A molecular approach to the identification of cytotoxic T-lymphocyte epitopes within equine herpesvirus 1

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Equine herpesvirus 1 (EHV-1) causes respiratory and neurological disease and abortion in horses. Animals with high frequencies of cytotoxic T lymphocytes (CTL) show reduced severity of respiratory disease and frequency of abortion, probably by CTL-mediated control of cell-associated viraemia. This study aimed to identify CTL epitopes restricted by selected major histocompatibility complex (MHC) class I alleles expressed in the equine leukocyte antigen (ELA) A3 haplotype. Effector CTL were induced from EHV-1-primed ponies and thoroughbreds with characterized MHC class I haplotypes and screened against P815 target cells transfected with selected EHV-1 genes and MHC class I genes. Targets that expressed EHV-1 gene 64 and the MHC B2 gene were lysed by effector CTL in a genetically restricted manner. There was no T-cell recognition of targets expressing either the MHC B2 gene and EHV-1 genes 2, 12, 14, 16, 35, 63 or 69, or the MHC C1 gene and EHV-1 genes 12, 14, 16 or 64. A vaccinia virus vector encoding gene 64 (NYVAC-64) was also investigated. Using lymphocytes from ELA-A3 horses, the recombinant NYVAC-64 virus induced effector CTL that lysed EHV-1-infected target cells; the recombinant virus also supplied a functional peptide that was expressed by target cells and recognized in an MHC-restricted fashion by CTL induced with EHV-1. This construct may therefore be used to determine the antigenicity of EHV-1 gene 64 for other MHC haplotypes. These techniques are broadly applicable to the identification of additional CTL target proteins and their presenting MHC alleles, not only for EHV-1, but for other equine viruses.

INTRODUCTION

The alphaherpesvirus equine herpesvirus 1 (EHV-1) infects horses throughout the world, causing respiratory and neurological disease, abortion in pregnant mares, and establishing latency in the trigeminal ganglion and lymphocytes (Allen et al., 1999; Edington et al., 1994; Slater et al., 1994; Welch et al., 1992). It is responsible for substantial economic losses within the bloodstock industry and in addition has serious welfare implications. To control the disease, it will be necessary to reduce nasopharyngeal virus shedding and cell-associated viraemia, and this reduction is likely to be achieved by the stimulation of virus-neutralizing antibody and cytotoxic T lymphocytes (CTL) (Allen, 2002; Allen et al., 1999). While current vaccines containing inactivated virus stimulate virus-neutralizing antibody (Heldens et al., 2001) and reduce the amount and duration of infectious virus, which is shed from the nasopharynx (Hannant et al., 1993; Heldens et al., 2001), they fail to prevent infection. Sporadic outbreaks of EHV-1-related abortion and neurological disease therefore continue to occur (Patel & Heldens, 2005). These outbreaks are likely caused by failure of the available vaccines to stimulate CTL and thereby control cell-associated viraemia.

EHV-1-specific CTL can recognize and lyse virus-infected cells in a genetically restricted, antigen-specific manner (Allen et al., 1995) and are important in control of infection. Ponies with high pre-infection frequencies of CTL, as measured by limiting dilution analysis, show a reduction in clinical and virological signs of disease, including abortion (Kydd et al., 2003; O’Neill et al., 1999). Therefore the stimulation of CTL by vaccination is an important aim. However, EHV-1 has a large genome containing 76 open reading frames. Some EHV-1 proteins and/or transcripts...
have potentially deleterious effects in vitro such as immunosuppression (Charan et al., 1997; Hannant et al., 1999) and downregulation of MHC class I molecules (Ambagala et al., 2004; Rappocciolo et al., 2003). Therefore the identification of CTL-target proteins and their inclusion in vaccines that present antigen by the endogenous route are essential for improved vaccination strategies (Minke et al., 2004). Early data suggest that CTL-target proteins are encoded by genes of the immediate early (IE) and early classes of EHV-1 (Allen et al., 1995). Recently, Soboll et al. (2003) have shown that the single IE gene of EHV-1 (gene 64) contains CTL epitopes that are presented by an MHC class I allele or alleles carried on the serologically defined A3 haplotype. Their approach involved the biolistic transfection of dendritic cells with plasmids encoding individual EHV-1 gene products. These transfected dendritic cells were then used to induce effector CTL from primed ponies, which were then tested against whole virus-infected target cells. However, the allele(s) responsible for presentation of the EHV-1 peptide(s) encoded by gene 64 was not identified in that study. In contrast, the current paper takes a molecular approach, enabling identification of EHV-1 proteins that are recognized by CTL and the equine MHC class I genes responsible for their presentation. In particular, it is important to identify the MHC class I alleles involved in the presentation of gene 64 epitopes, to characterize further their interactions and enable construction of MHC class I–peptide tetramers to characterize immune responses ex vivo.

