Efficient downregulation of major histocompatibility complex class I molecules in human epithelial cells infected with cytomegalovirus

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Liver and intestinal epithelial cells are a major target of infection by cytomegaloviruses (CMV), causing severe disease in affected organs of immunocompromised patients. CMV downregulates major histocompatibility complex class I (MHC-I) molecule expression in fibroblasts in order to avoid lysis by CD8⁺ cytotoxic T lymphocytes. However, MHC-I expression in human cytomegalovirus (HCMV)-infected hepatic tissue was reported to be increased. As it is unclear at present whether HCMV affects MHC-I expression in epithelial cells, new cell culture models for HCMV infection of differentiated hepatobiliary cell lines were established. HCMV immediate early gene expression was achieved in 60 to 95% of cells. Progression of the HCMV replication cycle differed from prototypic infection of fibroblasts, since structural early and late proteins were produced at low levels and HCMV progeny yielded much lower titres in hepatobiliary cells. In contrast, HCMV glycoproteins, gpUS2, gpUS3, gpUS6 and gpUS11, that downregulate MHC-I expression were synthesized with temporal kinetics and in a similar quantity to that seen in fibroblasts. As a result, HCMV infection led to a drastic and selective downregulation of MHC-I expression in epithelial cells and was uniformly observed irrespective of the hepatic or biliary origin of the cells. The new models document for the first time a stealth function of HCMV in epithelial cells and indicate that the downregulation of MHC-I expression by HCMV can occur in the virtual absence of virus replication.

Introduction

Cytomegaloviruses (CMV) represent prototype viruses of the β-subgroup of the family Herpesviridae. Human cytomegalovirus (HCMV) gene expression in infected organs has been documented in a broad array of cell types and tissues, in particular in epithelial and endothelial cells, but also in fibroblasts, smooth muscle cells and macrophages (Sinzger et al., 1995, 1999). HCMV infection in the immunocompetent host does not in most cases cause a significant illness, although a mononucleosis-like syndrome, including a benign cause of hepatitis, has been observed (Snover & Horwitz, 1984; Ten Napel et al., 1984). Immune control does not achieve complete clearance of the virus. Instead, the viral genome persists in a nonproductive form. From latency, CMV replication is frequently reactivated and can result in recurrent infection and virus shedding. Impairment of cellular immune control represents a prime risk factor for CMV recurrence and disease (Britt & Alford, 1996). In AIDS patients, persistent virus replication leads to tissue injury in some but not all organs, of which the colon represents the most common gastrointestinal site of disease (Burakoff & Eglow, 1995). In organ transplant recipients, CMV infection frequently complicates post-transplantation (Payá et al., 1989; Arnold et al., 1992; Lautenschlager et al., 1997). After liver transplantation, CMV may cause severe hepatitis and cholangitis, leading to allograft dysfunction and, if untreated, to death (Stratta et al., 1989; Lautenschlager et al., 1990).
Previous work has demonstrated that CMV downregulates the surface expression of major histocompatibility complex class I (MHC-I) molecules (Del Val et al., 1992; Beersma et al., 1993; Yamashita et al., 1993). In productively infected fibroblasts, presentation of antigens to MHC-I-restricted CD8+ T cells is abolished (Del Val et al., 1992; Warren et al., 1994; Hengel et al., 1995). Analysis of HCMV deletion mutants led to the identification of genes within the short segment of the HCMV genome, i.e. US2, US3, US6 and US11, all of which can mediate this effect independently (Jones et al., 1995, 1996; Hengel et al., 1996, 1997; Wiertz et al., 1996a, b; Ahn et al., 1996, 1997; Lehner et al., 1997). These genes belong to two related gene families encoding type I transmembrane glycoproteins. The common phenotype of transfected cells expressing these genes is a drastic reduction of MHC-I molecules on the cell surface. HCMV proteins employ sophisticated molecular mechanisms to interrupt the MHC-I pathway at distinct checkpoints. gpUS2 and gpUS11 displace nascent MHC-I molecules from the endoplasmic reticulum (ER) back to the cytosol for rapid proteolytic degradation by proteasomes (Wiertz et al., 1996a, b). gpUS3 was shown to retain assembled MHC-I complexes in the ER, thus preventing transport to the cell surface (Ahn et al., 1996; Jones et al., 1996). The US6-encoded glycoprotein does not directly interact with MHC-I molecules, but inhibits the transporter associated with antigen processing-mediated transport of cytosolic peptides into the ER, thereby blocking the assembly of the peptide containing ternary MHC-I complexes (Hengel et al., 1997; Ahn et al., 1997; Lehner et al., 1997). To cover all phases of the protracted HCMV replication cycle, the synthesis of genes that control MHC-I surface expression is tightly regulated. Whereas the immediate early (IE) gene US3 is transcribed from 3 to 6 h post-infection (p.i.) (Ahn et al., 1996), US2 and US11 expression peaks in the early phase, between 24 and 48 h p.i., of virus replication (Jones & Muzithras, 1991). gpUS6 is the most abundant protein in the late phase of the HCMV replication cycle (Hengel et al., 1997).

