Identification of equine herpesvirus-1 antigens recognized by cytotoxic T lymphocytes

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Equine herpesvirus-1 (EHV-1) causes serious disease in horses throughout the world, despite the frequent use of vaccines. CTLs are thought to be critical for protection from primary and reactivating latent EHV-1 infections. However, the antigen-specificity of EHV-1-specific CTLs is unknown. The aim of this study was to identify EHV-1 genes that encode proteins containing CTL epitopes and to determine their MHC I (or ELA-A in the horse) restriction. Equine dendritic cells, transfected with a series of EHV-1 genes, were used to stimulate autologous CTL precursor populations derived from previously infected horses. Cytotoxicity was subsequently measured against EHV-1-infected PWM lymphoblast targets. Dendritic cells were infected with EHV-1 (positive control) or transfected with plasmids encoding the gB, gC, gD, gE, gH, gI, gL, immediate-early (IE) or early protein of EHV-1 using the PowderJect XR-1 research device. Dendritic cells transfected with the IE gene induced CTL responses in four of six ponies. All four of these ponies shared a common ELA-A3.1 haplotype. Dendritic cells transfected with gC, gD, gI and gL glycoproteins induced CTLs in individual ponies. The cytotoxic activity was ELA-A-restricted, as heterologous targets from ELA-A mismatched ponies were not killed and an MHC I blocking antibody reduced EHV-1-specific killing. This is the first identification of an EHV-1 protein containing ELA-A-restricted CTL epitopes. This assay can now be used to study CTL specificity for EHV-1 proteins in horses with a broad range of ELA-A haplotypes, with the goal of developing a multi-epitope EHV-1 vaccine.

INTRODUCTION

EHV-1 is a viral pathogen with a high prevalence in domestic horses worldwide (Allen *et al.*, 1999; Bryans & Allen, 1989). EHV-1 infection has enormous economic impact on the breeding, competitive and recreational horse industries, as losses through abortion, respiratory disease, neurological disorders and death of full-term newborns occur perennially (Allen *et al.*, 1999; Gilkerson *et al.*, 1998). The high prevalence of the virus results from difficulty in preventing primary infections and the subsequent establishment of latent infection in the lymphocytes and neuronal tissues (Baxi *et al.*, 1995; Chesters *et al.*, 1997; Edington *et al.*, 1994). Reactivation of latent infections can result in new primary infections.

Prevention of EHV-1 infection currently depends heavily on vaccination, but available vaccines are of limited efficacy (Allen *et al.*, 1999; Burki *et al.*, 1990). The most significant obstacle to the development of effective vaccines has been a lack of understanding of what constitutes protective immunity. There is a brief window of 3–6 months after natural EHV-1 infection when horses are protected from re-infection (Bryans & Allen, 1989). It is likely that this protection depends on the complex interaction of different immune mechanisms including serum and mucosal antibodies and CTLs (Allen *et al.*, 1999). Because CTLs can control intracellular pathogens and are important for control of cell-associated viraemia (Goldsby *et al.*, 2000), EHV-1 research has recently focused on the protective role of CTLs. These studies have shown CTLs to have an important role in controlling both primary and latent EHV-1 infection (Allen *et al.*, 1995; Smith *et al.*, 1998), and

EHV-1 CTL precursor frequency is a good correlate for protection from challenge infection (O'Neill *et al.*, 1999).

Generating CTL responses by vaccination is difficult to accomplish using inactivated vaccines (Audibert & Lise, 1993). Strategies using modified live viruses are typically more successful (Ellis, 1999). However, the only available modified live EHV-1 vaccine induces only limited cellular responses (Ellis *et al.*, 1995). In addition, modified live vaccines are potentially dangerous in pregnant animals (Oehen *et al.*, 1991), which are an important equine population requiring protection from EHV-1 infection (Allen *et al.*, 1999). A number of strategies employing recombinant vectors and DNA vaccination techniques are now available that have the potential to generate potent CTL responses (Fu *et al.*, 1999). In addition, there are minimal safety concerns, and production of DNA vaccines is relatively easy and economical (Cohen, 1993; Hassett & Whitton, 1996). DNA vaccines have been successfully used in the horse to protect against equine influenza virus (Lunn *et al.*, 1999), and initial studies have shown the potential of DNA vaccines for prevention of EHV-1 infection (Ruitenberg *et al.*, 1999a, b, 2000a, b; Walker *et al.*, 1997). DNA vaccination can also be combined with recombinant vector vaccines to generate extremely potent CTL responses (Allen *et al.*, 2000; Ramsay *et al.*, 1999).

