Immediate-early protein of equid herpesvirus type 1 as a target for cytotoxic T-lymphocytes in the Thoroughbred horse

Julia H. Kydd,† Ruth Case, Julius Minke, Jean-Christophe Audonnet, Bettina Wagner and Douglas F. Antczak

1Animal Health Trust, Lanwades Park, Kennett, Newmarket, Suffolk CB8 7UU, UK
2Merial SAS, R&D, 254 rue Marcel Merieux, Lyon, France
3Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York, NY 14853, USA
4Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, New York, NY 14853, USA

Cytotoxic T-lymphocytes (CTLs) are associated with protective immunity against disease caused by equid herpesvirus type 1 (EHV-1). However, the EHV-1 target proteins for CTLs are poorly defined. This limits the development of vaccine candidates designed to stimulate strong CTL immunity. Here, classical CTL assays using lymphocytes from horses of three defined MHC class I types that experienced natural infection with EHV-1 and a modified vaccinia virus construct containing an EHV-1 gene encoding the immediate-early (IE) protein are reported. Horses homozygous for the equine leukocyte antigen (ELA)-A2 haplotype, but not the ELA-A5 haplotype, produced MHC-restricted CTL responses against the IE protein. Previously, horses homozygous for the ELA-A3 haplotype also mounted CTL responses against the IE protein. Both haplotypes are common in major horse breeds, including the Thoroughbred. Thus, the IE protein is an attractive candidate molecule for future studies of T-cell immunity to EHV-1 in the horse.

Equid herpesvirus type 1 (EHV-1) can cause late gestation abortion in pregnant mares, and respiratory and neurological disease in all equids (Ma et al., 2013; Minke et al., 2004). Current vaccines against EHV-1 contain either live attenuated or killed virus, and both types provide partial clinical and virological protection (Minke et al., 2004). However, periodic abortion storms and outbreaks of neurological disease highlight the need for improved vaccines. Protection against EHV-1 in horses is associated with high titres of neutralizing antibody (Hannant et al., 1993; Heldens et al., 2001) and high frequencies of cytotoxic T-lymphocytes (CTLs) (Allen, 2008; Kydd et al., 2003; O'Neill et al., 1999). Ideal vaccines might consist of defined antigens which can stimulate protective cellular and humoral immunity in all horses, and thus eliminate the negative effects of viral proteins which modulate the host’s immune response (Ambagala et al., 2005; Griffin et al., 2010; van der Meulen et al., 2006), including downregulation of MHC class I (Rappocciolo et al., 2003; Said et al., 2012).

To develop novel vaccines against EHV-1 that stimulate protective cellular immune responses, it is necessary to identify conserved and immune-dominant viral proteins, and determine the distribution of MHC class I molecules within and between horse breeds. MHC class I molecules act as restriction elements that present viral peptides to the antigen-specific receptors on T-cells. The equine MHC region has been defined using serological assays (Lazary et al., 1988) and molecular techniques that have taken advantage of resources of the Horse Genome Project (Gustafson et al., 2003; Ramsay et al., 2010; Tallmadge et al., 2005, 2010). MHC typing using microsatellites (Tseng et al., 2010) has confirmed earlier serological studies (Antczak et al., 1986) which indicated that within the Thoroughbred breed, a limited number of stable MHC haplotypes [equine leukocyte antigen (ELA)-A2, -A3, -A5 and -A10] represent the majority of the genetic diversity in this region of the genome. The economic importance of the Thoroughbred justifies a closer examination of the genetic basis of immunity in this breed.

To date, although high frequencies of CTL precursors are associated with reduced clinical signs (Allen, 2008; Kydd et al., 2003), the only CTL target protein which has been identified for EHV-1 is the immediate-early (IE) protein, which is encoded by gene 64 (Soboll et al., 2003). Epitopes of this protein are presented by the B2 allele of the ELA-A3 haplotype (Kydd et al., 2006), which is also known as
ELA-A3.1 (Tallmadge et al., 2005). Vaccination of ELA-A3 ponies which expressed the B2 allele with a construct that expressed the IE protein resulted in the stimulation of IFN-γ+ lymphocytes in peripheral blood, which are associated with CTL activity (Paillot et al., 2006) and a reduction in cell-associated viraemia (Soboll et al., 2010), indicating partial virological protection.

