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Feline Herpesvirus-1 Down-Regulates MHC Class I Expression in an Homologous Cell System

S. Montagnaro,[1*] M. Longo,[1] M. Pacilio,[1] P. Indovina,[2,3] A. Roberti,[2,4] L. De Martino,[1] G. Iovane,[5] and U. Pagnini[1]

[1] Department of Pathology and animal Health, School of Veterinary Medicine, University of Naples "Federico II", Via Delpino n° 1, 80137 Naples, Italy

[2] Department of Human Pathology and Oncology, University of Siena, Siena, Italy

[3] CROM—Center of Oncology Research Mercogliano, Avellino, Italy

[4] Sbarro Institute for Cancer Research and Molecular Medicine, Center for Biotechnology, College of Science and Technology, Temple University, Philadelphia Pennsylvania

[5] Experimental Zooprophylactic Institute of Southern Italy, Portici, Naples, Italy

ABSTRACT

Cytotoxic T lymphocytes (CTLs) are an essential component of the immune defense against many virus infections. CTLs recognize viral peptides in the context of the major histocompatibility complex (MHC) class I molecules on the surface of infected cells. Many viruses have evolved mechanisms to interfere with MHC class I expression as a means of evading the host immune response. In the present research we have studied the effect of in vitro Feline Herpesvirus 1 (FeHV-1) infection on MHC class I expression. The results of this study demonstrate that FeHV-1 down regulates surface expression of MHC class I molecules on infected cells, presumably to evade cytotoxic T-cell recognition and, perhaps, attenuate induction of immunity. Sensitivity to UV irradiation and insensitivity to a viral DNA synthesis inhibitor, like phosphonacetic acid, revealed that immediate early or early viral gene(s) are responsible. Use of the protein translation inhibitor cycloheximide confirmed that an early gene is primarily responsible. J. Cell. Biochem. 106: 179–185, 2009. © 2008 Wiley-Liss, Inc.

KEY WORDS: FeHV-1; MHC CLASS I MOLECULES; DOWNREGULATION

Feline Herpesvirus type 1 (FeHV-1), a member of the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, is considered to be one of the most important pathogens in cats causing, primarily, an upper respiratory tract disease known as feline viral rhinotracheitis (FVR) [Crandell and Despeaux, 1959]. Ocular disease, abortion in pregnant queens, neurological disorders and virus generalization, with mortality reaching 50% in kittens, have also been observed [Povey, 1979].

FeHV-1 is transmitted to susceptible cats via close contact and transmission of bodily fluids, particularly respiratory, and ocular secretions [Stiles, 2003; Gaskell et al., 2007].

The majority of cats become infected via mucous membranes as young, immunologically naïve kittens. The virus rapidly replicates in epithelial cells at these sites; especially the conjunctiva and then ascends via axons of sensory neurons to establish lifelong latency within the trigeminal ganglia [Gaskell et al., 2007].

Herpesviruses are distinguished by their ability to establish lifelong infection cycling between lytic and latent phases. One challenge to this lifestyle is that the immune system of the vertebrate hosts has the opportunity to be repeatedly primed, thereby increasing the potential for the host to eradicate the pathogen. To cope with this challenge, herpesviruses have evolved multiple mechanisms to evade immune detection or clearance. These mechanisms target all aspects of the immune response, including antibodies, chemokines, cytokines, natural killer (NK) cells, CD4, and CD8 T cells [Alcami and Koszinowski, 2000; Tortorella et al., 2000].

Major histocompatibility complex (MHC) class I molecules are present on virtually all somatic cells. These molecules are generated by a highly intricate multi-step antigen-presentation pathway, which involves proteasomal degradation of cytosolic proteins (including viral proteins) into short peptides, ATP-dependent transport of these peptides from the cytosol into the lumen of the ER by the transporter associated with antigen processing (TAP), binding of peptides by class I molecules and egress from the ER via the Golgi apparatus for expression on the cell surface [Williams et al., 2002].