In order to use recombinant or DNA vaccination technologies, it is essential to identify EHV-1 proteins containing CTL epitopes. In the horse, significant progress has been made in defining the CTL epitopes of equine infectious anaemia virus (EIAV) (McGuire *et al.*, 2000; Ridgely & McGuire, 2002; Zhang *et al.*, 1998). However, little is known about EHV-1 CTL antigens. Because CTLs do not recognize protein antigens in their native form, but only as short processed peptides of 8–10 amino acids in length presented by highly polymorphic MHC I molecules, it is important to determine which EHV-1 CTL epitopes are presented by which MHC I haplotypes. The best-characterized and most polymorphic equine class I gene is encoded by the ELA-A locus (Antczak, 1992). CTLs recognize epitopes in the context of a particular MHC I molecule, and different MHC I molecules vary in their peptide-binding repertoires. Therefore, a CTL epitope highly immunogenic in horses expressing one ELA-A allele might not induce CTLs in a horse expressing a different ELA-A allele. Studies of EHV-1 CTL epitopes need to determine the ELA-A restriction of specific CTL responses so that future vaccines can be formulated to include epitopes that induce strong CTLs in a genetically diverse population of horses.

Selection of EHV-1 proteins to screen for CTL epitopes is complex. The genome of EHV-1 contains 76 different open reading frames encoding gene products (Allen *et al.*, 1999). These gene products are classified as immediate-early (IE), early, or late (which includes the major glycoproteins). In contrast to other herpesviruses, EHV-1 has only one IE gene. It has been proposed that EHV-1 proteins expressed early in the replication cycle, such as those encoded by the IE and

early (UL5) genes, are likely to be important CTL targets (Koen *et al.*, 2000; Ruitenberg *et al.*, 1999b; Siedek *et al.*, 1999; Smith *et al.*, 1998). In addition, the glycoproteins of herpesviruses have been identified as containing CTL epitopes (Gallichan *et al.*, 1993), and several studies in mice have reported protection from EHV-1 infection by vaccination with gB, gC, gD, gH and gL glycoproteins (Allen & Yeargan, 1987; Crabb *et al.*, 1991; Flowers *et al.*, 1995; Kukreja *et al.*, 1998; Osterrieder *et al.*, 1995; Stokes *et al.*, 1996; Wellington *et al.*, 1996a, b; Whalley *et al.*, 1995). The aim of our study was to identify EHV-1 genes that encode proteins containing CTL epitopes recognized by horses and to determine the ELA-A restriction of these CTL responses. A study was performed using CTL precursor populations derived from ponies recently infected with EHV-1. Autologous equine dendritic cells were transfected with plasmids encoding the gB, gC, gD, gE, gH, gI, gL, IE or UL5 genes of EHV-1, using the PowderJect XR-1 research device (PowderJect Vaccines). These transfected dendritic cells were then used to stimulate CTL precursor populations and cytotoxicity was measured against EHV-1-infected PWM lymphoblast targets. This method was chosen because dendritic cells have been recognized as extremely potent antigen-presenting cells for the generation of CTL responses (Banchereau & Steinman, 1998) and will be an important target for future vaccine delivery.

METHODS

Experimental animals. Six gelded ponies between 2 and 3 years of age were used in this study. Ponies were housed in a semi-isolation unit, fed twice a day with a diet of hay and pelleted concentrate and maintained according to the animal care guidelines of the Research and Animal Resources Committee, University of Wisconsin-Madison.

EHV-1 preparation and isolation. This experiment used EHV-1 strain Army 183 (EHV-1/A183). The virus was propagated in equine dermal cells (ATCC CCL-57), and cell-free virus-laden supernatant used for challenge infections and *in vitro* assays.