While several studies have demonstrated downregulation of MHC-I molecules in productively CMV-infected fibroblasts leading to the abrogation of T cell recognition (Del Val et al., 1992; Beersma et al., 1993; Yamashita et al., 1993), presentation of murine CMV (MCMV) peptides by MCMV-infected macrophages to CD8+ cytotoxic T lymphocytes (CTL) was highly efficient and MHC-I expression in infected macrophages was essentially unaffected (Hengel et al., 2000). Apparently, the type of cell used to analyse CMV infection may profoundly affect many characteristics of the infection process, including viral gene expression and protein trafficking. Previous reports have suggested an increase of MHC-I expression in HCMV-infected liver (Steinhoff et al., 1988; Arnold et al., 1991, 1992) and biliary epithelial cells in vitro (Scholz et al., 1997). We have established new cell culture models for HCMV infection of liver and biliary cells to analyse the fate of MHC-I molecules. The data document a strong and selective loss of MHC-I assembly and surface expression on infected epithelial cells. Despite a very low level of HCMV replication, all HCMV proteins that affect MHC-I expression were efficiently synthesized with similar kinetics to those seen in fibroblasts, indicating that MHC-I modulation is not linked to productive infection.

### Methods

#### Cells

MRC5 foetal human lung fibroblasts (passages 6–20) (BioWhittaker) were grown in Dulbecco’s minimal essential medium (DMEM) (Gibco BRL) supplemented with 10% foetal calf serum (FCS), penicillin, streptomycin and 2 mM glutamine. Human foreskin fibroblast (HFF) cells were established following standard procedures by mincing the skin and subsequently stirring it in pre-warmed trypsin. Recovered fibroblasts were passaged in DMEM supplemented with 10% FCS, penicillin, streptomycin and 2 mM glutamine and used between culture

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**Table 1. Efficiency of HCMV infection of epithelial cell lines and HFF**

Cells were infected with HCMV strain AD169 at an m.o.i. of 10, as described in Methods. Immunofluorescence microscopy was performed 72 h p.i. after staining the cells with an IE1/pp72-specific MAb. Evans blue was used as the counterstain. A total of 100 individual cells was analysed in at least two independent experiments.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Description</th>
<th>Source</th>
<th>IE1/pp72-positive cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFF</td>
<td>Primary fibroblast</td>
<td>Hengel et al. (1995)</td>
<td>&gt; 95</td>
</tr>
<tr>
<td>Huh7</td>
<td>Hepatoma</td>
<td>Nakabayashi et al. (1982)</td>
<td>&gt; 90</td>
</tr>
<tr>
<td>MzChA2</td>
<td>Biliary carcinoma</td>
<td>Knuth et al. (1985)</td>
<td>&gt; 80</td>
</tr>
<tr>
<td>Hep3B</td>
<td>Hepatoma</td>
<td>Aden et al. (1979); HB-8064 (ATCC)</td>
<td>&gt; 75</td>
</tr>
<tr>
<td>Caco2</td>
<td>Colon carcinoma</td>
<td>HTB-37 (ATCC)</td>
<td>&gt; 60</td>
</tr>
<tr>
<td>MzChA1</td>
<td>Biliary carcinoma</td>
<td>Knuth et al. (1985)</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>PLC/PREF/5</td>
<td>Hepatoma</td>
<td>Gabay et al. (1995)</td>
<td>&lt; 20</td>
</tr>
<tr>
<td>HepG2</td>
<td>Hepatoma</td>
<td>HB-8065 (ATCC)</td>
<td>&lt; 10</td>
</tr>
</tbody>
</table>
Fig. 1. HCMV gene expression in HFF, Huh7 and MzChA2 cells. HFF (a), Huh7, (b) and MzChA2 (c) cells were infected with HCMV strain AD169 at an m.o.i. of 5. At 24 h.p.i., monolayers were fixed, permeabilized and labelled for the presence of nuclear IE1/pp72 antigen using a pp72-specific MAb and an FITC-conjugated goat anti-mouse IgG. The distribution of pp72 was visualized by confocal laser scanning microscopy (i) and the cell morphology of the same cells visualized by counterstaining with Evans blue (ii) is shown.