*Correspondence to: Dr. S. Montagnaro, Department of Pathology and Animal Health, School of Veterinary Medicine, University of Naples "Federico II", Via Delpino 1, 80137 Napoli, Italy. E-mail: semontag@unina.it

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Thus, interference with any of the steps in the class I antigen presentation pathway provides the virus with a means of escape from elimination by the host immune system.

MHC class I molecules are a particularly attractive target for immune evasion by viruses, because decreasing expression and/or antigen presentation by MHC class I can attenuate CD8 T-cell-mediated recognition of infected cells [Fruh et al., 1999; Yewdell and Hill, 2002].

Many viruses have evolved mechanisms to interfere with MHC class I expression as a means of evading the host immune response. This is particularly true for herpesviruses and may relate to the need to establish latency [Ploegh, 1998]. Infection of appropriate host cells with herpes simplex virus (HSV), human and mouse cytomegalovirus (HCMV, MCMV), varicella-zoster virus (VZV), Bovine herpesvirus 1 (BHV-1), Equine herpesvirus 1 (EHV-1) and pseudorabies virus (PrV) all result in down-regulation of MHC class I expression [Mellencamp et al., 1991; Cohen, 1998; Johnson and Hill, 1998; Koppers-Lalic et al., 2001; Rappocciolo et al., 2003]. However the mechanisms involved are only clearly understood and well characterized in few herpesvirus infections. These mechanisms include interference with peptide transport, retention of MHC class I heavy chains in the endoplasmic reticulum, direction of mature heavy chains to the endocytic pathway and rapid degradation of MHC class I at the cell surface. A number of different viral genes are involved in these processes, but not all related viruses carry homologous genes [Rappocciolo et al., 2003].

In the present study, the expression of MHC-I molecules on CrFK cell line was investigated to indicate the possible effects of FeHV-1 infection. The data generated may shed light on the mechanisms used by FeHV1 to subvert the host immune response.

MATERIALS AND METHODS

CELLS AND CULTURE CONDITION

Crandell Feline Kidney (CrFK) cells (American Type Culture Collection CCL94) were maintained in complete medium (CM) consisting of Dulbecco Minimal Essential Medium (DMEM) containing 10% foetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37°C in a CO_2 atmosphere.

VIRUS

The FeHV-1 strain Ba/91 (kindly provided by Prof. C. Buonavoglia, School of Veterinary Medicine of Bari) used in this study was propagated and titrated on Crandell's Feline Kidney cells to obtain stocks with titres of 10^7 $TCID_{50}$/ml. Cell extracts, obtained by three cycles of freezing and thawing, were pooled, collected, and stored in aliquots at −80°C until used. In some experiments FeHV-1 was inactivated by a photochemical treatment [Pagnini et al., 2005] that includes an incubation with a psoralen compound (trioxsalen) and an exposure to UV light. The trioxsalen (4,5,8-trimethylpsoralen) (Sigma, Milan, Italy) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 200 μg/ml. Just before irradiation, this solution was added to the FeHV-1 suspension (10^8 $TCID_{50}$/ml) to obtain a final concentration of 1 mg of trioxsalen/ml. One milliliter of this viral suspension was then irradiated for 4 min with UV light. UV inactivation was confirmed by the absence of cytopathology following adsorption on to CrFK cells and subsequent lack of expression of FeHV-1 antigens.

INFECTION OF CELLS AND TEMPORAL CONTROL OF VIRAL PROTEIN EXPRESSION

To restrict viral gene expression to immediate-early (IE) and early (E) genes, cells were infected or mock-infected in the presence of the viral DNA synthesis inhibitor phosphonoacetic acid (PAA; Sigma) at a concentration of 300 $\mu g\,ml^{-1}$, followed by incubation in fresh medium also containing PAA.