Preparation of EHV-1-encoding plasmids. The EHV-1 glycoproteins (gB, gC, gD, gE, gH, gI, gL), the IE protein and the UL5 early protein have been characterized extensively (Allen & Yeargan, 1987; Audonnet *et al.*, 1990; Birch-Machin *et al.*, 2000; Crabb *et al.*, 1991; Elton *et al.*, 1991; Flowers *et al.*, 1995; Koen *et al.*, 2000; Kukreja *et al.*, 1998; Osterrieder *et al.*, 1995; Stokes *et al.*, 1996; Tewari *et al.*, 1994; Wellington *et al.*, 1996a, b; Whalley *et al.*, 1995). The genes encoding the gC, gD, gE, gH, gI and gL glycoproteins, the IE protein and the UL5 protein were cloned into the PCR3.1 plasmid (Invitrogen). The gB glycoprotein was cloned into the pRCCMV plasmid (Invitrogen) (Munro *et al.*, 1999). All plasmids were purified for PowderJect-XR1 particle mediated DNA transfection by anion exchange chromatography (Qiagen) and coated on to gold beads at a concentration of 2·5 µg DNA (mg gold beads)$^{-1}$, as previously described (Lunn *et al.*, 1999).

To verify the utility of these plasmids and this transfection system for expression of EHV-1 proteins, Chinese hamster ovary (CHO) cells were transfected with plasmids encoding the gB, gC and gD glycoproteins using the PowderJect XR-1 research device (PowderJect Vaccines). These plasmids were selected for testing because of the availability of suitable monoclonal antibodies (mAbs) for detection of

protein expression. Controls included CHO cells transfected with empty vector plasmid as a negative control. After 24 h, cells were stained with mAbs recognizing the gB, gC and gD proteins, or with positive and negative anti-EHV-1 equine serum. Antibody binding was detected using anti-murine or anti-equine immunoglobulin–peroxidase conjugates (Jackson Laboratories) as appropriate, in combination with 3-amino-9-ethylcarbazole substrate solution. Results of staining confirmed expression of the gB, gC and gD glycoproteins, based on positive staining with mAbs and equine anti-EHV-1 serum.

EHV-1 infection of ponies and sample collection. All ponies received two EHV-1 infections with a 6 month interval. For each infection, 2×10^7 p.f.u. EHV-1/A183 in 1 ml tissue culture supernatant was instilled intranasally. At 14, 21 and 28 days after the second infection, blood was collected for cryopreservation of PBMC containing CTL precursors. Starting not less than 4 weeks after the last infection, blood was collected from ponies for preparation of dendritic cells for use in CTL stimulator cultures, as described below.

Physical examinations were conducted throughout the experiment at all sample collection times and daily for 21 days after challenge infection. Serum was prepared from blood samples collected by jugular venipuncture. For the isolation of dendritic cells, precursor CTLs and autologous lymphoblast targets, blood was collected into heparin by jugular venipuncture. For ELA-A typing, blood was collected into ACD blood collection tubes (Becton Dickinson).

Examination of latent EHV-1 infection status of ponies. To determine whether ponies were latently infected with EHV-1, cellular nucleic acid was isolated from 1×10^7 PBMC per pony using the High-Pure Template Preparation Kit (Roche Diagnostics). Latent infection was identified using a nested PCR, as described previously (Borchers & Slater, 1993). This PCR used two primer pairs specific for the EHV-1 gB gene. On the basis of this experiment, all six ponies were latently infected before the start of the experiment.

ELA-A typing. PBMC of all ponies were ELA-A typed by microcytotoxicity assays using previously described antibody reagents (Bailey et al., 1984). The equine ELA-A locus includes the internationally accepted alleles A1–A10, A14, A15 and A19 and the provisionally accepted alleles W16, W17, W18 and W20 (Antczak, 1992; Bailey et al., 2000). Available antisera at the time of typing included those specific for A1–A10, as well as for the less well characterized W11.

Virus neutralizing assay. Virus neutralizing antibody titres in serum were determined at the Wisconsin State Veterinary Diagnostic Laboratory (Madison, WI). A 50 µl volume of a serial dilution of each sample from 1:2 to 1:4096 was added to 96-well tissue culture plates (Corning Inc.) and incubated with 50 µl EHV-1 (12 000 p.f.u. ml^{-1}) at 37 °C, 5 % CO_2 for 1 h. Subsequently a Madin–Darby bovine kidney cell suspension was added and plates were incubated for a further 4 days at 37 °C, 5 % CO_2, then examined for cytopathic effects. Testing of each sample group included EHV-1-positive and -negative sera as controls. Titres were defined as the highest dilution of the sample at which a cytopathic effect could be seen.

