

HLA Class I Molecules Are Not Transported to the Cell Surface in Cells Infected with Herpes Simplex Virus Types 1 and 2¹

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To assess the effect of herpes simplex virus (HSV) on assembly and transport of class I MHC molecules, we compared class I MHC immunoprecipitated from metabolically labeled infected and uninfected human dermal fibroblasts. The immunoprecipitates were analyzed by isoelectric focusing, allowing identification of individual class I alleles and assessment of transport through the Golgi apparatus by the sialation of carbohydrate residues. In cells infected with wild-type HSV, class I synthesis was reduced or abolished because of the host protein synthesis shutoff function of the UL41 gene product. In cells infected with mutant viruses of both HSV-2 strain G and HSV-1 strain 17 that lack the UL41 gene, class I HLA molecules failed to become sialated, suggesting that they were not transported to the Golgi apparatus. In contrast, transferrin receptor was normally sialated in both infected and uninfected cells. Drug treatments of cells to restrict viral gene expression suggested that an early gene or genes were responsible for the effect. A pulse chase showed that class I molecules were synthesized in normal amounts in infected cells, but that heavy chains were retained in a sialyl transferase negative compartment either stably associated with $\beta 2m$ or as free heavy chain in a pattern that is characteristic for each class I allele. HSV is thus the fourth example of a DNA virus that interferes with class I assembly or transport. *Journal of Immunology*, 1994, 152: 2736.

CTLs form an essential part of immune defence against many virus infections. For intracellular viral Ag to be presented to CTLs, it must be degraded in the cytosol, transported into the endoplasmic reticulum (ER)³ and bound to nascent class I MHC heavy chains, which can then form a stable complex with $\beta 2$ -microglobulin ($\beta 2m$). Normally, only heavy chains that have stably bound peptide and $\beta 2m$ can leave the ER and appear on the cell surface (1, 2).

Although rapidly mutating RNA viruses may evade immune responses primarily by antigenic variation, many

large DNA viruses have evolved specific measures to avoid or modify host immune effectiveness, including expression of cytokine-like factors, Fc receptors, and interaction with the complement cascade (3). At least three DNA viruses have been described to interfere specifically with class I MHC transport to the cell surface. Adenovirus 2 encodes a 19 kDa protein that retains class I in the ER (4). Two herpesviruses, murine cytomegalovirus (MCMV) (5) and human cytomegalovirus (HCMV) (6, and A. Warren, personal communication) also prevent class I leaving the ER, by currently unidentified mechanisms.

Herpes simplex virus (HSV), an important human pathogen, is also known to interfere with CTL-recognition of virally-infected cells by decreasing surface expression of class I MHC (7, 8), and inhibiting recognition of infected fibroblast targets by both HSV-specific and alloreactive CTLs (8, 9). The precise mechanism of these effects is unclear and may be multifactorial. The inability of CTL to lyse HSV-infected fibroblasts appears a result, at least in part, of a specific disarming of CTL effector capability (9). Reduction of MHC cell surface expression, which is seen late in infection, may be partly a result of the effect of the HSV UL41 gene product in shutting down

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³ Abbreviations used in this paper: ER, endoplasmic reticulum; HSV, herpes simplex virus; MCMV, murine cytomegalovirus; HCMV, human cytomegalovirus; fb, fibroblast; Tr, human transferrin receptor; PAS, protein A-Sepharose; IE, immediate early; PAA, phosphonoacetic acid; MOI, multiplicity of infection.

host protein synthesis (10); the surface $t_{1/2}$ of class I MHC is approximately 8 to 16 h (11). However, presentation of viral Ag to CTL depends not on the total surface class I, but on the ability of class I heavy chains present in the endoplasmic reticulum while viral proteins are being synthesized to bring peptides derived from viral proteins to the cell surface. As infective viral progeny are released 18 to 20 h after infection (10), effective CTL control of HSV replication would rely on effective Ag presentation well within this time period. We have investigated HLA class I synthesis, assembly, and transport in human dermal fibroblasts infected with HSV types 1 and 2 during this critical period for Ag presentation. We now report that in cells infected either with mutant viruses lacking the UL41 gene or with wild-type viruses, HLA class I molecules fail to become sialated: indicating retention in a sialyl transferase-negative intracellular compartment.