To distinguish between involvement of IE and E genes, cells were infected with FeHV-1 and incubated in the presence of the protein synthesis inhibitor cycloheximide (CX; Sigma) at a concentration of 100 $\mu g\,ml^{-1}$. After 5 h, cells were washed in PBS and fresh medium was added containing the transcription inhibitor actinomycin-D (Act-D; Sigma) at 5 $\mu g\,ml^{-1}$ to allow translation of accumulated IE mRNA, while preventing further transcription. Controls comprised infected cells without addition of CX/Act-D, mock-infected cells with and without CX/Act-D, and infected and mock-infected cells with CX alone, for both 5 and 12 h (data not shown).

MONOCLONAL ANTIBODIES (MABS)

An anti-FeHV-1 polyclonal antibody conjugated to fluorescein isothiocyanate (FITC) of feline origin (210-49-FVR), recognizing feline rhinotracheitis virus purchased for VMRD, Inc. (Pullman, WA).

Anti-sheep MHC class I MCA897 (Serotec Ltd, Oxford, UK), which recognizing a monomorphic determinant expressed on the heavy chain of sheep MHC class I was used. This clone is reported to cross react whit feline MHC class I [Bishop et al., 1996]. Goat anti-mouse IgG antibody conjugated whit phycoerythrin (SC3738, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), was used as secondary antibody.

FLOW CYTOMETRY

CrFK cells were counted and a population of approximately 7×10^6 cells were infected with FeHV-1 Ba/91 strain (10 multiplicity of infection, m.o.i.), which had been previously propagated and titrated in CrFK cells. After 2 h of virus adsorption, the medium was removed and replaced with fresh DMEM containing 10% FCS. Control cultures were inoculated with the equivalent amount of cellular extracts collected from uninfected CrFK cells, manipulated and then stored in the same way as those containing the virus. Both infected and control (negative control) cells (equal in number) were incubated at 37°C under 5% CO^2. At 3, 6, 12, and 24 h p.i., cells were harvested, stained by immunofluorescence and analyzed by flow cytometry. Simultaneous analysis of MHC class I and FeHV-1 antigens expression was performed by flow cytometry in a Parteck Flow Cytometer. In brief, mock-infected and infected cells (1×10^7) were washed twice in PBS/BSA and then probed with an anti-FeHV-1 polyclonal antibody conjugated to fluorescein isothiocyanate of feline origin (210-49-FVR, VMRD, Inc.), and with anti-sheep MHC class I MCA897 (Serotec Ltd) for 30 min at room temperature, in the dark. The antibodies were washed off twice and replaced with PE-conjugated goat secondary anti-mouse antibody (SC3738, Santa Cruz Biotechnology, Inc.) under the same incubation conditions. The

secondary antibody was washed off three times, the cells were resuspended in PBS/BSA and then analyzed by flow cytometry.

RESULTS

INFECTION WITH FeHV-1 LEADS TO DOWN-REGULATION OF MHC CLASS I AT THE CELL SURFACE

To define whether infection by FeHV-1 could be associated with the ability of the virus to interact with MHC class I antigen presentation pathway, we evaluated the effect of Ba/91 strain on expression of MHC-I on cell surface. At this purpose, confluent monolayers of CrFk cells were either mock-infected or infected with FeHV-1 for 2 h at m.o.i. 10. At 3 h, 6h, 12 h, and 24 h p.i., cells were harvested, stained and analyzed by flow cytometry.

Strong cell-surface staining of MHC-I was observed on all mock-infected cells (Fig. 1). No MHC-I down-regulation on cell surface was detected at m.o.i. of 10 at 3 h p.i.. At 6 h p.i., a decrease in staining was observed in FeHV-1-infected cells (Fig. 1B) and by 12–24 h p.i. there was substantial loss of MHC class I expression (Fig. 1C). A similar trend was noted at m.o.i. 1 but with lower levels of MHC-I down-regulation than at m.o.i. 10 (data not shown).

