Down-regulation of MHC class I expression by equine herpesvirus-1

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There is good evidence that cytotoxic T lymphocytes play an important role in the clearance of equine herpesvirus-1 (EHV1) in horses. We have demonstrated that, in common with other alphaherpesviruses, EHV1 infection can lead to dramatic down-regulation of MHC class I expression at the cell surface, a common strategy for pathogen evasion of the host immune response. This down-regulation is specific for MHC class I and does not reflect a general shut-off of host-cell protein synthesis. The use of monoclonal antibodies that recognize different MHC class I epitopes has demonstrated that the effect may be allele- or locus-specific. Use of the viral DNA synthesis inhibitor phosphonoacetic acid, which prevents late viral gene expression, showed that the effect is mediated by an immediate-early or early viral gene, and use of the protein translation inhibitor cycloheximide confirmed that an early gene is primarily responsible. The data indicate that EHV1 infection results in enhanced endocytosis of MHC class I from the cell surface; the only other herpesvirus reported to use this mechanism is human herpesvirus-8. Elucidation of the precise mechanisms used by EHV1 in this process and identification of the genes responsible may lead to improved vaccination strategies.

INTRODUCTION

Equine herpesvirus-1 (EHV1), a member of the subfamily Alphaherpesvirinae, is the cause of an acute and highly contagious infection in the horse. The virus is transmitted by aerosol inhalation and replicates in the epithelium of the upper respiratory tract (Patel et al., 1992) causing respiratory disease, neurological disorders and late-onset abortion in mares (Allen & Bryans, 1986). Following primary infection, the virus can become latent, and intermittent reactivation can occur resulting in excretion of virus. Vaccines currently in use elicit strong humoral immune responses, but antibody in itself does not appear to be fully protective, as breakthrough infections commonly occur in vaccinated horses (Mumford et al., 1987). During the intracellular phase of virus replication when virus may be transported from the respiratory tract to the pregnant uterus, humoral immunity is largely ineffective, but cell-mediated immunity has been shown to have an important role (Allen et al., 1999).

CD8\(^+\) cytotoxic T lymphocytes (CTLs) form an essential part of the immune defence against many virus infections. CTLs recognize viral peptides presented at the surface of infected cells by major histocompatibility complex (MHC) class I molecules (Townsend & Bodmer, 1989). This is the result of a complex process of antigen processing involving a number of different molecules and cellular compartments (Pamer & Cresswell, 1998). 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 of 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 (BHV1) and pseudorabies virus (PrV) all result in down-regulation of MHC class I expression (Cohen, 1998; Johnson & Hill, 1998; Koppers-Lalic et al., 2001; Mellencamp et al., 1991). However the mechanisms involved are only clearly understood and well characterized in HSV and CMV 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.

In vitro assays show that EHV1-specific, MHC class I-restricted CTLs are detectable in the peripheral blood of EHV1-seropositive horses (Allen et al., 1995). Given that current vaccination strategies do not appear to elicit an effective cell-mediated response to EHV1, we have studied the effect of in vitro EHV1 infection on MHC class I expression. The data generated may shed light on the
mechanisms used by EHV1 to subvert the host immune response.

**METHODS**

**Cells.** NBL-6, an equine skin fibroblast cell line, was obtained from ATCC and maintained as a monolayer in EMEM supplemented with 10% foetal calf serum, 29 mg l-glutamine ml⁻¹, 10 units penicillin G ml⁻¹, 10 mg streptomycin sulphate ml⁻¹ and 1% non-essential amino acids (Gibco BRL). EEL, an equine embryonic lung cell line (a gift from J. Kydd, Animal Health Trust, Newmarket, UK), was cultured under the same conditions as NBL-6 cells. Stable mouse P815 transfectants expressing equine MHC class I genes (Holmes & Ellis, 2000) were maintained in RPMI 1640 supplemented as above, with the addition of 0·5 mg ml⁻¹ G418 sulphate (Gibco BRL).