Viruses, infection conditions and virus titration. Virus stocks were prepared by propagating HCMV strains AD169, Towne, Toledo and a low passage clinical isolate, UL1271, derived from a congenitally infected patient in MRC5 cells. Infectious supernatants were harvested when 100% of cells showed cytopathic effects. Virus titres were determined by standard plaque assay; the titre of each virus stock was between approximately 1 x 10^5 and 5 x 10^7 p.f.u./ml. HCMV infection was enhanced by centrifugation at 800 g for 30 min. To achieve cell-cycle synchronization, subconfluent monolayers of HFF, Huh7, Hep3B, MzChA2 and Caco2 cells were maintained in serum-free DMEM for 24 h before cells were infected with HCMV at an m.o.i. of 5 or 10. In all experiments, control settings were included to ensure IE1/pp72 expression, which was assessed by immunofluorescence microscopy (Table 1). Cells were infected with HCMV strain AD169 at an m.o.i. of 5 to determine the growth kinetics of HCMV in HFF and epithelial cells. Input virus was removed by exchanging the culture medium. Supernatant cells were collected at the indicated time-points p.i. After homogenizing the cells, HCMV infectivity was determined in p.f.u./ml by endpoint titration in MRC5 fibroblasts.

Reagents and antibodies. The antibodies used in this study are: monoclonal antibody (MAb) W6/32 (HB-95; ATCC) recognizing HMC-1 heavy chain/β2 microglobulin (Parham et al., 1979); polyclonal rabbit antisera recognizing calnexin (StressGen Biotechnologies); anti-HCMV gpUS6 MAb (Hengel et al., 1997); anti-HCMV gpUS2 MAAb (a kind gift from E. Wiertz, Bilthoven, The Netherlands; Wiertz et al., 1996); anti-HCMV gpUS11 and gpUS3 MAb (a kind gift from K. Früh, San Diego, CA, USA; Ahn et al., 1996); anti-HCMV IE antigen pp72/pp86 MAb (Du Pont NEN); anti-HCMV IE antigen pp72 MAb 63-27 (Andreoni et al., 1989); anti-HCMV IE antigen pp86 MAb 295 (Plachter et al., 1993); anti-HCMV glycoprotein B (gB) MAb 27156 (Spaete et al., 1988); anti-HCMV pp28 MAb (ABI); anti-HCMV major capsid protein (MCP) UL86 MAb 28-4 (Sanchez et al., 1998); fluorescein-conjugated mouse anti-IE antigen pp72 MAb (Chemicon); anti-intercellular adhesion molecule-1 (ICAM-1) (CD54) MAb; anti-CD44 MAb; anti-CD49a MAb (Dianova); fluorescein-conjugated goat anti-mouse IgG (Sigma); indocarbocyanin (Cy5)-conjugated goat anti-mouse IgG (Dianova); and peroxidase-conjugated affinity pure goat anti-mouse IgG (H+L) (Dianova).

Western blot analysis. Following serum starvation, cells were either infected or mock-infected with virus at an m.o.i. of 10. Cells were then detached with trypsin, washed in ice-cold PBS, pelleted and lysed in lysis buffer containing 0.125 M Tris, pH 6.8, 2% SDS, 20% glycerol, 1% mercaptoethanol and 0.5% bromophenol blue. After heating the samples at 95 °C for 5 min, lysates were separated by 10% SDS-PAGE, transferred to nitrocellulose and probed with the Epp72/pp86 and anti-calnexin MAb, respectively. Peroxidase-conjugated antibodies, used as the secondary reagent, were detected with the ECL plus detection kit (Amersham–Pharmacia).