TIME COURSE OF VIRUS PRODUCTION AND VIRAL PROTEIN EXPRESSION

We next determined whether down-regulation of MHC class I was related to a significant level of viral production. For this purpose, infection of FeHV-1-infected CRFK was evaluated as $TCID_{50}$. Figure 2 shows a time course of virus yield, in supernatants and cell associated virus, following exposure to FeHV-1 for 2 h at a m.o.i. 10. Productive infection was clearly demonstrated by a progressive increase in released virus with the ongoing culture. Replication of FeHV-1 in CRFK cells was also confirmed using a FeHV-1-specific primary polyclonal antibody and analysis by flow cytometry. Using this technique to detect virus replication, the first appearance of FeHV-1 proteins was at 3 h p.i., with a high percentage of cells staining for viral proteins at 12 and 24 h p.i. (Fig. 2).

PENETRATION OF VIRUS PARTICLES IS REQUIRED FOR FeHV-1-INDUCED MHC DOWN-REGULATION

The relationship between down-regulation of MHC I and virus replication was then investigated. Since attachment of FeHV-1 is mediated through interactions of viral glycoproteins with heparin like moieties [Maeda et al., 1997], we investigated the effect of heparin, a well-known inhibitor of FeHV-1 attachment, on the ability of FeHV-1 to modulate MHC I expression in CRFK cells. In the absence of heparin, as expected, we observed a significant down-regulation of MHC I of infected cultures (Fig. 3A) while cultures containing heparin showed no MHC I down-regulation.

UV-inactivated FeHV-1 was used to determine whether de novo viral protein synthesis was required for MHC I down-regulation. The effects of virion associated proteins can be distinguished from those requiring de novo expression by their lack of sensitivity to UV irradiation, which prevents viral gene expression by cross-linking the viral DNA [Jing et al., 2001].

Figure 3B shows that infection of CRFK cells with UV-inactivated virus at m.o.i. of 10 resulted in no MHC I down-regulation. No expression of FeHV-1 proteins was detected (data not shown). This data indicate that FeHV-1-induced down-regulation required de novo gene expression and is therefore not mediated by virion-associated proteins.

AN FeHV-1 E GENE(S) IS ESSENTIAL FOR MHC CLASS I DOWN-REGULATION

To determine the class of viral protein involved in the down-regulation, cells were infected in the presence of PAA, which inhibits late viral gene expression. Figure 4 shows that MHC class I down-regulation was still observed with or without PAA. These results indicate that the gene(s) responsible for MHC class I down-regulation is an IE or E FeHV-1 gene. This was investigated further with the use of the protein synthesis inhibitor cycloheximide.

Addition of CX to CRFK cells, either alone or followed by Act-D, effectively abrogated down-regulation of MHC class I by the virus. Since removal of CX and addition of Act-D should allow translation of IE genes, this result strongly suggests that an FeHV-1 E gene, either alone or associated with other genes, is responsible for the observed down-regulation of MHC class I.

DISCUSSION

Major histocompatibility complex [MHC] class I molecules are a particularly attractive target for immune evasion by viruses, because decreasing expression and/or antigen presentation by MHC class I can attenuate CD8 T-cell-mediated recognition of infected cells [Fruh et al., 1999; Yewdell and Hill, 2002]. Inhibition of MHC class I antigen presentation is a hallmark of the herpesvirus family with all family members having at least one mechanism to achieve this. For example, the murine cytomegalovirus (MCMV) m152 gene product gp40 binds to the MHC class I/peptide complex in the ER/cis-Golgi compartment preventing export to the cell surface [Ziegler et al., 1997, 2000]. The human cytomegalovirus [HCMV] US11 gene product binds nascent MHC class I heavy chain in the endoplasmic reticulum and targets it to the cytosol for proteasomal degradation [Jones et al., 1995; Wiertz et al., 1996].

Our data demonstrate down-regulation of MHC class I expression in FeHV1-infected feline cell lines. UV inactivation of virus showed that viral protein synthesis was required for this to occur. This conclusion was supported by the use of CX to block viral protein synthesis.