Materials and Methods

Cell lines, viruses, and antibodies

Adult human dermal fibroblast (fb) lines were established by outgrowth of adherent cells from forearm skin biopsies from 2 tissue-typed volunteers, J.B. (HLA A3, A29, B7, B51, Cw7) and J.S. (HLA A1, A3, B7, B35, Cw4, Cw7). The JSfb line was the kind gift of Dr. A. Warren (University of Wales College of Medicine, Cardiff, UK). The cells were cultured in Dulbecco's modified Eagles's medium (J.S.) or RPMI (J.B.) supplemented with 10% FCS, penicillin, and streptomycin, and were used between passages 5 and 10.

HSV-2 strain G (HSV-2(G)), HSV-1 strain 17 (HSV-1(17)), and two mutants with deletions of the UL41 gene (12) (HSV-2(G:UL41⁻) and HSV-1(17:UL41⁻) were kindly provided by R. Everett (Medical Research Council Virology Unit) and were propagated and titrated as described (12). Viral stocks were stored at -80°C .

The mAbs W6/32 (13), recognizing $\beta 2\text{m}$ -associated human class I, and HC10 (14), recognizing free human heavy chains mostly of B and C locus alleles, were purified on a protein A-Sepharose column and used at 15 $\mu\text{g}/\text{ml}$. OKT-9, recognizing human transferrin receptor (Tr) (15), kindly provided by A. Warren, was used as ascitic fluid at 10 $\mu\text{l}/\text{ml}$. HSV-1/2 cross-reactive mAbs 10176 (anti-IE175), 7381 (anti-UL29), and 2153 (anti-gB) were the generous gift of A. Cross (Medical Research Council Virology Unit) and were used as ascitic fluid at 10 $\mu\text{l}/\text{ml}$.

Metabolic labeling and pulse-chase analysis

Subconfluent monolayers of fbs were infected with HSV at multiplicity of infection (MOI) 10 to 40. At the time described, uninfected and infected cells were labeled with [^{35}S]methionine either in the plate or in suspension. For plate labeling (used in experiments in Figs. 1 and 3a), plates were washed twice with PBS and 7 ml methionine-free RPMI supplemented with 1 mCi [^{35}S]methionine (Translabel, Flow, Irvine, Scotland) added for 1 or 2 h. To conserve [^{35}S]methionine, label was first added to uninfected plates and then transferred to infected plates, as previously described (6). For suspension labeling, adherent cells were washed in the plate twice with PBS, once with trypsin/EDTA (GIBCO BRL, Gaithersburg, MD), and incubated with trypsin/EDTA until just nonadherent, collected in a centrifuge tube, washed twice in RPMI/10% FCS and once in methionine-free RPMI/5% FCS. Washes were conducted at room temperature. Equal numbers of cells were then incubated at 37°C in methionine-free RPMI supplemented with [^{35}S]methionine. For long labeling experiments, the cells were labeled for 1 to 2 h and then sedimented and lysed in ice-cold lysis buffer (0.5% NP40, 20 mM Tris, 10 mM EDTA, 0.1 M NaCl pH7.5, supplemented with 1 mM PMSF and 10 mM iodoacetamide from stocks dissolved in acetone) at not more than 10^7 cells/ml. For the pulse-chase experiment the cells were labeled for 30 min and then diluted into 10 times their volume of warm RPMI containing 2 mM unlabeled methionine, and divided into three aliquots. One was lysed immediately as above and two were further incubated at 37°C for