This study aimed to assess the EHV-1 IE protein as a CTL target in horses of defined MHC haplotypes and which had been exposed to a virulent strain of EHV-1 during a field outbreak. The preliminary data suggested that the IE protein acted as a CTL target in two mares carrying the ELA-A2 MHC haplotype, but not in a mare homozygous for the ELA-A5 haplotype. The data enhanced our knowledge of CTL target proteins for EHV-1 – a finding critical to the rational development of novel vaccines.

The mares used in this study (Table 1) were members of an experimental herd at Cornell University, USA that experienced a natural outbreak of disease caused by EHV-1. Some mares had been vaccinated with an inactivated vaccine against EHV-1 and EHV-4, according to the manufacturer's instructions (Pneumabort K; Zoetis). This product stimulates high titres of virus-neutralizing antibody and detectable IFN-γ mRNA (Holmes et al., 2006) which in challenge experiments are associated with a reduction in nasal shedding and cell-associated viraemia (Goehring et al., 2010). One additional mare (Esther) was located at the Animal Health Trust, UK. This mare was hyper-immune to EHV-1 following an experimental herd at Cornell University, USA that experienced a natural outbreak of disease caused by EHV-1. The mares used in this study (Table 1) were members of the local diagnostic laboratory, based on clinical signs, virus isolation and complement fixing (CF) antibody titres. One mare (AM) displayed transient ataxia. The remaining Cornell mares had high titres of CF antibody (>1:80) at the time of first sampling for this study, regardless of vaccination status.

Peripheral blood mononuclear cells (PBMCs) collected by jugular venepuncture were isolated at Cornell University by density-gradient centrifugation over Ficoll, washed, and resuspended at 108 ml-1 in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated FCS, 2 mM l-glutamine, HEPES buffer, 100 U penicillin ml-1 and 100 μg streptomycin ml-1. Cells were transported to the UK at ambient temperature over 48 h under a government permit. On arrival, cells were recounted and either used immediately in induction cultures or alternatively cryopreserved in liquid nitrogen as described previously (Allen et al., 1995) and thawed as required. Due to the difficulty of harvesting and transporting such large numbers of cells internationally, each experiment was performed once only. Viability in all samples as determined by Trypan blue exclusion was >90%.

An EHV-1-specific assay of CTL activity was performed as described previously (Allen et al., 1995; Kydd et al., 2006). Effector cells were induced with live EHV-1 and screened against a variety of autologous or heterologous target cells that were either EHV-1-infected, mock-infected or infected with a modified vaccinia virus construct (NYVAC) expressing EHV-1 gene 64, which encodes the IE protein (see Kydd et al., 2006; Paillot et al., 2006 for construct details). The optimum m.o.i. for infection of target cells by the construct was determined by titration (m.o.i. 5, 2 and 1) and screened against effector lymphocytes from a mare (Esther) with an ELA-A3/x haplotype that had been hyper-infected with EHV-1. Results were expressed as percentage specific lysis, calculated according to a standard formula: (experimental c.p.m.–spontaneous c.p.m.)/(c.p.m. total release–c.p.m. spontaneous release) × 100. Data were expressed as the mean of three replicates. Unfortunately, the empty NYVAC construct was unavailable for screening.