Many viruses and some bacteria have evolved the ability to inhibit class I antigen presentation on infected cells [Lorenzo et al., 2001]. For example, in BHV1 infection, it has been reported that down-regulation of MHC class I starts to occur as early as 3 h p.i., increasing to maximum down-regulation at 8 h p.i. [Koppers-Lalic et al., 2001]. Also EHV-1 has been demonstrated to down-regulate MHC class I expression starting from 8 h p.i., with maximum down-regulation occurring by 24 h [Ambagala et al., 2003] while PrV infection induces MHC class I down-regulation in pig cells by 12 h p.i. [Mellencamp et al., 1991; Ambagala et al., 2000].

In contrast to BHV1, we saw little effect of FeHV-1 infection on MHC class I expression at 6 h p.i., with maximum down-regulation occurring by 12 h p.i.

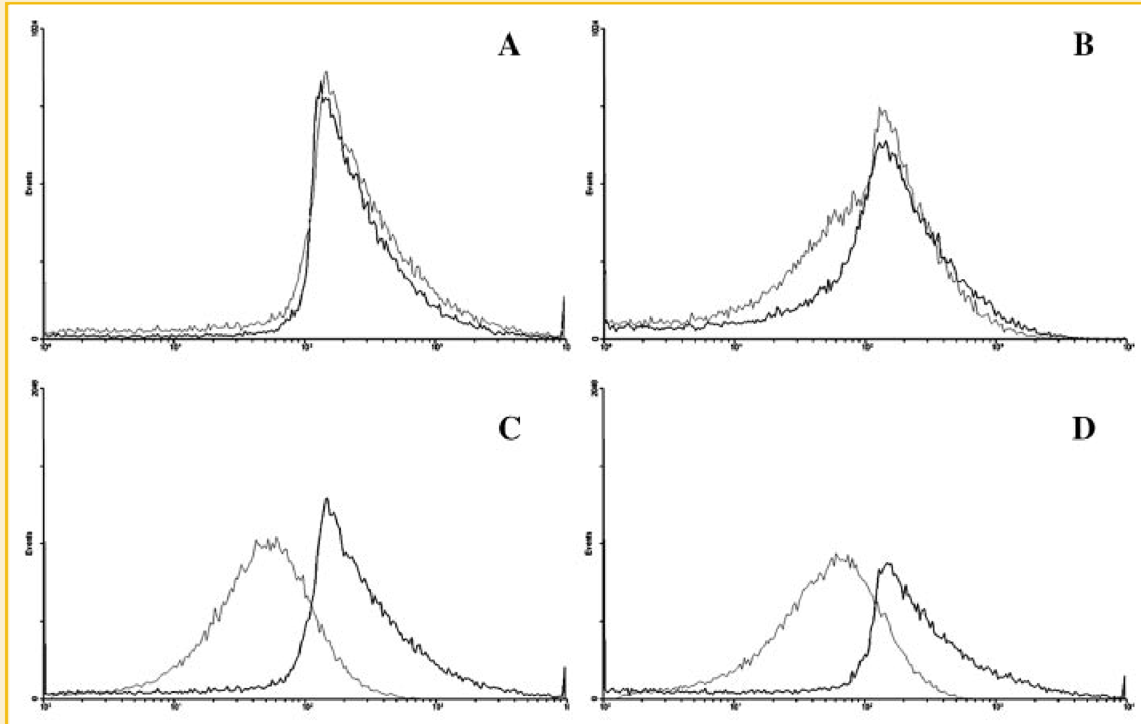

Fig. 1. MHC class I expression is down-regulated following FeHV-1 infection. Crfk cells were infected with FeHV-1 (gray lines) or mock-infected (black lines). Cells were stained with anti-MHC class I mAbs at 3 h (A), 6 h (B) 12 (C) or 24 h (D) p.i. Surface expression was measured by flow cytometry, histogram overlays of MHC class I expression detected by the mAb MCA897 are shown. Results of one representative experiment of three are shown.