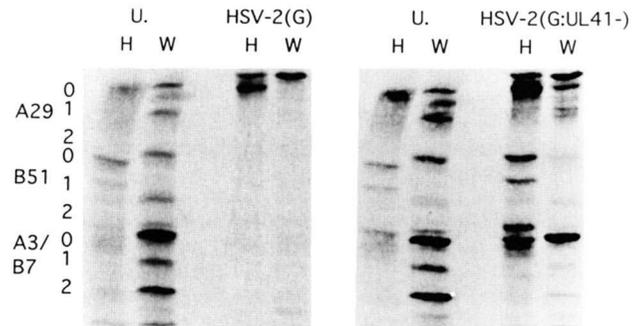


FIGURE 1. MHC Class I is not sialated in cells infected with HSV2. JBfb cells were infected with HSV-2(G) or HSV-2(G:UL41⁻) at a MOI of 40 for 2 h and then labeled in the plate with [^{35}S]methionine for 2 h. Uninfected JBfb were similarly labeled. Immunoprecipitations were conducted with HC10 (lanes H) or W6/32 (lanes W). Isoelectric focusing positions of class I alleles A29, B51 and A3/B7 (which focus in the same position) are shown; the 0, 1, and 2 sialic acid forms are designated accordingly. The two bands (a) migrating above A29:0 in infected cells are coprecipitating HSV proteins that are not seen when more stringent washing conditions are used are probably not specifically associated with class I. The effect of HSV can be seen by comparing the W6/32-precipitated A29 bands: in uninfected cells both asialo (b) and sialated (c) bands are found; in HSV2(G:UL41⁻) infected cells asialo A29 (d) is found but no sialation is seen (e). Similarly, asialo A3B7 bands are found in both uninfected (f) and infected (g) cells: fully sialated bands are seen in uninfected cells (h) but not in infected cells (i).

times indicated before lysis. Lysates were kept at 4°C throughout the preclearing and immunoprecipitation procedures. After 30 min, nuclei were sedimented and lysates precleared with 250 μl 10% Staph A cells (Pansorbin, Calbiochem, Nottingham, UK) turning end over end overnight. The long preclearing was used to allow loosely assembled (peptide free) heavy-chain $\beta 2\text{m}$ complexes that are found in the ER to dissociate (16). The next day lysates were divided into two portions if necessary and immunoprecipitated with W6/32 and HC10 for 1 h, BSA added to 1% and 100 μl 5% protein A-Sepharose (PAS) beads in lysis buffer added and turned end over end for another hour. The PAS beads were pelleted and the lysates reserved. PAS beads were washed twice with lysis buffer containing 0.1% SDS and 1% BSA, once with 10% lysis buffer in 0.5 M NaCl, and once in lysis buffer. They were then frozen at -70°C or resuspended in sample buffer for isoelectric focusing. For certain experiments, lysates were precleared again twice for 1 h with 200 μl 10% Staph A cells, and immunoprecipitated with OKT9 or anti-HSV mAbs.

Drug treatment of HSV-infected cells

Cells were treated to allow selective expression of immediate early (IE) HSV genes or to suppress late gene expression. For IE gene expression, cells were pretreated for 1 h with 140 $\mu\text{g}/\text{ml}$ cycloheximide (Sigma Chemical Co., St. Louis, MO) and infected for 2 h in the presence of cycloheximide. Under these conditions no viral protein is made and only IE genes are transcribed. They were then washed four times with ice-cold PBS containing 2.5 $\mu\text{g}/\text{ml}$ actinomycin D (Sigma: stock solution of 1 mg/ml in ethanol stored at 4°C), and incubated for 3 h at 37°C in normal medium containing 10 $\mu\text{g}/\text{ml}$ actinomycin D. Because actinomycin D inhibits transcription, under these conditions only IE gene products are

synthesized. Three hours after release from cycloheximide blockade, the cells were washed and trypsinized in the presence of 2.5 $\mu\text{g/ml}$ actinomycin D, and labeled in suspension for 2 h in the presence of 10 $\mu\text{g/ml}$ actinomycin D.

To inhibit late gene expression, cells were preincubated in phosphonoacetic acid (PAA) (Sigma) 300 $\mu\text{g/ml}$ for 1 h before infection in the same concentration of PAA.

Isoelectric focusing

Isoelectric focusing gels were run exactly as previously described (17).