### Table 1. Details of horses used as blood donors for CTL assays

<table>
<thead>
<tr>
<th>Horse</th>
<th>Age (years)</th>
<th>ELA serological haplotype*</th>
<th>Vaccination status†</th>
<th>Reciprocal CF antibody titre versus EHV-1‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>13</td>
<td>A2</td>
<td>NV</td>
<td>640</td>
</tr>
<tr>
<td>Y2K</td>
<td>5</td>
<td>A2</td>
<td>V</td>
<td>320</td>
</tr>
<tr>
<td>FTM</td>
<td>11</td>
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<td>V</td>
<td>80</td>
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<td>17</td>
<td>A3</td>
<td>NV</td>
<td>80</td>
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<td>4</td>
<td>A3</td>
<td>V</td>
<td>20</td>
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<td>BT</td>
<td>12</td>
<td>A3</td>
<td>NV</td>
<td>320</td>
</tr>
<tr>
<td>FW</td>
<td>14</td>
<td>A5</td>
<td>V</td>
<td>320</td>
</tr>
<tr>
<td>Esther</td>
<td>13</td>
<td>A3/x</td>
<td>NV</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Homozygous unless stated otherwise.
†V, vaccinated with inactivated virus using Pneumabort K; NV, non-vaccinated.
‡Samples collected on a single occasion as part of the outbreak’s diagnostic investigation. ND, Not done.
The first aim of this study was to identify horses of well-characterized MHC class I types with high levels of EHV-1-specific CTL activity; these were used for future screening against target cells presenting only the EHV-1 IE protein encoded by gene 64. Six of seven horses tested had detectable levels of virus-specific CTL activity, with low levels of lysis (<6%) against mock-infected autologous target cells (Fig. 1). One mare (G, ELA-A2) had insufficient effector cells and virus-specific lysis of <12% at 100:1 effector:target ratio (data not shown) and so was discarded from future experiments. The remaining six mares all had high levels of virus-specific lysis when tested on infected autologous cells (range >29–80.7% at effector:target ratios of 100:1). The effector CTLs were also tested against virus-infected target cells from the other mares. High levels of target cell lysis (>20%) were observed only when the CTLs and target cells carried the same MHC type, thus demonstrating classical MHC-restricted lysis.

Having identified mares with detectable CTL activity, additional PBMCs from three mares with representative MHC class I haplotypes (FTM and Y2K both ELA-A2, and FW ELA-A5) were collected and transported as described previously for further experiments designed to determine the role of the IE protein as a CTL target. For logistical reasons, the second sample was collected 1 year later and thus measurement of EHV-1-specific CTL activity was repeated at that time.

To determine the optimum m.o.i. of NYVAC-gene 64 with which to infect target cells, effector CTLs from an experimental mare, Esther, which carried the ELA-A3 haplotype were used. This A3/x mare was hyper-immune to EHV-1 and had CTL activity against the IE protein. This titration demonstrated that m.o.i. 2 was sufficient to produce detectable target cell lysis (Fig. 2a).

Next, the CTLs from three mares, Y2K and FTM (both ELA-A2) and FW (ELA-A5), were tested against target cells infected with NYVAC-gene 64 at m.o.i. 2 (Fig. 2). All mares showed CTL activity against autologous virus-infected target cells, but not against mock-infected targets. For the NYVAC-gene 64 infection, insufficient autologous target cells were available from the mare FTM (ELA-A2); therefore FTM effectors were tested against target cells from Y2K (ELA-A2). Effector CTLs from the two ELA-A2 mares lysed ELA-A2+ target cells infected with NYVAC-gene 64, but not NYVAC-gene 64-infected targets of the ELA-A3 haplotype. Mare FW (ELA-A5) failed to lyse either ELA-A3+ or ELA-A5+ cells infected with NYVAC-gene 64. Cumulatively, the data suggest that the IE protein provides peptides recognized by CTLs in EHV-1–primed horses of the ELA-A2 MHC class I haplotype, but not the ELA-A5 haplotype. Ideally, inclusion of the empty NYVAC construct as a control would have excluded any possibility of a non-specific response against antigens in the NYVAC construct. Nevertheless, there is a high likelihood that the CTL activity against target cells infected with the NYVAC-gene 64 construct is specific for the IE protein, because the lysis demonstrated genetic restriction.

This study provides new data on the viral proteins that stimulate CTL activity in horses after natural infection with EHV-1. The use of a unique herd of MHC homozygous horses permitted association between CTL response and MHC haplotype. Previous studies indicated that the EHV-1 IE protein encoded by gene 64 is the source of peptides that bind to the ELA-A3.1 gene (alias B2) of the ELA-A3 haplotype (Kydd et al., 2006; Soboll et al., 2003). Here, the data suggest that the IE protein also contains a peptide(s) that is presented by a MHC class I gene of the ELA-A2 haplotype, but not of the ELA-A5 haplotype. This information adds to our understanding of the targets of cellular immune responses against this important equine viral pathogen. Further study will be required to identify which ELA-A2 MHC class I gene presents peptide(s) from the IE protein. Five MHC class I genes have been identified in the ELA-A2 haplotype, with two showing properties of classical, polymorphic, antigen-presenting molecules (Tallmadge et al., 2010).