The MHC of the horse is known by its species-specific designation as the equine leukocyte antigen (ELA-A) region. Serological reagents identify 17 internationally accepted ELA specificities that correspond to haplotypes (Antczak, 1992; Antczak et al., 1982; Bailey et al., 2000). However, the serological reagents fail to distinguish the different gene products expressed on these haplotypes or subgroups of the haplotypes. The first physical map of the equine MHC has been defined through a contig of bacterial artificial chromosome (BAC) clones produced using DNA from a horse homozygous for the ELA-A3 serological haplotype (Gustafson et al., 2003). Recently, the genomic sequences of 15 horse MHC class I genes and pseudogenes were obtained from these BAC clones (Tallmadge et al., 2005). Seven of these genes are transcribed and predicted to be expressed. These include four genes that have the characteristics of classical MHC class I genes (designated 3-1, 3-2, 3-3 and 3-4, where ‘3’ indicates the haplotype of origin and the number of a fully sequenced gene) and three so-called non-classical class I genes (3-5, 3-6 and 3-7). Four of these seven genes have been described previously from cDNA clones (Ellis et al., 1995). B2 (classical gene), A1, C1 and E1 (all non-classical genes) of Ellis et al. (1995) correspond to 3-1, 3-5, 3-6 and 3-7, respectively (Tallmadge et al., 2005). The present study focused on the classical B2 gene (equivalent to 3-1) and the C1 non-classical gene (equivalent to 3-5), which are expressed by the ELA-A3 haplotype. The ELA-A7 haplotype, which expresses classical B1 and B4 genes but not the C1 non-classical gene, was used as a control. This model was studied because the B2 and C1 genes have been cloned and expressed (Ellis et al., 1995), and the ELA-A3 haplotype (as defined serologically) is expressed by a large proportion of the thoroughbred population (Bodo et al., 1994), which forms a major market for vaccines. The ELA-A3 serological specificity is apparently heterogeneous, however, since effector CTL from ponies typed as subgroup A3-1 failed to lyse targets from a pony typed as subgroup A3-2 (Soboll et al., 2003).

The work described in this paper used a molecular approach to screen a selection of EHV-1 genes in order to identify the CTL-target proteins of EHV-1 presented by the MHC class I B2 molecule. It also examined the role of the C1 gene. The data derived from these studies are an essential prerequisite for the identification of EHV-1 target peptides and ultimately the synthesis of novel reagents such as MHC–peptide tetramers to characterize immune responses in vivo. The technique is also applicable to other equine viruses for which CTL-target proteins and their presenting MHC alleles are unknown.