Flow cytometry. To analyse MHC-I surface expression, HFF, Huh7, Hep3B and Caco2 cells were infected with HCMV strain AD169 at an m.o.i. of 10 and incubated for 3 days. After harvesting, cells were pre-incubated in 10% goat serum (Dianova) and immunostained with MAb W6/32. As a control, cells were immunostained with an anti-ICAM-1 MAb. Bound antibodies were visualized by the addition of fluorescein-conjugated goat anti-mouse IgG. As a negative control, cells were incubated with only the fluorescein-conjugated goat anti-mouse IgG antibody. A total of 10^4 cells was analysed for each fluorescence histogram on a FACScan IV (Becton Dickinson). Double-stain analysis of fluorescein-conjugated MHC-I surface expression and HCMV IE1/pp72 nuclear expression was performed as follows: 3 days after infection at an m.o.i. of 10, cells were harvested, pre-incubated with 10% goat serum and immunostained with MAb W6/32. Bound antibodies were visualized by the addition of Cy5-conjugated goat anti-mouse IgG antibody and fixed in 3% paraformaldehyde. After permeabilization in cold methanol, cells were centrifuged and resuspended in 0.2 ml nuclear extraction buffer (1 μM NaCl, 0.5 μM MgSO4, 0.6% NP-40, 2% FCS and 0.01 M PBS) (Thornthwaite et al., 1980) for 2 min. The cells were then immunostained with the fluorescein-conjugated anti-IE1/pp72 MAb and fixed in 3% paraformaldehyde. As a negative control, cells were incubated with only the Cy5-conjugated goat anti-mouse IgG antibody. A total of 10^4 cells was analysed for each fluorescence profile. Discrimination between expression levels was performed with quadrant statistics using the Cell Quest software (Becton Dickinson). After 72 h of incubation, Huh7 and HFF cells were harvested and double-stained for MHC-I surface expression and IE1/pp72 nuclear expression.
Cloning procedures and transfectants. The HCMV US6 and US11 genes were cut from pcDNAI constructs (Hengel et al., 1996, 1997) and subcloned into the Sall site of the p845-Neo vector (Ohe et al., 1995). Huh7 cells were transfected with plasmid DNA using the SuperFect transfection reagent (Qiagen). Cell clones were selected in the presence of 0.5 mg/ml G418 and tested for gpUS6 and gpUS11 protein expression, respectively, with specific antibodies.

Metabolic labelling and immunoprecipitation. Subconfluent monolayers of HFF, Huh7 and MzChA2 cells were infected with HCMV strain AD169. At 24, 48 and 72 h p.i., MHC-I complexes and HCMV proteins gpUS2, gpUS3 and gpUS11 were immunoprecipitated. HCMV-infected cells were washed and starved in cysteine- and methionine-free RPMI 1640 before labelling with [35S]methionine and [35S]cysteine (1200 Ci/mmol) (Amersham–Pharmacia) at a concentration of 140 mM NaCl, 20 mM Tris, pH 7.6, 5 mM MgCl2 and 0.2 mM PMSF with 1% NP-40. As a control, mock-infected cell lysates were precipitated with each antibody. Detection of gpUS6 and gpUS11 was controlled by the precipitation of proteins from lysates of Huh7 cells stably transfected with pB45-US6 and pB45-US11. The incorporation of [35S]S into proteins was quantified in all experiments by liquid scintillation counting of the lysate. All lysates used were adjusted to ensure comparability in quantitative terms. After the removal of cell nuclei by centrifugation, lysate. All lysates used were adjusted to ensure comparability in quantitative terms. After the removal of cell nuclei by centrifugation.

Establishment of cell culture models for HCMV infection in epithelial cells from the liver and colon

Tissue tropism of HCMV in vivo is broad and includes epithelial cells from different organs (Sinzger et al., 1995). To develop new cell culture models for HCMV infection of cells with obvious pathophysiological significance, we systematically screened a panel of epithelial cell lines derived from the liver, gut, kidney, exocrine glands and placenta for their susceptibility to HCMV infection. The expression of non-structural HCMV IE1/pp72 nuclear antigen was visualized by immunofluorescence using an IE1/pp72-specific MAb. The proportion of pp72-positive cells varied widely between cell lines, exceeding 50% in the case of differentiated Huh7 and Hep3B hepatoma cells and less-well differentiated biliary adenocarcinoma MzChA2 cells. To further improve efficiency, selected parameters of infection were modified. Among these, serum starvation leading to the synchronization of the cell cycle and centrifugal enhancement of infection significantly increased the number of cells infected with HCMV. Depending on the cell line, between 60 and 95% of cells were found to be IE1/pp72-positive (Table 1 and Fig. 1).