Results and Discussion

Human adult dermal fibroblasts from a donor of HLA type A3, A29, B7, B51, Cw7 (JBfb) were infected with HSV-2(G), wild type; or HSV-2 (G:UL41⁻), a mutant virus in which the UL41 gene that shuts off host protein synthesis has been deleted(12). Analysis by isoelectric focusing of HLA class I synthesized during a 2-h labeling period is shown in Figure 1. Class I heavy chains detected by HC10 are B and C locus products that were unfolded in the ER or were of the "fall apart" phenotype; i.e., having associated with $\beta 2\text{m}$ but not with a high enough affinity peptide to give the complex sufficient stability to remain W6/32-reactive after 16 h in a dilute detergent lysate (16). Conversely, W6/32 detects heavy chains of all loci that are $\beta 2\text{m}$ -associated and are presumed to have bound peptide. Two hours after infection, no newly synthesized class I was detected in cells infected with the HSV2(G) wild-type virus. In cells infected with the UL41⁻ mutant virus, class I was synthesized but was not sialated in the normal way. Additionally, more class I was recovered as free heavy chain (HC10-reactive) rather than $\beta 2\text{m}$ -associated (W6/32-reactive). HLA class I molecules undergo a single N-linked glycosylation in the ER and subsequent modification of this carbohydrate includes the addition of two sialic acid residues by the enzyme sialyl transferase in the trans-Golgi apparatus (18). Resistance to the effects of the enzyme endoglycosidase-H is acquired when the carbohydrate is modified in the medial Golgi apparatus, and is often used as an indicator of transport through the Golgi. In a similar fashion, the detection of sialation can be used to monitor transport through the Golgi apparatus, and by implication to the cell surface, and is often employed in the study of MHC molecules, because the isoelectric focusing technique used to detect it also allows separate analysis of individual allelic products (6, 17, 19). The failure to detect sialated class I in HSV-2-infected cells suggests that class I MHC is retained in an intracellular compartment before the trans-Golgi apparatus, both as free heavy chain and associated with $\beta 2\text{m}$.

The strong host protein shutoff function seen with wild-type HSV-2 may seem to obviate any advantage to the virus in additionally interfering with class I-restricted Ag presentation. In HSV-1 (strain 17), however, the UL41 gene function is relatively weak. In Figure 2 the same protocol was used to study cells infected with HSV-1(17), both wild-type and a UL41⁻ mutant virus. Class I MHC

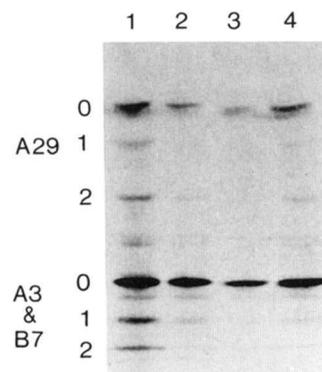


FIGURE 2. Intracellular retention of MHC Class I is also seen in HSV-1-infected cells. JBfb cells were infected with HSV-2(G:UL41⁻) (lane 2), HSV-1(17) (lane 3), or HSV-1(17:UL41⁻) at a MOI of 20 for 2 h. Infected or uninfected (lane 1) cells were labeled in suspension for 2 h and immunoprecipitated with W6/32.

continued to be synthesized in wild-type HSV-1-infected cells, although only at 50% of the level of uninfected cells; however both in wild-type and UL41⁻-infected cells the near absence of sialated class I MHC indicates that most class I had not reached a sialyl transferase positive compartment during the labeling period. The small amount of sialated material detected may have been because of failure to infect all the cells, or because of the continued transport of a small amount of class I.

The next set of experiments was designed to identify the phase of viral gene expression responsible for class I retention. HSV genes are expressed in a temporally regulated manner (20) and can broadly be divided into three main phases, immediate early (IE), early, and late. IE proteins are synthesized immediately after viral infection: their transcription is initiated by the structural virion polypeptide Vmw65 (α -TIF) and their synthesis is independent of the de novo synthesis of other viral proteins. In contrast, transcription of early and late genes requires IE gene products and the efficient expression of late genes additionally requires early protein and viral DNA synthesis(10). A time course experiment showed that class I retention was complete 2 h after infection with HSV-2 (Fig. 3a). Early gene expression was required for class I retention (Fig. 3b), indicating that the effect is not caused by nonspecific toxicity of the virus preparation and that neither structural proteins carried into the cell in the infecting virions nor IE gene products are responsible alone. Additionally, class I retention was complete in the presence of PAA, which inhibits viral DNA synthesis and thus late gene expression (Fig. 3c). Taken together the results suggest that an early gene causes class I retention.