METHODS

Cloning and expression of MHC class I genes and selected EHV-1 genes. The classical MHC class I B2 gene and the non-classical C1 gene were cloned as described previously (Ellis et al., 1995) into the pcDNA3 mammalian expression vector (Invitrogen). Each gene was stably transfected by electroporation into the murine mastocytoma P815 cell line (H-2d; European Cell and Culture Collection, Centre for Applied Microbiology and Research, Porton Down, Wiltshire), and expression was confirmed by using indirect immunofluorescence and the anti-horse MHC class I monoclonal antibody (mAb) CZ3. A total of eight EHV-1 genes were chosen for screening as CTL targets (Table 1). Seven of these were amplified from DNA derived from EHV-1 strain Ab4/13, a plaque-purified clone of wild-type strain Ab4/8 (Telford et al., 1992) that was isolated in the UK. Primers were designed from the published sequences to include restriction enzyme sites (Table 1) at the 5’ and 3’ ends, and the amplicon cloned into pcDNA6/V5-His (V5 tag at C terminus; Invitrogen). A plasmid encoding open reading frame (ORF) 16 (glycoprotein C; a kind gift from Professor Hartwig Huemer, Institute of Hygiene, University of Innsbruck, Austria) was subcloned into pcDNA6 (Invitrogen). All constructs were sequenced prior to their further use. A panel of stable P815 transfectants expressing the class I allele B2 and each of the eight EHV-1 genes was generated using electroporation, limiting dilution cloning and selection with G418 (Calbiochem), and blasticidin S (Invitrogen). In addition ORFs 12, 14 and 64 were transfected into P815 cells expressing the class I allele C1. Expression of ORF 16 was confirmed using the murine mAbs 8F8 and 7F11 specific for gC (Sinclair et al., 1989) and indirect immunofluorescence. Expression of the other seven EHV-1 genes was confirmed by Western blot analysis using a mAb to the V5 tag (Invitrogen).

Donors of peripheral blood mononuclear cells (PBMC), lymphocyte isolation and priming by EHV-1 infection. Two groups of EHV-1-primed animals were selected as donors of PBMC, and MHC class I haplotypes were identified by serological typing (Antczak et al., 1986). Group 1 comprised four Welsh Mountain ponies, while group 2 consisted of four thoroughbred horses (Table 2). The ponies were 6C31 (A2/A3), 302A (A7/A7), 6132 (A3/A3) and her son 3862 (A3/A3). The ELA-A3 haplotype encodes the
### Table 1. Details of the EHV-1 genes, including the primer sequences, that were used for PCR amplification prior to cloning into pcDNA6/V5

The constructs were co-transfected with MHC class I B2 gene into P815 target cells and screened against EHV-1-specific, effector CTL. The percentage specific lysis against double transfectants is shown. Single transfectants consistently showed specific lysis <5%.

<table>
<thead>
<tr>
<th>ORF no.*</th>
<th>Function* (HSV-1 homologue)</th>
<th>Size (bp)</th>
<th>Primer sequences† (restriction enzyme site)</th>
<th>Representative specific lysis (%) at 100:1 effector-to-target ratio‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Unknown (Not applicable)</td>
<td>617</td>
<td>F5'-GATAAGTTACATGGACGCACCCGG-3' (HindIII)</td>
<td>3·4±0·3</td>
</tr>
<tr>
<td>12</td>
<td>Tegument [UL48 (VP16, ICP25)]</td>
<td>360</td>
<td>R5'-GCCCTGAGCTGCATGCCCTTTCCAACCC-3' (XhoI)</td>
<td>0·5±0·7</td>
</tr>
<tr>
<td>14</td>
<td>Tegument [UL46 (VP11/12)]</td>
<td>2243</td>
<td>F5'-GCAGTTCTAGCTACAATGGACGGAG-3' (EcoRI)</td>
<td>8·7±0·7</td>
</tr>
<tr>
<td>16</td>
<td>Glycoprotein C (UL44)</td>
<td>1406</td>
<td>R5'-GCAGTTCTACAGCGAGCTACCGCT-3' (EcoRI)</td>
<td>1·7±2·0</td>
</tr>
<tr>
<td>35</td>
<td>Protease/minor capsid scaffold protein [UL26 (VP24/VP21)]</td>
<td>1940</td>
<td>F5'-GGCTGATTGGACGTACACCGTG-3' (HindIII)</td>
<td>-4·2±1·0</td>
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<tr>
<td>63</td>
<td>Transcriptional activator [RL2 (ICP0)]</td>
<td>1598</td>
<td>R5'-CTCGAGCCGAGCGCTACATCTG-3' (XhoI)</td>
<td>3·2±5·0</td>
</tr>
<tr>
<td>64</td>
<td>Transcriptional activator (IE gene) [RS1 (ICP4)]</td>
<td>4463</td>
<td>F5'-CTCGAGCCGAGCGCTACATCTG-3' (EcoRI)</td>
<td>52·6±2·0</td>
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<tr>
<td>69</td>
<td>Protein kinase (US3)</td>
<td>1148</td>
<td>R5'-CTCGAGCTACACCGTACCGCT-3' (HindIII)</td>
<td>2·4±4·2</td>
</tr>
</tbody>
</table>