Viral protein synthesis and productivity of HCMV-infected epithelial cells

Biosynthesis of HCMV marker proteins of the IE, early and late replication phase was analysed. Using Western blots, IE1/pp72 was detected in AD169-infected Huh7, MzChA2 and Hep3B cells at similar levels from 12 h p.i. to 168 h p.i., similar to that seen in permissive MRC5 fibroblasts (Fig. 2a). IE2/pp86 expression in MRC5 fibroblasts continued to increase over time. In MzChA2 and Huh7 cells, the level of IE2/pp86 required for its detection in Western blots was not reached (Fig. 2a). In AD169-infected Hep3B cells, IE2/pp86 was detected between 24 and 168 h p.i., albeit at significantly lower levels compared to MzChA2 and Huh7 cells (Fig. 2b). In all cell lines, the level of pp72 nuclear antigen was visualized by IE1/pp72-specific MAb. The incorporation of [35S]S into proteins was quantified in all experiments by liquid scintillation counting of the lysate. All lysates used were adjusted to ensure comparability in quantitative terms. After the removal of cell nuclei by centrifugation, lysate.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>MRC5</th>
<th>Huh7</th>
<th>MzChA2</th>
</tr>
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<tbody>
<tr>
<td>72</td>
<td>0</td>
<td>72</td>
<td>0</td>
</tr>
<tr>
<td>120</td>
<td>0</td>
<td>72</td>
<td>0</td>
</tr>
<tr>
<td>168</td>
<td>0</td>
<td>72</td>
<td>0</td>
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Fig. 2. Synthesis of HCMV proteins in infected epithelial cells. (a) MRC5 fibroblasts, HFF, Huh7, Hep3B and MzChA2 cells were infected with HCMV strain AD169 at a m.o.i. of 10. Cell lysates were harvested at the indicated time-points and adjusted for protein content. IE1/pp72 and IE2/pp86 were detected with IE-specific MAbs in Western blots. As a control, calnexin was detected using a specific antiserum. At 72 h p.i., cells were metabolically labelled for 60 min and lysates precipitated with antibodies specific for gb (b), pp65 (c) and pp28 (d) before being separated by SDS–PAGE. Note the background band in (d) migrated more slowly than the band corresponding to pp28, which is only detectable in HCMV-infected HFF cells.
lower levels as compared to MRC5 cells. This type of result was also found during immunoprecipitation studies of metabolically labelled cells using IE1/pp72- and IE2/pp86-specific antibodies (data not shown). In metabolically labelled cells, expression of the matrix phosphoprotein UL83/pp65 (Fig. 2c) and UL86/MCP (data not shown) was clearly lower in all epithelial cells as compared to fibroblasts, while synthesis of gB in epithelial cells reached almost similar amounts to those seen in fibroblasts (Fig. 2b), but depended on the virus titre used for infection. To test the biosynthesis of a true late protein, UL99/pp28 was precipitated 4 days p.i. While readily found in fibroblasts, pp28 synthesis could not be detected in any of the epithelial cells (Fig. 2d). These findings suggested that the HCMV replication cycle may be less frequently completed in epithelial cells compared with fibroblasts. Quantitative assessment of AD169 progeny in epithelial cells revealed a more delayed kinetics of virus production in liver and colonic cell lines and reached titres of varying magnitude, but were significantly lower than levels seen in fibroblasts (Fig. 3). To test whether infection of epithelial cells with a low passage clinical isolate strain of HCMV might be more productive, we plotted single-step growth curves of UL1271, a clinical isolate derived from a congenitally infected infant. As depicted in Fig. 3, productivity of infection was not increased compared with AD169. From these data, we concluded that infection of Huh7, Hep3B and MzChA2 cells with HCMV is efficient, although productivity is significantly less (Hep3B, MzChA2) or virtually abortive (Huh7) compared with fibroblasts.