This variation in the time taken for MHC class I down-regulation to occur following infection may be a result of the different viral mechanisms responsible, but could also reflect varying rates of MHC synthesis/turnover in different cell lines. In fact, it has been demonstrated that MHC class I stability is very variable and that molecules can remain on the cell surface for 20 h [Su and Miller, 2001].

Many different strategies have been documented that are used by alphaherpesviruses to evade the host immune system. However the mechanisms involved are only clearly understood and well characterized in few herpesvirus infections.

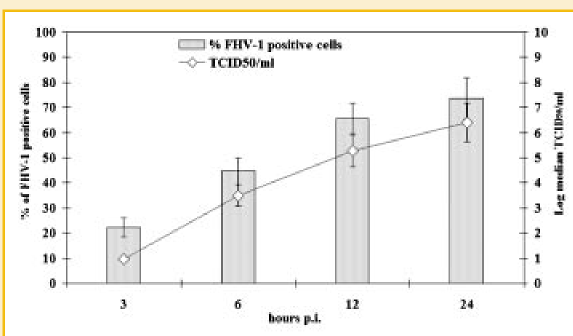

Fig. 2. Time course of virus production and viral protein expression in Crfk cells infected with FeHV-1. Virus production was titrated at 3, 6, 12, and 24 h after infection with FeHV-1 at a m.o.i. of 10 (Lines) and expression of viral proteins, following staining with anti-FeHV-1 polyclonal antibody evaluated by flow cytometry (Bars). Results are expressed as Mean $\pm$ SD of three

For example, EHV-1 infection results in enhanced endocytosis of MHC class I from the cell surface [Rappocciolo et al., 2003]. It has also been demonstrated that EHV-1 inhibits transport of peptides by TAP as early as 2 h post-infection (p.i.) [Ambagala et al., 2004]. Complete shutdown of peptide transport was observed by 8 h p.i. Furthermore, pulse–chase experiments revealed that maturation of class I molecules in the endoplasmic reticulum (ER) was delayed in EHV-1-infected cells, which may be due to reduced availability of peptides in the ER as a result of TAP inhibition. Metabolic inhibition studies indicated that an early protein(s) of EHV-1 is responsible for this effect [Ambagala et al., 2004]. Moreover, it has been reported that also BHV-1 uses at least two different strategies to interfere with the expression of antigen loaded MHC class I molecules at the cell surface [Hariharan et al., 1993; Nataraj et al., 1997; Hinkley et al., 1998; Koppers-Lalic et al., 2001; Gopinath et al., 2002]. This effect was shown to be partly due to the virion host shut-off [vhs] protein and to UL49.5 gene-product [Koppers-Lalic et al., 2001, 2005].

No data are available on the mechanisms involved in FeHV-1-induced MHC-I down-regulation, and it is clearly important to determine which one(s) is used by FHV1 and to identify the gene(s) responsible. Like in other herpesviruses, FeHV-1 gene expression occurs in a cascade fashion. The IE or α genes are expressed first, which induce the expression of early or β genes. The expression of early genes induces the onset of the viral DNA replication which is