CTL target proteins have been identified in other herpesviruses. For example, in varicella-zoster virus, tegument proteins encoded by ORF4, 10, 62, 63 and gI act as CTL targets (Arvin et al., 1991; Bergen et al., 1991; Sadzot-Delvaux et al., 1997). In human cytomegalovirus, phosphoprotein 65, a major late matrix protein, is recognized by CTLs from HLA-A2 individuals (Kern et al., 2002; McLaughlin-Taylor et al., 1994; Wills et al., 1996). In bovine herpesvirus type 1 (BHV-1), CTL clones lysed BHV-1-infected target cells in a genetically restricted, virus-specific manner (Splitter et al., 1988), although whether gB, gC and gD are the targets is controversial (Hart et al., 2011; Levings & Roth, 2013).

In the horse, CTL target proteins and their genetic restriction elements have also been identified for equine infectious anemia virus (EIAV). These include Gag p26 which is presented by ELA-A5.1 and ELA-A9, and peptides of the Env protein, which are ELA-A1-restricted (Zhang et al., 1998). Detailed studies of the interaction between viral epitopes and MHC class I alleles have revealed a remarkable degree of complexity. In horses, there is clearly subhaplotypic variation (Chung et al., 2003) and this may be a reflection of polymorphism at alleles encoded by classical MHC class I loci. Additionally, horse MHC haplotypes appear to have differing numbers of classical MHC class I genes – a feature not found in humans or mice (Tallmadge et al., 2010). This diversity by genes expressed on classical MHC class I loci is consistent with the host’s need to generate immune responses and to retain the capacity for flexibility in defence against attack by pathogens, but complicates vaccine design.

Despite this diversity of MHC class I loci and alleles, there is also evidence that certain viral proteins behave in an immunodominant fashion and stimulate CTLs from horses carrying several different MHC haplotypes. For example, studies of CTL targets in six horses that had been infected with EIAV showed that Gag gene products, which encode
matrix and capsid proteins, were consistently recognized by various serological MHC class I haplotypes. However, no identical peptides within these proteins were recognized consistently (Zhang et al., 1998). The elegant work of Mealey et al. (2006) showed that within the ELA-A1 haplotype, a single amino acid difference in the α2 domain between the MHC class I genes 7-6 and 141 resulted in the ineffective presentation of the Gag GW-12 peptide by gene 141. As a consequence, there was a functional alteration in the ability of horses carrying the 141 gene to recognize peptides. Modelling suggested that the mechanism was related to the 114–Gag Gw12 complex not being recognized.

Fig. 1. MHC restriction of CTL killing of EHV-1-infected target cells. CTLs from six MHC homozygous horses [(a) one ELA-A5, (b, c) two ELA-A2 and (d–f) three ELA-A3] were tested against EHV-1-infected target cells (inf) of each of the three MHC haplotypes plus mock-infected autologous control cells (m). Effector : target cell ratios were 100 : 1, 50 : 1, 25 : 1 and 12 : 1 as indicated by the shading. Genetically restricted, virus-specific CTL activity was detectable in all mares as demonstrated by lysis of virus-infected target cells from mares which shared the same haplotype. Levels of lysis were low in autologous, mock-infected and heterologous target cells collected from mares which did not share the same haplotype. Results are expressed as mean percentage specific lysis of three replicate wells; bars, sd.
by the T-cell receptor. In the ELA-A3 serological haplotype, two subtypes, A3.1 and A3.2, have been revealed with functional differences, namely only ponies with A3.1 recognized the IE protein as a CTL target (Soboll et al., 2003). These subtle yet important functional differences need further investigation if subunit vaccines are to become practical in an outbred population.

In the current study, the degree of lysis of target cells infected with NYVAC-gene 64 was consistently lower than that of target cells infected with EHV-1 virus. Soboll et al. (2003) reported a similar phenomenon using single gene products. In the MHC homozygous horses studied here, the lower CTL activity to the IE protein is probably a reflection of the single target protein presented. The equine CTL response to EHV-1 is undoubtedly complex and will include recognition of peptides derived from different EHV-1 proteins.

In summary, the current data in EHV-1-infected mares with defined MHC class I haplotypes suggest that peptides of the IE protein are presented by an allele(s) of the ELA-A2 serological haplotype, but not ELA-A5. The IE protein therefore acts as a CTL target protein in two MHC class I haplotypes, ELA-A2 and ELA-A3.1, which are common in the Thoroughbred breed, thereby strengthening the argument for considering its inclusion in future novel vaccines.

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**References**