**HCMV infection prevents surface expression and assembly of MHC-I complexes in hepatobiliary and colonic cells**

HCMV infection of fibroblasts results in a strong and selective downregulation of MHC-I surface expression (Beersma et al., 1993; Yamashita et al., 1993; Warren et al., 1994; Hengel et al., 1995). In contrast, immunocytochemical analysis of MHC-I expression in the liver of CMV-infected patients (Steinhoff et al., 1988; Arnold et al., 1991, 1992) and cytofluorometric analysis of infected epithelial cells (Scholz et al., 1997) suggested an upregulation of MHC-I expression, implying that MHC-I expression in these cells was resistant to the effects of HCMV. In view of the conflicting reports, we determined MHC-I surface expression in Huh7, Hep3B and MzChA2 cells during the course of HCMV infection. Similar to HFF cells, all epithelial cells had a large decrease in MHC-I surface density compared with mock-infected cells following AD169 infection (Fig. 4a). Loss of MHC-I surface expression in hepatobiliary and colonic cells was almost as efficient as that seen in HFF cells and occurred selectively, since other molecules expressed on the cell surface, e.g. ICAM-1, CD44 and CD49a, were essentially not affected by HCMV infection (Fig. 4a; data not shown). Likewise, MHC-I downregulation was observed in epithelial cells infected with the HCMV strains Towne and Toledo (data not shown). In HFF cells, ICAM-1 surface expression was upregulated following HCMV infection, as reported previously (Ito et al., 1995). To analyse the fate of newly synthesized MHC-I molecules, the formation of MHC-I complexes in the ER was compared in AD169-infected HFF, Huh7, Hep3B and MzChA2 cells during the course of infection. Metabolic labelling and immunoprecipitation with the conformation-dependent MAb W6/32 revealed a higher level of constitutive MHC-I complex assembly in HFF cells compared with hepatobiliary cells (Fig. 4b). As expected, MHC-I complexes are greatly diminished in HFF cells upon HCMV infection and remained at low levels throughout the complete replication cycle. This phenotype was perfectly reproduced in HCMV-infected Huh7 and MzChA2 cells (Fig. 4b) as well as in Hep3B cells (data not shown) at all time-points p.i., suggesting that genes that downregulate MHC-I expression, other than IE US3, are synthesized. To prove that cells exhibiting a reduced MHC-I phenotype were indeed HCMV-infected, double-staining for HCMV IE1/pp72 and surface MHC-I molecules was performed. As seen in Fig. 5, pp72 antigen was detected in 97.9% of Huh7 cells and in 92.2% of HFF cells. Among Huh7 cells,
Fig. 4. HCMV infection downregulates MHC-I surface expression in Huh7, Hep3B and MzChA2 cells. (a) HFF, Huh7, Hep3B and MzChA2 cells were infected with HCMV strain AD169 at an m.o.i. of 10. At 72 h p.i., mock-infected (thin lines) and infected (bold lines) cells were harvested and stained with MAb W6/32, specific for MHC-I complexes. Bound antibodies were visualized by the addition of FITC-conjugated goat anti-mouse IgG. As a negative control, mock-infected (dotted lines) and HCMV-infected (dashed lines) cells were stained with the second antibody alone. Likewise, cells were stained with an ICAM-1-specific MAb (CD54). (b) Inhibition of MHC-I complex formation by HCMV in hepatobiliary cells. HFF, Huh7 and MzChA2 cells were infected with HCMV strain AD169 at an m.o.i. of 10 for 24, 48 and 72 h or left untreated before metabolic labelling for 60 min. Cell lysates were precipitated with MAb W6/32 and immunoprecipitates were separated by 11.5–13.5% gradient SDS–PAGE. MHC-I heavy chains (HC) and β2-m are indicated by arrowheads.

Fig. 5. Huh7 cells exhibiting a reduced MHC-I phenotype express HCMV IE1/pp72. Huh7 and HFF cells were infected with HCMV strain AD169 at an m.o.i. of 10 or mock-infected. After 72 h, cells were harvested and stained with MHC-I-specific MAb W6/32 and Cy5-conjugated goat anti-mouse IgG. Next, cells were fixed, permeabilized and incubated with an FITC-conjugated IE1/pp72-specific MAb. Quadrant statistics were performed using the Cell Quest software. The percentage of cells is indicated in each rectangle. 80.7% were pp72-positive and exhibited a reduced MHC-I phenotype, whereas 88.8% of all HFF cells showed this pattern. Taken together, these data show that HCMV efficiently controls MHC-I expression in infected liver epithelial cells, despite comparatively poor or absent HCMV replication.