**Equine herpesvirus-1.** The strain of EHV1 used was AB4/14 (a gift from J. Kydd). Virus was grown on EEL cells to obtain stocks with titres of 2 x 10⁶ p.f.u. ml⁻¹, which were stored in aliquots at −80 °C. For UV inactivation, 1 ml undiluted virus stock was placed in a Petri dish under a Universal UV source (Gelman-Camag) set at 254 nm at a distance of 5 cm for 15 min. UV inactivation was confirmed by the absence of cytopathology following adsorption on to NBL-6 cells and subsequent lack of expression of EHV1 glycoprotein C.

**Monoclonal antibodies (mAbs).** The anti-equine MHC class I mAbs CZ3 and CZ6 were a gift from D. F. Antczak (Cornell University, USA). The anti-porcine class I mAb PT85A was a gift from W. C. Davis (Washington State University, Pullman, USA; available from VMRD, Pullman, WA). The anti-EHV1 glycoprotein C mAb, 8F8, was a gift from J. Kydd. The anti-CD44 mAb CVS18 was purchased from Serotec. The isotype control mAb (IgGa2a), TRT6, recognizes turkey rhinotracheitis virus and was raised at the Institute for Animal Health, Compton. Goat anti-mouse Ig, conjugated to fluorescein isothiocyanate (FITC), was purchased from Southern Biotechnology Associates.

**Infection of cells and temporal control of viral protein expression.** Subconfluent cell cultures were washed once, then infected with EHV1 at an m.o.i. of 10 for 90 min at 37 °C in a CO₂ incubator. If the cells were to remain in culture for more than 90 min, they were washed with PBS and given fresh growth medium. Mock infections were carried out in parallel using cell-free supernatant (collected from EEL cell cultures) in place of the virus medium. Mock infections were carried out in parallel using cell-free supernatant (collected from EEL cell cultures) in place of the virus medium.

**RESULTS**

Infection with EHV1 leads to down-regulation of MHC class I at the cell surface

Confluent monolayers of NBL-6 cells were either mock-infected or infected with EHV1 for 90 min at an m.o.i. of 10. At 90 min, 8 h and 24 h p.i., cells were harvested, stained by immunofluorescence and analysed by flow cytometry. Strong cell-surface staining was seen with mAb CZ3 (recognizing horse MHC class I) on all mock-infected cells (Fig. 1). At 8 h p.i., a small decrease in staining was observed in EHV1-infected cells (Fig. 1b) and by 24 h p.i. there was substantial loss of MHC class I expression (Fig. 1c). A similar loss of MHC class I was observed following infection of EEL cells with EHV1 (data not shown). The level of staining for MHC class I was similar when cells were permeabilized with saponin (data not shown), but since the precise nature of the epitope recognized by CZ3 is not known, this result is open to a number of interpretations. Staining of both cell lines with mAb 8F8 (recognizing EHV1 glycoprotein C) demonstrated that the cells were infected with EHV1 (Fig. 2).

In order to demonstrate that the down-regulation of MHC class I is specific and not a result of general inhibition of protein synthesis, staining of NBL-6 and EEL cells was carried out with CVS18, a mAb recognizing equine CD44. Fig. 3 shows that no difference in CD44 expression was seen between mock-infected and EHV1-infected NBL-6 cells, while MHC class I detected using mAb CZ3 was clearly reduced. A similar result was seen with EEL cells (data not shown).