To determine whether the aberrant processing of class I molecules was simply a result of a generalized disruption or 'takeover' by viral proteins of the protein processing pathways, the experiments were conducted using Tr as a

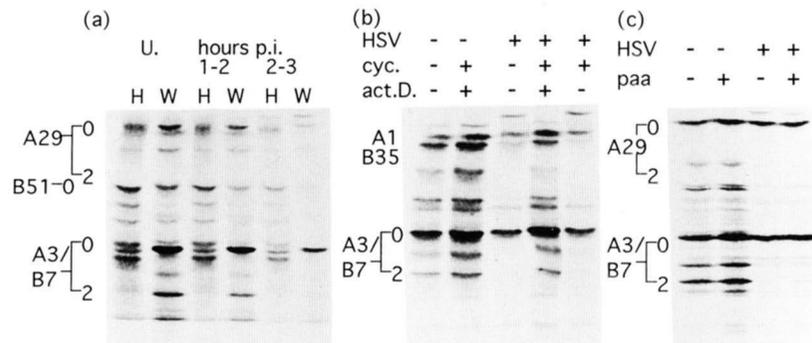


FIGURE 3. Intracellular retention of class I MHC is complete within 2 h of infection, requires HSV early gene expression, and is not inhibited by PAA. (a) JBfb cells were infected with HSV-2(G:UL41⁻) at MOI of 40. Labeling in the plate was performed first on uninfected cells for 1 h, and the label then was transferred to infected cells to label sequentially 1 to 2 and 2 to 3 h post-infection samples. Immunoprecipitation was with HC10 (H) and W6/32 (W). (b) Drug treatment was used to regulate the phase of HSV gene expression. JSfb cells were used; 2 h labeling in suspension and immunoprecipitation with W6/32. Lanes 1 and 2: uninfected. Lanes 3, 4 and 5: infected with HSV-2(G:UL41⁻), MOI of 20. Lanes 1 and 3: no drug treatment. Lanes 2 and 4: Treated with cycloheximide (CY) followed by actinomycin D (AD) to allow only IE gene expression. Lane 5, treated with cycloheximide as for lanes 2 and 4 but without actinomycin D. After preclearing and immunoprecipitation with W6/32, the lysates were further precleared with Staph A cells and immunoprecipitated with mAb directed against IE175 (an IE protein), gB and UL29 (early proteins). SDS-PAGE analysis confirmed that for the lysate in lane 4, only IE gene products were seen, whereas early gene products were detected for the lysates in lanes 3 and 5 (data not shown). (c) To assess the effect of late HSV gene expression HSV-2(G:UL41⁻) infection was conducted in the presence or absence of phosphonoacetic acid (PAA) 300 μ g/ml. Lanes 1 and 3: no PAA. Lanes 2 and 4: plus PAA. Lanes 1 and 2: uninfected. Lanes 2 and 4: infected. Labeling for 2 h was conducted in suspension 3 h post-infection. Immunoprecipitation was with W6/32.

control. Figure 4 shows a pulse chase experiment of infected and uninfected cells, performed in the presence of PAA to diminish nonspecific effects of late HSV genes on host protein synthesis. After immunoprecipitation of class I, Tr was immunoprecipitated from the same lysates. Tr contains three N-linked glycosylation sites, giving multiple acidic bands with sialation. Acquisition of sialated sugars by Tr occurred at the same rate in infected and uninfected cells. The demonstration that transport and glycosylation of Tr is intact in HSV-infected cells rules out a generalized disruption or 'takeover' by viral proteins of these pathways as the cause of retention of class I MHC.

The pulse chase shows that with PAA treatment, equivalent amounts of class I were synthesized in HSV-2(G:UL41⁻)-infected and -uninfected cells. However, only in uninfected cells did class I acquire sialated sugars, indicating assembly and transport through the Golgi apparatus. This experiment also demonstrated the heterogeneity in assembly behavior among class I alleles previously described (17, 19). For instance, although HC10 does not detect free A29 heavy chain (hc), assembly of A29 hc with β 2m is observed in this protocol by the increase in asialo A29 W6/32-reactive material after 30 min of chase. The majority of A29 was fully sialated after 60 min. In the infected cells, some increase in asialo A29 was seen after 30 min, but there was no sialation; the stability of the β 2m-association after overnight preclearing of detergent lysates implies that A29 was retained in HSV-infected cells in a peptide loaded, β 2m-associated form. We have previously described the slow assembly of B51, whose