Sequential expression of HCMV proteins that affect MHC-I expression

In highly permissive fibroblasts, HCMV genes that downregulate MHC-I expression are synthesized in a multi-step order (Jones & Muzithras, 1991; Ahn et al., 1996). Therefore, we analysed the synthesis and kinetics of glycoproteins affecting MHC-I expression in weakly permissive Huh7 and MzChA2 cells. Expression of gpUS3 shows IE kinetics and peaks a few hours after infection of fibroblasts (Ahn et al., 1996). Infection at an m.o.i. of 10 led to the synthesis of both US3 products of 23 and 17 kDa, respectively, at similar levels in Huh7 and HFF cells, but at a slightly lower level in MzChA2 cells. Likewise, the expression of gpUS11 and gpUS2 in Huh7, MzChA2 (Fig. 6) and Hep3B cells (data not shown) was comparable to that seen in HFF cells after 24 and 48 h of infection and decreased at later time-points in each cell type. Moreover, synthesis of gpUS11 in infected Huh7 cells exceeded that in a stable Huh7-US11 transfectant (Fig. 6) showing a strong reduction of MHC-I molecules on the cell surface (data not shown). gpUS6 is characterized by early–late kinetics, reaching the highest expression levels 72 h p.i. (Hengel et al., 1997). Remarkably, the 21 kDa gpUS6 early late protein was detected at comparable amounts in HFF, Huh7 and MzChA2 cells, reaching levels similar to those found in a stable Huh7-US6 transfectant (Fig. 6). Overall, the rate of synthesis of
MHC-I downregulating proteins correlated with the virus titre used for infection. In conclusion, all HCMV proteins that affect MHC-I expression are abundantly synthesized in infected hepatobiliary epithelial cells irrespective of their temporal classification.

Discussion

In this study, we have demonstrated that HCMV infection inhibits MHC-I surface expression in epithelial cells of hepatic and biliary origin. The loss of MHC-I surface expression in HCMV-infected epithelial cells reproduces the phenotype observed in fibroblasts. Indeed, all CMV genes that encode MHC-I downregulating proteins, i.e. US3, US2, US11 and US6, were expressed in the same temporal order and at similar quantitative levels in epithelial cells to those seen in the prototypic infection of highly permissive fibroblasts. Since Huh7, Hep3B, MzChA2 and Caco2 cells are little, if at all, productive for HCMV replication, this finding indicates that, in principle, the loss of MHC-I expression does not correlate with CMV replication and is not necessarily restricted to productively infected cells.

CMV exhibits broad cell tropism in vivo. In infected organs, CMV was primarily identified in epithelial cells, including hepatocytes, bile duct cells and colon mucosa as well as endothelial cells (Ten Napel et al., 1984; Paya et al., 1990; Theise et al., 1993; Barkholt et al., 1994; Sizinger et al., 1995, 1999; Podlech et al., 1998). Currently, most in vitro studies concerning HCMV–host cell interactions are conducted with embryonic or dermal fibroblasts. Only a few attempts of HCMV infection of differentiated epithelial tissues like retinal epithelial cells or syncytiotrophoblasts have been reported in the past (Tugizov et al., 1997; Hemmings et al., 1998; Halwachs-Baumann et al., 1998; Sizinger et al., 1999). A small number of primary epithelial cells has been evaluated for supporting the growth of HCMV, but the preparation of cells from organs is cumbersome and the purity and characterization of these cells is limited (Hemmings et al., 1998; Sizinger et al., 1999). This holds particularly true for the liver, which consists of hepatocytes and bile duct epithelial cells, but in addition, of stromal, Kupffer and Ito cells. The complexity of primary liver cell preparations and the poor availability of organ material from healthy donors are major obstacles for experimental analysis of the effects of the virus on defined cell types. To generate epithelial cell culture models of HCMV infection, we systematically screened a panel of differentiated cell lines for their susceptibility to HCMV–host cell interactions and varied infection conditions to optimize infection efficiency. In this way, we were readily able to select liver and intestinal cell lines that supported infection with all laboratory HCMV strains and clinical isolates. The cells produced HCMV proteins IE1/pp72, IE2/pp86, pp65, gB and UL86/MCP, which represent different temporal classes of proteins, but with variable rates of synthesis compared with those seen in fibroblasts. Notably, the liver cell lines
Hep3B and MzChA2 supported HCMV production at least as efficiently as reported recently in primary hepatocytes (Sinzheimer et al., 1999). Reflecting the high tropism of HCMV for these epithelial cells, these cell lines provide suitable models for studying HCMV pathogenesis at a molecular level in vitro. Our finding that CaCo2 cells are readily infected and exhibit a reduced MHC-I phenotype after HCMV infection (unpublished observation) adds to recent reports that demonstrate that HCMV enters the basolateral surface of polarized CaCo2 cells (Jarvis et al., 1999; Esclatine et al., 2000).