**MHC class I down-regulation may be locus or allele-specific**

Two additional mAbs were used to study MHC class I expression on EHV1-infected cells, PT85A and CZ6. PT85A was raised against pig MHC class I and recognizes a conformation-dependent epitope that is present on class I molecules in a wide range of species including horse. There are no data to show whether or not the epitope recognized by PT85A is present on all horse MHC class I alleles; however, given its broad species distribution, it is generally
assumed to recognize a monomorphic epitope. CZ6 was raised against horse MHC class I and its staining patterns suggests that it also recognizes a conserved epitope. Analysis of the CZ6 and PT85A staining pattern using horse MHC class I transfectants demonstrated that both mAbs recognize a range of classical class I molecules. Fig. 4(a) shows that PT85A and CZ6 recognized mouse P815 cells transfected with a representative classical class I gene, B2, but CZ6 did not recognize P815 cells transfected with the non-classical class I gene C1 (Ellis et al., 1995) and PT85A showed only very weak staining of C1 (Fig. 4b).

NBL-6 cells demonstrated almost complete loss of expression of the MHC class I epitope(s) recognized by CZ6 and PT85A at 24 h p.i. (Fig. 4d, e), whereas CZ3 staining, while demonstrating clear down-regulation, showed that the majority of cells were still expressing a low level of MHC class I (Fig. 4c). This result indicates that the viral mechanism is targeting specific allelic groups or products of one or more class I locus.

An EHV1 E gene(s) is responsible for MHC class I down-regulation

UV-inactivated EHV1 was used to determine whether de novo viral protein synthesis was required for MHC class I down-regulation. Fig. 5(a) shows that infection of NBL-6 cells with UV-inactivated virus at an m.o.i. of 10 resulted in no loss of class I expression. No expression of EHV1 glycoprotein C was detected (data not shown). To determine the class of viral protein involved in the down-regulation,

Fig. 1. MHC class I expression is down-regulated following EHV1 infection. NBL-6 cells were infected with EHV1 (grey lines) or mock-infected (black lines). Cells were stained with CZ3 at 90 min (a), 8 h (b) or 24 h (c) p.i., or with an isotype control mAb, TRT6 (grey shading). Surface expression was measured by flow cytometry.

Fig. 2. NBL-6 cells become infected with EHV1. NBL-6 cells were infected with EHV1 (grey line) or mock-infected (black line). Cells were stained with mAb 8F8 at 24 h p.i., or with an isotype control mAb, TRT6 (grey shading). Surface expression was measured by flow cytometry.
cells were infected in the presence of PAA, which inhibits late viral gene expression. Fig. 5(b) shows that expression of glycoprotein C (a late viral protein) was inhibited in the presence of PAA, but a similar pattern of MHC class I down-regulation was observed with or without PAA, as measured by CZ3 (Fig. 5c). The only difference was that a very small population of cells that were still expressing MHC class I was observed in the PAA-treated sample. A similar result was seen when MHC class I was measured using CZ6 and PT85A (data not shown). These results indicate that the gene(s) responsible for MHC class I down-regulation is an IE or E EHV1 gene.

This was investigated further with the use of the protein synthesis inhibitor CX. After 24 h growth in medium containing CX, NBL-6 cells showed down-regulation of surface MHC class I expression, which was largely restored following addition of Act-D. Fig. 5(d) shows this result as measured by CZ3; similar down-regulation was observed with PT85A (data not shown). It was assumed that the significant level of class I expression remaining at the surface following CX treatment was due to long-lived MHC class I molecules. This result was supported by an additional experiment using brefeldin A to block transport of newly synthesized protein to the cell surface, which also demonstrated that a significant level of class I expression remained unaffected (data not shown). The fact that the class I down-regulation observed following CX treatment was not as great as that seen following EHV1 infection (Fig. 1c, 4e) indicates that the virus is doing more than preventing transcription, translation or transport of newly synthesized class I to the cell surface.

Addition of CX to NBL-6 cells, either alone or followed by Act-D, effectively abrogated the 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 EHV1 E gene, either alone or in conjunction with other genes, is responsible for the observed down-regulation of MHC class I.