*Telford et al. (1992).
†F, Forward primer sequences; R, reverse primer sequences. Restriction enzyme sequences are underlined.
‡Effector CTL lysis of P815 target cells doubly transfected with the horse MHC class I B2 gene and a single EHV-1 gene. Results shown are representative of three separate experiments.

### Table 2. MHC class I haplotypes and EHV-1 infection histories of ponies and thoroughbred horses used in this study

X, Unknown haplotype.

<table>
<thead>
<tr>
<th>Animal identity</th>
<th>Age (years)</th>
<th>ELA (MHC) haplotypes</th>
<th>EHV-1 infection history</th>
<th>CF antibody*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1: ponies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6C31</td>
<td>12</td>
<td>A3/X</td>
<td>3 experimental†</td>
<td>Not done</td>
</tr>
<tr>
<td>302A</td>
<td>11</td>
<td>A7/A7</td>
<td>2 experimental†</td>
<td>Not done</td>
</tr>
<tr>
<td>3862</td>
<td>7</td>
<td>A3/A3</td>
<td>2 experimental†</td>
<td>Not done</td>
</tr>
<tr>
<td>6132</td>
<td>11</td>
<td>A3/A3</td>
<td>Field infection‡</td>
<td>Not done</td>
</tr>
<tr>
<td><strong>Group 2: thoroughbred horses</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>4</td>
<td>A3/A3</td>
<td>Field infection, January 2005</td>
<td>10^1·3</td>
</tr>
<tr>
<td>BT</td>
<td>12</td>
<td>A3/A3</td>
<td>Field infection§</td>
<td>10^2·5</td>
</tr>
<tr>
<td>CP</td>
<td>17</td>
<td>A3/A3</td>
<td>Field infection§</td>
<td>10^1·9</td>
</tr>
<tr>
<td>FW</td>
<td>24</td>
<td>A5/A5</td>
<td>Field infection§</td>
<td>10^2·5</td>
</tr>
</tbody>
</table>

*Complement-fixing antibody against EHV-1/－4 expressed as log_{10} reciprocal titre.
†Intranasal EHV-1 Ab4/15 at approximately annual intervals.
‡No experimental infections, but field infections were assumed in a mare of this age.
§Field infection within previous 3 months as indicated by CF antibody titre ≥10^1·6.
classical class I gene B2, several non-classical genes including C1, and additional classical class I genes (Ellis et al., 1995; Tallmadge et al., 2005). The ELA-A7 haplotype expresses the classical MHC class I genes B1 and B4 but not the non-classical C1 gene (Ellis et al., 1995). Expression of the B2 and C1 genes of the ELA-A3 haplotype and the B1 and B4 genes of the ELA-A7 haplotype was confirmed in group 1 ponies by PCR amplification and sequencing of expressed class I genes (Ellis et al., 1995). Ponies 6C31, 302A and 3862 were primed to EHV-1 by intranasal infections with strain Ab4/15 (a plaque-purified clone of the virulent Ab4/8 strain derived from a field outbreak in the UK; Telford et al., 1992) as described previously (Mumford, 1994). Pony 6132 had experienced only field infections with EHV-1.

