical Research Institute, Manassas, VA) and cultured in MEM (Life Technologies, Rockville, MD) supplemented with 10% FBS (HyClone, Logan, UT), penicillin (50 U/ml), and streptomycin (50 μg/ml).

Results

The HCMV gene products US3 and US6 independently down-regulate surface presentation of trophoblast class I Ags

Analyses of a series of HCMV deletion mutants identified a 7-kb unique short (US) region as being required for class I H chain down-regulation in HCMV-infected cells (28). Among the genes within this region, four of the encoded proteins, US2, US3, US6, and US11, are able to reduce classical class I expression on the cell surface when individually expressed (14–17, 19, 20). To determine whether these gene products also affect the trophoblast class I species HLA-G and HLA-C, we tried to express each US cDNA separately in cells of a tumor cell line of extra-embryonic origin, JEG-3. However, we failed to establish stable US transfectants in JEG-3, because the prolonged expression (>1 wk) of US protein resulted in cell death. To circumvent this low efficiency of transient transfection, we cotransfected JEG cells with US cDNA and a plasmid conferring ouabain resistance (18). The transfectants became enriched after being exposed to ouabain for 12 h before analysis.

Viruses and viral infection

Vaccinia virus (vv) recombinants expressing US3 and US6 were generated by homologous recombination essentially as described (26) and plaque purified three times in thymidine kinase-deficient 143B cells under bromodeoxyuridine selection (50 μg/ml). JEG-3 cells were infected with recombinant vaccinia virus at a multiplicity of infection of 25 for 1 h in 500 μl of PBS supplemented with 10% BSA (Sigma, St. Louis, MO) at 37°C. LCL 721.221 cells were infected with vaccinia virus at a multiplicity of infection of 50 for 2 h in 1 ml RPMI 1640 medium at 37°C.

Abs

MHC class I-specific antisera K455 was raised against purified human class I heterodimers with human β2m (27). K455 recognizes H chain and β2m in both assembled and nonassembled forms. Monoclonal Ab W6/32 recognizes only the complex of H chain and β2m, and mAb OKT4 recognizes human CD4. The monoclonal 87G Ab reacts specifically with HLA-G (22). Polyclonal antisera detecting US3 and US6 were raised against synthetic peptides corresponding to the luminal NH2-terminal portion of the proteins (16, 18).

Flow cytometric analysis

The expression of class I MHC glycoproteins on the membrane was determined by flow cytometry (FACScalibur; Becton Dickinson, Mountain View, CA) after indirect immunofluorescence staining using anti-class I (W6/32) mAb and FITC-conjugated goat anti-mouse Ab (Sigma). A total of 10,000 gated events were collected by the FACScalibur and analyzed using the CellQuest software (Becton Dickinson).

Metabolic labeling and immunoprecipitation

Cells were methionine-starved for 30 min in a methionine-deficient medium before pulse-labeling for 30 min using 0.1 mCi/ml of [35S]methionine (Translabel; Amersham, Arlington Heights, IL). The label was chased at various time points with MEM containing 10% FBS. After one wash with cold PBS, the cells were lysed using 1% Nonidet P-40 (Sigma) in PBS or 1% digitonin in PBS for 30 min at 4°C. After this incubation, protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) were added to the lysates. The protein binding beads were then washed four times with 0.1% Nonidet P-40 or 0.1% digitonin and the proteins eluted by boiling in SDS sample buffer. All immunoprecipitates were electrophoresed in gradient SDS-polyacrylamide gels. The gels were dried, exposed to BAS film, and analyzed using the Phosphor Imaging System (BAS-2500, Fuji Film Company). For endoglycosidase H (Endo H) treatment, the immunoprecipitates were digested with 3 μl of Endo H (Boehringer Mannheim, Mannheim, Germany) for 16 h at 37°C in 50 mM NaOAc, pH 5.6, 0.3% SDS, 150 mM β-ME.