**DISCUSSION**

These data demonstrate almost complete down-regulation of MHC class I expression in EHV1-infected equine 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. 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). This effect was shown to be partly due to the virion host shut-off (vhs) protein. EHV1 has a vhs homologue in the form of the gene 19-encoded protein; however, we saw no evidence that this was involved in the EHV1-induced down-regulation of MHC class I. In contrast to BHV1, we saw little effect of EHV1 infection on MHC class I expression at 8 h p.i., with maximum down-regulation occurring by 24 h p.i. PrV infection induces MHC class I down-regulation in pig cells by 12 h p.i. (Ambagala *et al.*, 2000). 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. 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 & Miller, 2001). Experiments with CX and brefeldin A suggest that a significant proportion of the MHC class I molecules expressed by equine NBL-6 cells are stable at the cell surface for more than 24 h.

Complete loss of MHC class I expression was seen in NBL-6 and EEL cells following EHV1 infection when monitored with the mAbs CZ6 and PT85A, whereas only partial (though significant) down-regulation was seen when monitored with
mAb CZ3. There is no information available regarding the MHC class I genes/alleles expressed by NBL-6 and EEL cells; however, previous work has demonstrated that horse lymphocytes express the products of two polymorphic classical class I loci and may also transcribe a number of putative non-classical class I genes (Barbis et al., 1994; Ellis et al., 1995). While expression of these latter genes has not been formally demonstrated, there is evidence that one of them, C1, is expressed. P815 and L cells express C1 protein at the cell surface following stable transfection with C1 cDNA in an appropriate expression vector (Holmes & Ellis, 1999; Fig. 4b). In addition, CTLs generated in vitro from C1-positive, EHV1-immune horses recognize and kill EHV1-infected L cells expressing C1 (S. A. Ellis & G. Rappocciolo, unpublished data). The mAb CZ3 recognizes a monomorphic epitope on equine class I molecules (including C1) that may not be dependent on correct conformation. In contrast, the mAbs CZ6 and PT85A appear to recognize a conformation-dependent polymorphic epitope(s) that is not found on the non-classical class I molecule C1 and that may also be missing from some classical class I molecules. Our results clearly demonstrate that infection with EHV1

**Fig. 4.** MHC class I down-regulation following EHV1 infection is not complete. P815 cells transfected with B2 (a) or C1 (b) were stained with mAbs CZ3 (black lines), CZ6 (dark grey lines, arrowed) or PT85A (pale grey lines). NBL-6 cells were infected with EHV1 (grey lines c–e) or mock-infected (black lines, c–e). Cells were stained with CZ3 (c), CZ6 (d) or PT85A (e) at 24 h p.i., or with an isotype control mAb TRT6 (grey shading). Surface expression was measured by flow cytometry.
causes only a partial loss of MHC class I molecules from NBL-6 and EEL cells; this may be the result of an allele- or locus-specific mechanism. It is less likely simply to reflect differences in the relative turnover/stability of different MHC molecules, since treatment of cells with CX or brefeldin A for 24 h did not result in complete loss of either PT85A or CZ6 recognition. A similar phenomenon has been reported in PrV infection of mouse cells, where H-2Dk and H-2Kk are differentially regulated due to the action of multiple viral genes acting in an allele-specific manner (Sparks-Thissen & Enquist, 1999). Allele-specific binding by viral proteins is also seen in adenovirus, HCMV and HSV.

Fig. 5. MHC class I down-regulation is dependent on actively replicating EHV1 and an early gene is responsible. (a) NBL-6 cells were infected with EHV1 (grey line), UV-inactivated EHV1 (pale grey line), or mock-infected (black line). The pale grey line (UV-inactivated virus) is superimposed on the black line (mock-infected). (b,c) Effect of PAA. Cells were infected (grey line) or mock-infected (black line) in the presence of PAA. Cells were stained with mAbs 8F8 (b), CZ3 (c) or with an isotype control mAb TRT6 (grey shading) at 24 h p.i. The grey line (infected) and black line (mock-infected) are superimposed on the isotype control in (b). (d,e) Effect of CX. Cells were mock-infected (d) or infected with EHV1 (e), and CZ3 staining was carried out on untreated cells (black line), cells treated with CX for 24 h (dark grey line) and cells treated with CX for 5 h followed by Act-D (pale grey line). 8F8 staining on cells treated with CX alone or in combination with Act-D was superimposed on the isotype control (grey shading). 8F8 staining on infected cells with no treatment is shown in Fig. 2. Surface expression was measured by flow cytometry.
(Beier et al., 1994; Hill et al., 1994; Machold et al., 1997). This is an important aspect of the immune system evasion mechanism of these viruses, since it has been suggested that by leaving some MHC class I alleles at the cell surface NK cell lysis of infected cells may be avoided (Sparks-Thissen & Enquist, 1999).