The four thoroughbred mares in group 2, AM, BT, CP (all ELA-A3/A3, B2+) and FW (ELA-A5/A5), were from the experimental herd of James A. Baker (Institute for Animal Health, Cornell University, Ithaca, NY, USA). All were primed to EHV-1 through field infection within the 6 months prior to this study (Dr Julia Flaminio, Cornell University, personal communication). Infection was diagnosed by clinical signs (including ataxia in mare AM) and significantly elevated titres (reciprocal titres of \( \geq 1 \times 10^5 \)) of EHV-1/4 cross-reactive complement-fixing antibodies (Thomson et al., 1976). Mares AM and FW were seropositive for EHV-1 glycoprotein G as measured by ELISA (Svanova Biotech) at the time of blood collection. Mare FW (ELA-A5/A5) donated blood for heterologous target cells only. PBMC from the Cornell mares were transported in complete RPMI (Sigma) to the Animal Health Trust (AHT) (UK) by air for testing.

PBMC were enriched from heparinised blood by centrifugation over ficoll (Pharmacia), washed in PBS and viable cells were counted and assessed for viability by trypan blue dye exclusion. Cells from the AHT ponies and Cornell horses were used fresh, stored in complete RPMI for up to 2 days at 4°C or cryopreserved (O’Neill et al., 1999) for later use in the CTL assay. All research studies involving animals were approved by the Ethical Review Committee of the AHT and complied with the Animals (Scientific Procedures) Act 1986.

**CTL assay.** A 4 h chromium release assay was used to detect EHV-1-specific, MHC class I-restricted CTL activity (Allen et al., 1995) that is mediated by CD8+ lymphocytes. The previously validated assay was modified slightly by using EHV-1 strain Ab4/15, a plaque-purified clone (Telford et al., 1992) to induce effectors and to infect target cells. Transfected P815 cells were also used as target cells. These were cultured in complete RPMI and split the day before radioisotope labelling. Effector CTL-to-target cell ratios ranged from 100:1 to 12:1 with three replicates each. Percentage spontaneous release was \( \leq 25\% \) in all experiments. Percentage specific lysis was calculated according to the standard formula:

\[
\text{Specific lysis} = \frac{\text{total release} - \text{spontaneous release}}{\text{total release}} \times 100
\]

In some experiments, the assay was adapted to determine the effects of several parameters on the percentage specific lysis. These included infection of target cells or induction of effectors with modified vaccinia virus (NYVAC) constructs; these are described in detail below. The results reported are each representative of at least three separate experiments and are expressed as the mean ± standard deviation.

**Origin of NYVAC constructs.** The recombinant virus constructs (vP1014 and vPR884) were generated by the insertion of the IE gene 64 or ORF 33 (encoding glycoprotein B), respectively, of EHV-1 (originating from the Kentucky D strain) into the highly attenuated vaccinia-derived New York (NYVAC) strain (Piccini et al., 1987). These recombinant viruses were developed by Virogenetics according to standard in vitro recombination techniques (Tartaglia et al., 1992). NYVAC is a registered trademark in the United States of Connaught Technology Corporation.

**Infection of target cells with NYVAC constructs.** Lymphoblast target cells were infected with either NYVAC gene 64 or NYVAC gB at an m.o.i. of 10 for 1–5 to 2 h prior to labelling with radioisotope and used in the CTL assay.

**Induction of effector CTL with NYVAC constructs.** To test the ability of EHV-1 gene 64 to induce CTL in vitro, PBMC from ponies primed by previous experimental infection to EHV-1 strain Ab4/13 were incubated with either NYVAC gene 64 or NYVAC gB at an m.o.i. of 0·3 or 0·6 for 6 or 7 days. Effector CTL were then tested against EHV-1-infected lymphoblasts or transfected P815 cells.

**RESULTS**

**Cloning and stable transfection of EHV-1 genes**

A total of eight EHV-1 genes were cloned into pcDNA6/V5/His (Table 1) and co-transfected into P815 cells that expressed the equine MHC class I B2 gene. Plasmids containing ORFs, 12, 14, 16 and 64 were also co-transfected individually into P815 expressing the non-classical C1 gene. Control P815 cells transfected with a single gene encoding either B2, C1 or EHV-1 gene 64 were also produced. These stably transfected cells were used as targets in the CTL assay to identify target proteins.