Peptide transport assay

Transport assays were performed as previously described (18). Briefly, the JEG-3 cells were trypsinized and washed twice with transport buffer (130 mM KCl, 10 mM NaCl, 1 mM CaCl2, 2 mM EGTA, 2 mM MgCl2, 5 mM HEPES, pH 7.3 adjusted with KOH) at 4°C and then permeabilized (107 cells/ml) by incubation in transport buffer containing 1 U/ml streptolysin-O for 20 min at 37°C. The permeabilized cells (107 cells/sample) were then incubated for 10 min at 37°C in 5 μM of a FITC-conjugated peptide (RYNAGTRL) in transport buffer containing 1 mM DTT and 10 mM ATP. Following lysis in 1% Nonidet P-40, the glycosylated peptide fraction was isolated with Con A-Sepharose beads (Pharmacia) and fluorescence (excitation, 485 nm; emission, 530 nm) on the peptides eluted from Con A was measured in a fluorescence reader (HTS 7000 Bio Assay Reader; Perkin Elmer, Norwalk, CT).

This kind of reduction was similar to the reduction seen in transient transfection, we cotransfected JEG cells with US cDNA and a plasmid conferring ouabain resistance (18). The transfectants became enriched after being exposed to ouabain for 12 h before analysis.

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Infection of JEG-3 cells either with vvUS3 or vvUS6 inhibits intracellular transport of both HLA-G and HLA-C

The choriocarcinoma cell line JEG-3 expresses HLA-G and HLA-Cw4, both of which are recognized by W6/32 (30). To further delineate the substrate specificity of US3 and US6 for trophoblast class I molecules and to evaluate potentially unique characteristics of the trophoblast class I molecules, we examined the effects of US3 and US6 on the intracellular transport and processing of HLA-G and HLA-C. In line with the previous finding by Rosenthal et al. (31), JEG-3 cells were nonpermissive for HCMV infection in our experimental conditions as well. Therefore, JEG-3 cells were infected either with a recombinant vaccinia virus driving the expression of the HCMV US3 (vvUS3) or with a recombinant vaccinia virus driving the expression of the HCMV US6 (vvUS6). The proteins in the infected cells were then labeled, chased for various times, detergent extracted, and finally treated for immunoprecipitation with W6/32. Before analysis by SDS-PAGE, half of each sample was treated with Endo H.

The pulse-chase analysis showed that similar amounts of W6/32-reactive class I molecules were synthesized during the 30-min labeling period (Fig. 2A). Interestingly, the intracellular transport rate of HLA-G, as analyzed by Endo H digestion, was unusually slow, and we consistently observed that some of the HLA-G molecules remained in the ER even after the 6-h chase point (Fig. 2A, lane 6). In contrast, all HLA-C molecules traveled through the Golgi within 30 min of chase (data not shown), which is typical for the kinetics of HLA-A and HLA-B transport (18). In cells infected with vvUS3, the intracellular transport of trophoblast class I MHC molecules. Identical experiments to those in A were performed using vvUS6. C, Expression of HCMV US proteins in JEG-3 cells infected with vvUS3 and vvUS6.
and HLA-C molecules in the vvUS6-infected cells were being degraded rather rapidly during the chase. We could not detect HLA-G and HLA-C molecules at the 360-min time point. This finding was in agreement with previous studies by Hughes et al. (32), where it was shown that peptide-deficient class I-b2m heterodimers induced by the TAP inhibitor ICP47 of HSV-1 were moved from the ER to the cytosol, where they were deglycosylated and subsequently degraded by proteasomes. As in the case of the misfolded class I proteins induced by ICP47, the peptide-deficient class I molecules made as a consequence of TAP blocking by US6 appeared to produce a similar result. Therefore, we concluded that the HCMV US3 and US6 gene products had a capability that prevented trophoblast class I products from leaving the ER.

The inhibition of the intracellular HLA-G transport by US3 and US6 is not cell type specific

To determine whether the ER retention of trophoblast class I molecules caused by US3 or US6 is limited to specific cell types and whether the distinct kinetics of HLA-G, in particular, are characteristic of the placenta cell type, we performed the identical experiments as conducted with JEG-3 cells using HLA-G-expressing LCL 721.221 cells (HLA-G/221). In wild-type vaccinia virus-infected cells (vvWT), the overall kinetics of the transport rate were similar to those observed in the JEG-3 cells (Fig. 3). The results suggest that the slow maturation and movement of HLA-G along the Ag presentation pathway is attributable to its own unique characteristics and is not cell type specific.