Applying the newly established cell culture models, we assessed the impact of HCMV infection upon MHC-I expression in cells of hepatic, biliary and colonic origin. Previous attempts to analyse MHC-I expression on cultured primary biliary epithelial cells revealed conflicting results to ours, suggesting that MHC-I surface expression is upregulated rather than downregulated (Scholz et al., 1997). However, HCMV gene expression had not been determined, which raises the question of whether efficient infection of cells was achieved or not. Our data reveal a clear reduction of MHC-I molecules upon HCMV infection at the level of both complex formation and surface expression. Most convincingly, we demonstrate the abundant expression of the HCMV proteins responsible for controlling the MHC-I pathway. Their molecular functions have been elucidated in great detail when expressed in isolation (Wiertz et al., 1996a, b; Ahn et al., 1996, 1997; Jones et al., 1996; Hengel et al., 1997; Lehner et al., 1997), but there is little knowledge of their biological significance in infected tissues so far. Our data show that the factors controlling MHC-I expression also operate in parenchymal cells of the liver and colon. It is plausible to assume that the HCMV genes that regulate MHC-I expression promote virus pathogenesis in epithelial tissues in vivo, since animal studies using MCMV mutants deficient in these gene functions showed that the proteins that affect the MHC-I pathway facilitate virus replication and contribute to CMV pathogenesis and virulence in the infected organs, including the liver (Krmpotic et al., 1999).

Sequential phases of productive and nonproductive infection characterize the natural life-cycle of CMV in vivo (Jordan, 1983). In light of our findings, it is therefore tempting to speculate that MHC-I control by CMV is not only used as a mechanism for escaping the immune system during productive infection but may also occur in latently infected cells and in those initiating the replicative program. Future analysis of the transcriptional programs during latency and reactivation of productive infection should therefore include the genes that regulate MHC-I expression. Mice infected with MCMV mutants deficient in MHC-I downregulating genes (Krmpotic et al., 1999) may provide a suitable model to test whether genes regulating MHC-I expression are beneficial in maintaining latency and supporting productive reactivation.

On the other hand, avoidance of CD8+ T cell recognition and MHC-I expression by CMV has remarkable exceptions. Macrophages, shown to be exempt from effective class I downregulation, were readily lysed by MCMV-specific CTL (Hengel et al., 2000). According to current concepts, nonhaematopoietic cells are unable to induce a primary CTL response but guide immune recognition of primed T cell responses (Sigal et al., 1999). Considering the fact that HCMV controls MHC-I expression in fibroblasts and epithelial cells, and MCMV controls expression in fibroblasts but not in macrophages, it appears that the effector rather than the induction phase of the antiviral CD8+ T cell response is counteracted by CMV. However, inhibition of antigen presentation of infected nonhaematopoietic cell types is also not perfect, but regulated by certain cytokines. Interferon (IFN)-γ possesses the capacity to restore MHC-I expression and peptide presentation of fibroblasts to CD8+ T cells (Hengel et al., 1994, 1995). In epithelial cells, this effect is particularly pronounced. While fibroblasts largely lost responsiveness to IFN-γ after infection (Miller et al., 1998), IFN-γ efficiently induced MHC-I expression in a significant number of pre-infected epithelial cells without affecting the expression of the relevant HCMV proteins (unpublished observation). Thus cytokines, like IFN-γ, could explain the paradoxical finding that hepatocytes in HCMV-infected liver tissue express higher levels of MHC-I compared with healthy controls (Steinhoff et al., 1988; Arnold et al., 1991, 1992).

In summary, this study shows for the first time that CMV infection downregulates MHC-I expression in a multitude of epithelial cell types even in the absence of efficient virus replication. This panel of hepatic and intestinal cell lines represents well-characterized and permissive cellular models for analysis of HCMV pathogenesis in distinct epithelial tissues of clinical importance. They will offer the opportunity to study whether natural HCMV infection may also deal with nonclassical MHC gene products exclusively expressed in these tissues (Balk et al., 1994; Groh et al., 1996). A further application is to test HCMV mutants with targeted gene disruptions in order to define the specific roles of CMV genes on tissue-specific pathogenesis.

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**References**


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