**Induction of virus-specific, genetically restricted effector CTL activity from PBMC**

The effector CTL activity induced from PBMC of EHV-1-primed, MHC-typed ponies and thoroughbreds was tested against autologous or heterologous virus-infected lymphoblasts. As shown in Fig. 1, effector CTL induced with EHV-1 from pony 6C31 (ELA-A3/x), pony 302A (ELA-A7/A7) and horse AM (ELA-A3/A3) killed autologous virus-infected targets. The killing was genetically restricted; no mare lysed heterologous, virus-infected target cells. Also, the killing was virus specific; mock-infected lymphoblast targets were not lysed by any mare. These results confirmed the presence of lytic activity by effector CTL that were subsequently used against P815 transfectant target cells. Levels of EHV-1-specific CTL activity were high in all experimentally infected ponies and thoroughbreds throughout the sampling period. CTL stimulated from ponies 6132 and 3862 and thoroughbreds BT and CP (all ELA-A3 homozygotes) showed an identical pattern of genetic restriction to 6C31 (data not shown). The percentage specific lysis against virus-infected, homologous targets at 100:1 effector-to-target ratio was 63.0±3.0% for pony 3862, 24.0±4.4% for pony 6132, 29.3±2% for horse BT and 44.3±1.9% for horse CP.

**Screening of selected EHV-1 genes encoding potential CTL-target proteins**

As shown in Table 1, EHV-1-specific, effector CTL from pony 6C31 recognized only one of the eight EHV-1 genes...
Identification of CTL epitopes within EHV-1

screened, when presented by the MHC class I B2 molecule. Lytic activity against double transfectants that expressed the B2 gene and an EHV-1 gene (ORFs 2, 12, 14, 16, 35, 63, 69) was < 10% at 100:1 effector-to-target ratio (Table 1). In contrast, the protein encoded by EHV-1 gene 64 was identified as a target antigen for MHC class I B2-specific effector CTL from pony 6C31 (ELA-A3/x) as shown by the lysis of double transfectants (Fig. 2a). The same effector CTL failed to lyse single transfectants expressing only the MHC class I gene B2 or the EHV-1 gene 64. Similar data were obtained from the other two ELA-B2+ ponies 3862 and 6132 (data not shown) and thoroughbred mare AM
(Fig. 2b). In contrast, effector CTL from the pony mare 302A (ELA-A7/A7), which does not carry the B2 gene, failed to lyse B2+/gene 64+ double transfectants (Fig. 2c). Transfected target cells expressing the non-classical C1 gene and EHV-1 gene 64 were not lysed by effector CTL from either pony 6C31 [ELA-A3/x (B2+)] or thoroughbred AM [ELA-A3/A3, (B2+)]. Thus, there was T-cell recognition of targets expressing the MHC class I B2 gene and the EHV-1 gene 64, but not targets expressing either the C1 gene and genes 12, 14, 16 and 64, or the B2 gene and genes 2, 12, 14, 16, 35, 63 and 69.