In either vvUS3- or vvUS6-infected cells, HLA-G was sensitive to Endo H treatment, as was evident throughout all the chases. This suggested that HLA-G was being retained in the ER. The kinetics of the inhibition of the intracellular transport were again comparable to those observed in either vvUS3- or vvUS6-infected JEG-3 cells. These results indicate that both the US3- and the US6-mediated inhibitions of the intracellular transport of HLA-G are independent of cell type.

US3 binds directly to HLA-G and HLA-C

The facts that US3 is a type I transmembrane glycoprotein localized in the ER (17) and that the trophoblast class I molecules of the vvUS3-infected cells were sensitive to Endo H digestion prompted us to look for a possible direct association between US3 and trophoblast class I molecules. JEG-3 cells were infected either with vvUS3 or with vvWT and metabolically labeled, after which they were lysed with digitonin buffer. As shown in Fig. 4, both HLA-G and HLA-C could be immunoprecipitated from the digitonin extract with anti-H chain antiserum (K455) (Fig. 4A, lanes 2 and 5), but not with anti-US3 antiserum. Using the K455 Ab, a 22-kDa protein coprecipitated with the class I molecules from the vvUS3-infected cells, but not from the vvWT-infected cells (Fig. 4A, compare lane 5 with lane 2). This 22-kDa protein comigrated with the protein band detected by the anti-US3 antiserum (Fig. 4A, lane 4, for the control see lane 1). Therefore, these data confirm the identity of the coprecipitated protein as being US3.
Because the K455 Ab recognizes both HLA-G and HLA-C, it is not clear whether US3 binds to both forms of the trophoblast class I species or only to one of them. To investigate this point, we used an HLA-G-specific mAb (87G) that exhibited no cross-reactivity with other class I molecules (22). In extracts of vvWT-infected JEG-3 cells, 87G immunoprecipitated only HLA-G in association with β2m (Fig. 4A, lane 3). However, in extracts of vvUS3-infected JEG-3 cells, the 87G immunoprecipitate contained HLA-G, β2m and US3 (Fig. 4A, lane 6). This indicates physical association of these three components. To then further look for a specific association between HLA-C and US3, any residual HLA-G molecules that could have remained in the supernatant after the first immunoprecipitation (Fig. 4A, lanes 3 and 6) were extracted by two more rounds of immunoprecipitation with excess amounts of 87G Ab. The resultant supernatant was then subjected to immunoprecipitation with anti-HC K455 Ab. For both vvWT- and vvUS3-infected cells, no more HLA-G was detectable (Fig. 4A, lanes 7 and 8), whereas the amount of HLA-C material recovered was still similar to that obtained by “direct immunoprecipitation” of the untreated, original cell extract (compare lanes 2 and 5 with lanes 7 and 8, respectively). Under these conditions, US3 could be coimmunoprecipitated from lysates of vvUS3- (lane 8) and not from vvWT-infected cells (lane 7). Therefore, the results clearly indicate that US3 associates physically with either of the class I proteins, HLA-G and HLA-C, retaining them in the ER.

NK cells are grouped into two types, namely NK1 and NK2 (8), depending on their ability to discriminate between two groups of HLA-C allotypes. It has been shown that the product of the HLA-C locus in JEG-3 cells is HLA-Cw*0401 (30) and that it can inhibit NK1 cells. To see whether US3 exhibited some allotype specificity for products of the HLA-C locus, we cotransfected mouse NIH3T3 cells with the US3 cDNA and HLA-Cw*0304 cDNA, the product of which is known to inhibit NK2 cells. The physical association between these gene products was then examined by coimmunoprecipitation. Both W6/32 and K455 immunoprecipitated from digitonin lysates contained HLA-Cw*0304, mouse β2m, and US3 (Fig. 4B, lane 1, 2, and 3, respectively). The binding of US3 to HLA-Cw*0304 was also observed in the reciprocal experiment in which US3 was immunoprecipitated with anti-US3 Ab (Fig. 4B, lane 3). Neither W6/32 nor K455 showed cross-reactivity with endogenous mouse class I molecules (data not shown). Despite the differences in the deduced amino acid sequence of the two HLA-C allotypes, it is unlikely that US3 exhibits HLA-C allotype specificity. It is much more likely that it recognizes conserved regions in the HLA-C molecules. In addition, these results show that MHC molecules are altered in their expression by the HCMV US3 gene product in different species of cells.