Many different strategies have been documented that are used by alphaherpesviruses to evade the host immune system. It is clearly important to determine which one(s) is used by EHV1 and to identify the gene(s) responsible. Infection of cells in the presence of PAA clearly demonstrated that E or IE genes must be responsible for MHC down-regulation by EHV1. The small cell population that remain MHC class I-positive in the PAA-treated sample may indicate that it is difficult to achieve 100% infection due to the PAA-induced inhibition of virus growth. It is possible that this could be overcome by using a higher m.o.i. Infection of cells in the presence of CX followed by Act-D strongly suggested that, while the IE genes are not involved, an E gene(s) is responsible for the observed MHC class I down-regulation.

Our data show that EHV1 infection may result in enhanced endocytosis of MHC class I molecules from the cell surface. This was demonstrated by the fact that EHV1 infection resulted in significantly greater loss of MHC class I molecules from the cell surface than either CX or brefeldin A treatment of uninfected cells. CD44 expression was not affected, indicating that MHC class I molecules were specifically targeted. Human herpesvirus-8 (HHV8) has been shown to down-regulate MHC class I via two genes, K3 and K5, which enhance endocytosis and direct internalized class I molecules to endolysosomal vesicles for degradation (Coscoy & Ganem, 2000). K3 and K5 act independently and each have specificity for a different set of MHC class I alleles (Lorenzo et al., 2002). This is of interest given the apparent class I allele- or locus-specific down-regulation by EHV1 observed in this study. HHV8 is the only herpesvirus shown to modulate class I down-regulation using this mechanism, although unrelated viruses, e.g. human immunodeficiency virus, encode proteins that function in a similar manner (Piguet et al., 2000).

There may in addition be mechanisms mediated by the same or a different EHV1 gene that result in less MHC class I reaching the cell surface. No genes have been identified in EHV1 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 (Ahn et al., 1997; Hill et al., 1995; Telford et al., 1992). The likelihood of an allele/locus-specific mechanism suggests that an EHV1 viral protein(s) may be interacting directly with the class I heavy chain. This could involve re-direction of newly synthesized heavy chains to endolysosomes for degradation, as in MCMV infection (gp48) (Reusch et al., 1999), or re-direction to cytosolic proteasomes, as in HCMV infection (US2 and US11 proteins) (Wiertz et al., 1996). It is also possible that a viral gene product is interfering with TAP, but that some MHC molecules are less dependent than others on TAP for loading of peptides.

Existing EHV1 vaccines that contain inactivated virus, while not leading to MHC class I down-regulation, would be unlikely to induce CTLs due to lack of endogenous processing. Attenuated EHV1 vaccines are likely to induce MHC class I down-regulation, which may also lead to inefficient CTL priming and result in incomplete protection. Understanding the mechanisms by which EHV1 exerts this effect and identification of the gene(s) responsible may ultimately lead to the development of improved vaccines.

ACKNOWLEDGEMENTS

We would like to thank D. F. Antczak, W. C. Davis and J. Kydd for providing mAbs for use in this study and N. Davis-Poynter and J. Kydd for constructive discussion of the data. This work was funded by the Horserace Betting Levy Board, UK, and the Biotechnology and Biological Sciences Research Council, UK.

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