**Use of NYVAC gene 64 to confirm gene 64 as a CTL-target protein**

To confirm that EHV-1 gene 64 was a target in our ponies with characterized MHC class I haplotypes and alleles, and to assess the usefulness of NYVAC constructs derived from Kentucky D strain of EHV-1, two approaches were used. First, CTL were induced with live EHV-1 virus (strain Ab4/13) and tested against target lymphoblasts from pony 6C31 [ELA-A3/x; (B2+)] and pony 302A [ELA-A7/A7; (B1+B4+, B2−)], which had been infected with either NYVAC gB or NYVAC gene 64. Target lymphoblasts infected with EHV-1 (Ab4/13) or mock infected were used as controls. The results demonstrated that EHV-1-specific, effector CTL from pony 6C31 (B2+) lysed only NYVAC gene 64 or EHV-1-infected target cells (Fig. 3). Killing of NYVAC gB or mock-infected homologous cells was low and lysis of heterologous cells infected with NYVAC gB or NYVAC gene 64 target cells was <10-9 % (data not shown). Second, NYVAC gene 64 was used to induce effector CTL from lymphocytes of pony 6C31 [ELA-A3/x; (B2+)]. As shown in Fig. 4(a), induction with NYVAC gene 64 stimulated effector CTL, which lysed both whole virus-infected autologous lymphoblasts and B2+/gene 64+ double transfectants. No lysis was detectable when either single transfectants (Fig. 4a) or mock-infected lymphoblasts (data not shown) were used. In contrast, induction with NYVAC gB resulted in no detectable lysis of the same target cells (Fig. 4b). The results confirmed that NYVAC gene 64 derived from Kentucky D strain induced CTL in vitro from ponies primed to EHV-1 by infection with strain Ab4/13 and lysed target cells infected with Ab4/13. Similarly, NYVAC gene 64-infected target cells were lysed by effector CTL from a B2+ pony primed in vivo to EHV-1 by experimental infection with strain Ab4/13. These data also demonstrate that at least for gene 64, strains of EHV-1 derived from North American or European isolates can stimulate cross-reactive CTL in vitro.

**DISCUSSION**

The data described in this paper extend previous studies demonstrating that EHV-1 gene 64, the IE gene, is a target for effector CTL expressing the MHC class I haplotype ELA-A3 (Soboll et al., 2003). Soboll and colleagues used dendritic cells transfected with plasmids encoding EHV-1 genes to induce CTL from EHV-1-primed, MHC class I-typed ponies. Effector CTL from ponies with the ELA-A3 haplotype, which had been induced with gene 64, lysed whole virus-infected target cells. In contrast, using a murine mastocytoma cell line (P815) transfected with MHC class I and EHV-1 genes, we have demonstrated that the MHC class I gene B2 presents peptide(s) encoded by gene 64 to ELA-A3 effector CTL derived from EHV-1-primed ponies and thoroughbreds. Our work therefore, for the first time, identifies B2 as an MHC class I gene responsible for the genetic restriction of this viral gene product.

Equine MHC class I haplotypes are complex and the serological typing methods currently employed have limitations. These limitations have been shown by differences in CTL recognition between animals with the ELA-A3 (Soboll et al., 2003) and ELA-A5 (Zhang et al., 1998) serological haplotypes. For example, the anti-ELA-A3 antisera panel divided ponies into two subgroups, namely 1 and 2. EHV-1-specific CTL generated from subgroup 3 failed to lyse virus-infected targets from subgroup 3-2 (Soboll et al., 2003). Thus, further molecular analysis of the ELA-A3 haplotype in thoroughbreds should be performed on a representative sample to ensure that the haplotype has...
Identification of CTL epitopes within EHV-1

**Fig. 4.** Cytotoxic activity following induction of effector PBMC from pony 6C31 (ELA-A3/x; (B2+)) with (a) NYVAC gene 64 (vP1014) or (b) NYVAC gB (vP884) and testing against target cells shown on the x axis. Murne P815 target cells were either co-transfected with two plasmids expressing the MHC class I molecule B2 and EHV-1 gene 64 or singly transfected with either gene B2 or gene 64. Autologous lymphoblast targets (pony 6C31 inf) were infected with EHV-1. Effector-to-target ratios are shown in the box. Mean CTL activity ± standard deviation of at least three replicate cultures.