Peptide translocation by TAP is inhibited by US6 in JEG-3 cells

Using a peptide translocation assay (33), we and others have shown that HCMV US6 inhibited TAP-mediated peptide translocation in adherent cells (18–20) and that the peptide translocation in JEG-3 cells is readily measurable (34). To determine whether the impaired maturation of the trophoblast class I molecules in vvUS6-infected cells reflects a lack of available class I binding peptide in the lumen of the ER, we directly examined the effect of US6 expression on peptide translocation by TAP in JEG-3 cells. The rate of peptide translocation was compared between vvUS6- and control-infected cells (Fig. 5). No inhibition in TAP-mediated peptide translocation, as assessed by the glycosylation of FITC-conjugated peptides, was observed in both vvWT- and vvUS3-infected cells. In contrast, all peptide transport activity was completely abolished in vvUS6-infected cells, to an extent that is comparable to the one observed when ATP is removed. We con-

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** Peptide translocation in JEG-3 cells infected with vvWT or vvUS6. Translocation of the FITC-conjugated peptides into the ER of JEG-3 cells was measured as described in Materials and Methods. The peptide transport is expressed as a relative fluorescence value in comparison to vvWT-infected cells in the presence of ATP (100%) (■). No ATP was added for the control (□). Results are presented as means of duplicate experiments.
unique characteristics that allowed them to escape from the class I down-regulation generally associated with HCMV infection. However, this does not seem the case, because our data show that the US3 and US6 gene products of HCMV can also interrupt the surface expression of HLA-G and HLA-C, although the existence of still another gene product affecting the down-regulation of the trophoblast class I species cannot be excluded at this time. Therefore, our observations provide first evidence of a possible molecular mechanism underlying HCMV-related spontaneous pregnancy loss.

There must be several reasons why HCMV developed multiple mechanisms to prevent MHC class I presentation. One possible explanation could be that the selective pressure forcing the virus to cope with the presentation of a variety of MHC class I alleles may have favored diversification of the HCMV US genes and their functions. Down-regulation of the surface expression of the class I MHC molecules may be a mechanism by which the virus can escape the host’s CTL response, although there may still remain a potential risk, because the infected cell itself may become vulnerable to NK attack. Differential regulation of class I MHC molecules may thus allow virus-infected cells to avoid NK-mediated lysis. For instance, HIV-1 selectively down-regulates HLA-A and HLA-B but does not significantly affect HLA-C or HLA-E (35). The adenosine A3/19K protein binds more tightly to certain human class I MHC alleles during their retention in the ER than to others (36). Pseudo-rabies virus infection results in decreased expression of the D0 allele and an increased K0 allele expression in murine L929 cells (37). Furthermore, the US2 and US11 gene products exhibit substrate specificities for murine class I molecules, albeit they do not represent natural substrates for HCMV (38). These differential regulatory mechanisms may create a balance of conditions optimal for the escape of viruses from CTL and for protection against NK cells.

Peptide translocation happens independently of class I MHC molecules, because TAP inhibition occurs upstream of the class I MHC transport. Therefore, it is unlikely that HCMV US6 should exhibit class I substrate specificity, and we predict that HCMV US6 would also influence the surface expression of the HLA-E, which is nonclassical class I MHC molecule expressed in cytotrophoblast cells (39). However, we do not rule out the possibility that even in the presence of US6, a certain portion of class I MHC molecules, albeit not detectable in our assay systems, might acquire peptides via a TAP-independent peptide transport (40) before reaching the cell surface, where they could then play a role in inhibiting NK cell lysis. It seems that the ER retention associated with the HCMV US3 gene product could be a more general characteristic of all HLA locus products, because the US3 protein binds to a broad spectrum of HLA-A and HLA-B alleles with no particular preference (S. Lee and K. Ahn, manuscript in preparation). This is also supported by our finding that US3 did not differentially bind to either HLA-Cw*0401 or HLA-Cw*0304, which are the common inhibitory receptors for NK1 and NK2 cells, respectively. Furthermore, our data suggest that HCMV US3 has evolved to recognize both classical and nonclassical class I molecules.

Acknowledgments
We thank Dr. Sangduk Kim for critical review of the manuscript. We are also grateful to Dr. K. Fruh and Dr. Y. Yang for helpful discussions and their generous gifts of Abs and cDNAs.

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