heavy chain persists for long periods after synthesis in an HC10 reactive form (17). Very little asialo W6/32-reactive form of this allele is ever detected, assembled molecules apparently rapidly leaving the ER. In HSV-infected cells, B51 is found as free heavy chain throughout the chase; it does not become sialated, nor does it stably associate with β 2m. Similarly, with the A3/B7 bands (which focus in the same position), class I is detected both as free heavy chain and β 2m-associated. Thus the effect of HSV is not just to interfere with intracellular transport, it also prevents or delays assembly with β 2m, at least for some alleles. Class I assembly in the ER is assisted by the chaperone molecule IP90 (p88, calnexin)(21–23); which is found associated with class I heavy chains either β 2m-associated or free, and dissociates on the binding of peptide, allowing the complex to leave the ER(24). It is possible that HSV retains class I by interfering indirectly with a critical process in class I assembly and transport, possibly involving chaperone function.

The precise mechanism underlying the HSV-mediated effect on class I transport is unclear, and the HSV protein(s) mediating the aberrant processing of class I molecules are unknown. Analysis of the complete sequence of HSV-1 (25, 26) does not reveal obvious candidate proteins: for example, no proteins have recognized ER-retention motifs, or homology with the adenovirus 19K protein. Moreover, we have no evidence for an HSV protein that specifically coprecipitates with class I molecules. It is possible that the HSV mechanism of interference with class I

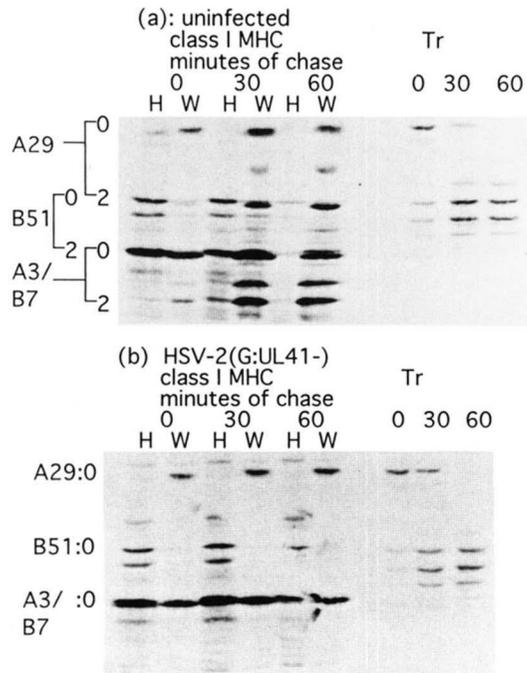


FIGURE 4. Pulse chase study of MHC and transferrin receptor in HSV infected and uninfected cells. To minimize the nonspecific effects in reducing host-protein synthesis caused by late HSV genes (10), this experiment was conducted in the presence of 300 $\mu\text{g/ml}$ PAA for both infected and uninfected samples. Equal numbers of uninfected cells and cells infected with HSV-2(G:UL41⁻) at a MOI of 100, 3 h previously, were labeled in suspension for 30 min and chased with cold methionine as described. After immunoprecipitation with W6/32 (W) and HC10 (H), the lysates for each time point were re-precleared and immunoprecipitated with OKT9 (recognizing Tr). *Panel a* shows the pulse chase for MHC on the left and Tr on the right for uninfected cells, *panel b* for infected cells.

MHC is more similar to that observed in other herpes viruses, in particular CMV, although HSV and CMV are only distantly related in evolutionary terms (27). In MCMV-infected cells it appears that fully peptide-loaded $\beta 2\text{m}$ -associated class I is retained in the ER (5). With HCMV, class I heavy chains are retained both $\beta 2\text{m}$ -associated and free, but are rapidly degraded (6). Both the HCMV and MCMV effects are also thought to be caused by early viral genes.

Thus, in conclusion HSV now forms a fourth example of a DNA virus that interferes with MHC class I assembly or transport. Isolation of the genes responsible for these effects should not only assist in understanding of the nature of the relationship between herpes virus infections and host immunity, but may also provide new insights into MHC class I assembly.

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