(a) Induction with NYVAC-64
(b) Induction with NYVAC-gB

<table>
<thead>
<tr>
<th>Target cells</th>
<th>Specific lysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2+64</td>
<td>100</td>
</tr>
<tr>
<td>B2</td>
<td>60</td>
</tr>
<tr>
<td>Gene 64</td>
<td>80</td>
</tr>
<tr>
<td>6C31 infected</td>
<td>40</td>
</tr>
<tr>
<td>B2+64</td>
<td>25</td>
</tr>
<tr>
<td>B2</td>
<td>12</td>
</tr>
<tr>
<td>Gene 64</td>
<td></td>
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</tbody>
</table>

the same composition in each case and to ensure the consistent presence of the B2 allele. More generally, sequencing of the equine MHC is needed to develop more sophisticated typing methods. Encouragingly, the MHC class I genes from an animal homozygous for a serologically defined ELA-A3 haplotype have recently been sequenced (Tallmadge et al., 2005), which confirmed the presence of the ELA-B2 allele but also revealed three further putative classical I genes that were shown to be transcribed. Earlier data (Ellis et al., 1995) suggest that most horse MHC class I haplotypes transcribe and express no more than two genes at easily detectable levels. Furthermore, B2 is expressed at a high level in our A3+ ponies and is transcribed at a significantly higher level than any other class I gene (Ellis et al., 1995). Thus, in addition to B2, it is possible that at least one of potentially three other ELA-A3 classical alleles may also present EHV-1 viral peptides, which may or may not be encoded by gene 64. Further work is clearly required to determine the functional relevance of the additional MHC class I genes and to determine if most or all haplotypes express four classical class I genes. It is likely that even within the ELA-A3 haplotype, further CTL-target proteins may require identification. Determination of CTL-target proteins for MHC class I alleles expressed by other major thoroughbred haplotypes, e.g., ELA-A2, -5 and -A9, would allow use of recombinant vaccines that could stimulate CTL responses in >95% of the thoroughbred population. The T-cell receptor repertoire may also differ between ELA-A3+ animals and this may affect the CTL response.

The NYVAC gene 64 construct proved useful in confirming that this antigen was genetically restricted by alleles expressed by the ELA-A3 haplotype, including the B2 gene. It also provided evidence of cross-reactivity of CTL responses stimulated by different strains of EHV-1, namely the Ab4/15 (isolated in the UK), Kentucky D (USA) and a North American field strain, suggesting that the immunogenic peptides are sufficiently similar for CTL recognition. This conclusion is substantiated by the high degree of conservation in the nucleotide sequences of Ab4/13 (Telford et al., 1992), KyA (Grundy et al., 1989) and KyD (Tartaglia et al., 1992), with only four base changes in gene 64, of which two result in an amino acid substitution at positions 1073 and 3700. If similar conservation is present in the strains of EHV-1 circulating in the field, CTL are likely to be cross-reactive and this is reassuring for future vaccine development in an international market. In addition, this construct may be used to screen animals of known MHC class I haplotypes in vitro for their response to gene 64. Recently, we demonstrated that vaccination of ponies that express the A3 haplotype with NYVAC gene 64, resulted in the stimulation of virus-specific effector CTL and interferon-gamma synthesis (Paillot et al., 2005, 2006). The product of gene 64 is therefore a strong candidate for inclusion in future vaccines designed to induce cellular immune responses, at least in the B2+ subpopulation of horses. Used in conjunction with other vaccines (e.g. either inactivated virus or glycoprotein subunits), which contain antibody epitopes, and administered intramuscularly and intranasally, there is a realistic chance that this vaccination strategy will improve protection.

In conclusion, we have identified the MHC class I molecule encoded by the B2 gene as being capable of presenting peptide from EHV-1 gene 64 epitopes to effector CTL. The system is applicable to other viruses and will enable the identification of CTL-target proteins and their genetic restricting elements. For EHV-1, the characterization of further CTL-target proteins and their restricting MHC class I alleles is dependent on the identification and cloning of additional equine MHC class I alleles and the application of molecular typing methods (Chung et al., 2003). Immediate future work will focus on the identification of the peptide(s) encoded by gene 64, which are presented by the B2 molecule. This will ultimately enable the construction of MHC class I–virus peptide tetramers as has been achieved successfully with Equine infectious anemia virus (Mealey et al., 2005) and many other pathogens (Klenerman et al., 2002). Further dissection of the in vivo immune response to EHV-1 using tetramers will yield valuable epidemiological...
information and ultimately lead to improved vaccination strategies.

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