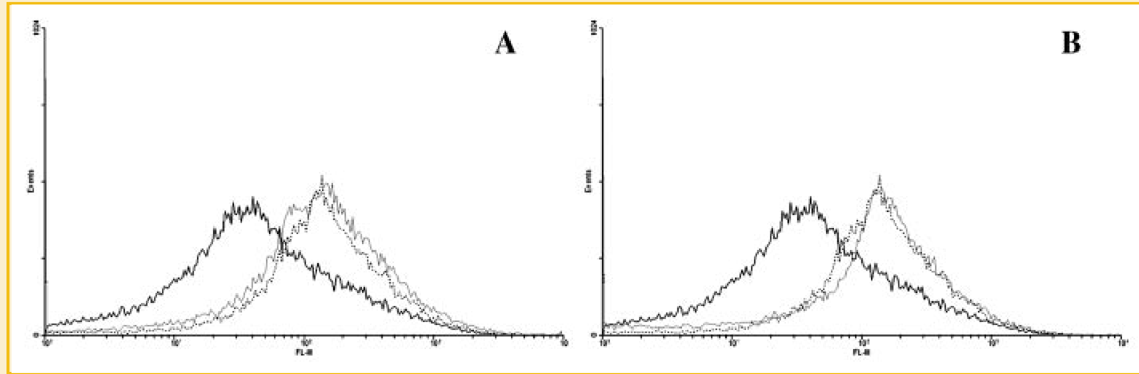

Fig. 3. MHC class I down-regulation is dependent on actively replicating FeHV-1. A: Crfk cells were infected with FHV1 (black line), in the presence of 10 UI/ml Eparin (gray line) or mock-infected (dashed line). Cells were stained with anti-MHC class I mAbs at 12 h p.i. The gray line (infected in presence of Eparin) and dashed line (mock-infected) are quite similar. B: UV-inactivated virus. Cells were infected with FeHV-1 (black line), infected with UV-inactivated FeHV-1 (gray line), or mock-infected (dashed line). The gray line (UV-inactivated virus) is superimposed on the dashed line (mock-infected). Surface expression was measured by flow cytometry, histogram overlays of MHC class I

followed by late or γ gene expression. Early gene products are mostly involved in genome replication, whereas the late gene products are mainly structural.

Lack of down-regulation of class I molecules in cells treated with UV-inactivated virus or live virus in the presence of CX, a protein synthesis inhibitor, clearly indicated that virus replication was essential for class I down-regulation.

The suppression of late protein expression by PAA could not restore the expression of class I to normal levels, indicating that the late proteins are highly unlikely to be responsible for class I down-regulation and demonstrating that E or IE genes must be responsible for MHC down-regulation by FHV1.

Infection of cells in the presence of CX followed by Act-D strongly suggests that, while the IE genes are not involved, an E gene(s) is responsible for the observed MHC class I down-regulation.

This was further demonstrated by the fact that FHV1 infection resulted in significantly greater loss of MHC class I molecules from the cell surface than CX treatment of uninfected cells (data not shown) suggesting that FHV1 infection may result in enhanced endocytosis of MHC class I molecules from the cell surface.

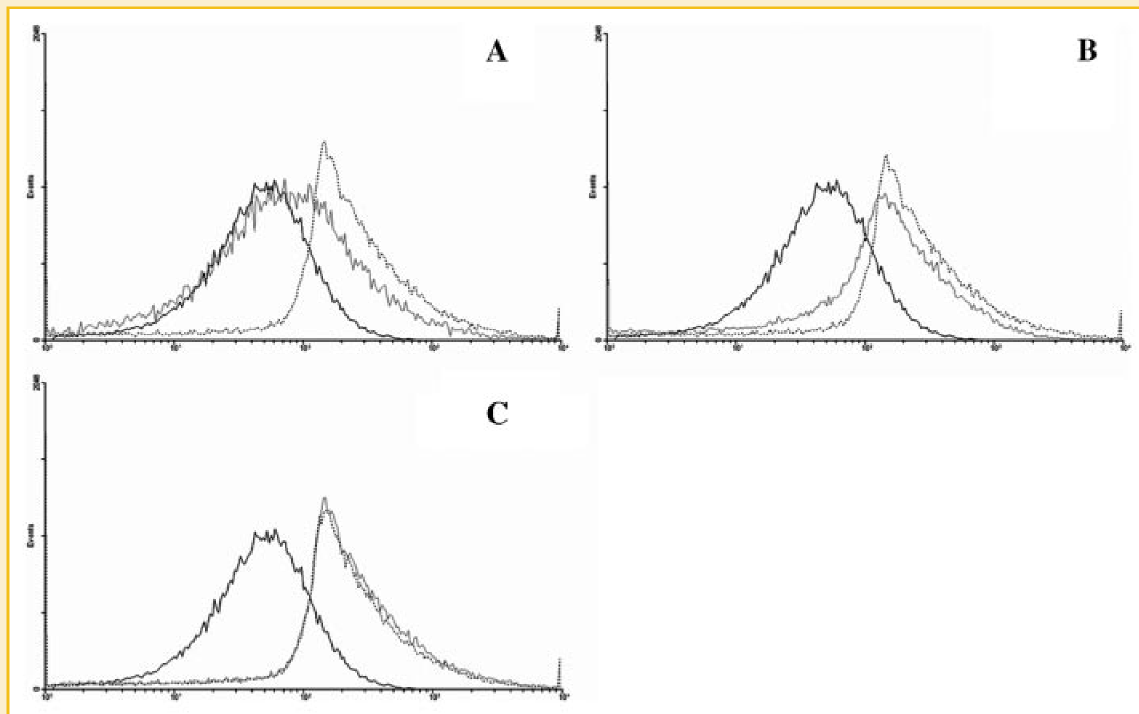

Fig. 4. An early gene is responsible for MHC class I down-regulation in FeHV-1 infected cells. Cells were mock-infected (dashed line) or infected with FeHV-1 (black line), and MHC class I surface expression was measured by flow cytometry on untreated cells, cells treated with phosphonoacetic acid for 12 h (PAA) (gray line) (A), cells treated with cycloheximide for 12 h (CX) (gray line) (B) cells treated with cycloheximide for 5 h followed by actinomycin-D (Act-D) (C). Results of one representative experiment of three are

The various mechanisms by which herpesviruses down-regulate class I target virtually all stages of the class I expression pathway, including synthesis [Hirata et al., 2001], peptide transport [Hill et al., 1995; Ahn et al., 1997; Zeidler et al., 1997; Hinkley et al., 1998; Jugovic et al., 1998; Ambagala et al., 2000], transit to [del Val et al., 1992; Campbell and Slater, 1994; Ahn et al., 1996; Jones et al., 1996; Wiertz et al., 1996; Jones and Sun, 1997; Reusch et al., 1999; Abendroth et al., 2001; Hudson et al., 2001] and stability on the cell surface [Ishido et al., 2000]. Although our data do not directly address mechanisms for class I down-regulation by FHV1, our observation that the kinetics of class I loss in response to virus infection closely parallels the response to cycloheximide is consistent with a block to repopulation of surface class I; however, as multiple FHV1 genes may be involved, a detailed understanding of their mechanisms awaits identification of the viral genes and independent analyses of their effects.

No genes have been identified in FHV1 that are homologues of those shown to be involved in MHC down-regulation in other viruses, for example, ICP47 in HSV and US6 in HCMV, both of which interfere with TAP, although by completely different mechanisms [Telford et al., 1992; Hill et al., 1995; Ahn et al., 1997]. Ambagala et al. [2004] did not find a US6 or ICP47 homologue in the BHV-1, PrV, or EHV-1 genomes, but several study [Ambagala et al., 2000; Koppers-Lalic et al., 2001] indicate that an early protein(s) of EHV-1, BHV-1, and PrV is responsible for TAP inhibition. Therefore, it is likely that FHV1, which belongs to the genus Varicellovirus, possess as genes encoding for homologues of ICP47 and US6 and that these proteins that are able to interfere with TAP.

Identification of the genes and of the protein(s) responsible for FHV1 MHC down-regulation is currently underway in our laboratory.

In conclusion, our results demonstrate down-regulation of MHC class I expression in FHV1-infected feline cell lines. Sensitivity to UV irradiation and insensitivity to a viral DNA synthesis inhibitor, like phosphonacetic acid, revealed that immediate early or early viral gene(s) are responsible. Use of the protein translation inhibitor cycloheximide confirmed that an early gene is primarily responsible.

Understanding the mechanisms by which FeHV-1 exerts this effect and identification of the gene(s) responsible may ultimately lead to the development of improved vaccines.

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