Generation and transfection of equine dendritic cells. Equine dendritic cells were prepared from each pony as previously described (Hammond et al., 1999). Briefly, equine PBMC were isolated from 500 ml heparinized blood using density gradient centrifugation Histopaque-1077 (Sigma). PBMC were then subjected to a 4 h incubation in cRPMI-10 (RPMI 1640 containing 10 % autologous horse serum, 5 µg gentamicin ml^{-1}, 4 µM L-glutamine, 50 µM β-mercaptoethanol and 0·25 µg amphotericin B ml^{-1}) in 150 mm diameter tissue culture-treated dishes. Non-adherent cells were removed and the remaining adherent cells were washed with PBS

and incubated for 4 days in media supplemented with recombinant human GM-CSF (1000 U ml^{-1}; Sigma) and recombinant equine interleukin-4 (rEq-IL-4) that had been expressed in CHO cells (1000 U ml^{-1}) (Dohmann et al., 2000). The low-density dendritic cells were purified using density centrifugation in Nycoprep ($\delta = 1·068$; Accurate Chemicals). Flow cytometric analysis using mAbs demonstrated increased levels of MHC I, MHC II, CD44 and CD86 in dendritic cell preparations when compared with fresh PBMC. Characterization of the mAbs used for this analysis (CVS22, CVS10, CVS18 and VI CD86.6) has been reported previously (Hammond et al., 1999; Lunn et al., 1996). Mature dendritic cells were subsequently resuspended at a concentration of 10^7 cells ml^{-1} and 20 µl aliquots were plated into each well of a six-well tissue culture plate (Corning Inc.). For each transfection with empty vector plasmid or plasmids encoding EHV-1 genes, three 20 µl aliquots of dendritic cells were bombarded at 250 p.s.i. using the PowderJect XR-1 research device. Each bombardment delivered 1·25 µg DNA, resulting in a total of 3·75 µg of each DNA being delivered per $\sim 6 \times 10^5$ dendritic cells. Immediately after transfection the three dendritic cell aliquots were combined and resuspended in 3 ml cRPMI-10. As a positive control, 6×10^5 dendritic cells were infected with $1·5 \times 10^7$ p.f.u. EHV-1/A183. Following transfection or infection, the dendritic cells were incubated at 37 °C, 4 % CO_2 for 16–20 h to allow protein expression and antigen presentation. After the incubation period, the transfected dendritic cells were washed and used in induction flasks for stimulation of $\sim 3 \times 10^7$ autologous CTL precursors.

CTL responses. The ability of the different gene products to induce specific killing was examined in CTL assays using autologous targets infected with EHV-1/A183 as previously described (Allen et al., 1995). Dendritic cells transfected with individual EHV-1 plasmids, empty vector plasmid, or exposed to virus were incubated with precursor CTLs for 6 days at 37 °C, 4 % CO_2. In addition, CTL precursors were also incubated with live virus as a second positive control. Virus-specific killing was measured using virus or mock-infected autologous PWM lymphoblasts in a standard 4 h chromium release assay. In order to show that the killing was ELA-A-restricted, virus-stimulated effectors from each pony were tested against heterologous target cells (these heterologous targets were derived from the six ponies used for the CTL assays, plus two additional ponies). In addition, labelled target cells were incubated at 20 °C for 30 min with mAbs blocking MHC I (mAb CVS22), or MHC II (mAb CVS10) (Lunn et al., 1996) before the addition of CTLs, to assess whether cytotoxic activity was MHC-restricted.

Lymphoproliferative responses. Each of the different transfected dendritic cell populations used for stimulation of CTL activity was also assessed for its ability to stimulate lymphoproliferative responses in autologous PBMC. Positive controls included virus stimulation and stimulation with dendritic cells exposed to virus. Negative controls included media-only stimulation and stimulation with empty vector-transfected dendritic cells. The stimulator dendritic cells were co-cultured with autologous PBMC at stimulator to responder cell ratios of 1:10, 1:20, 1:40 and 1:80. Triplicates of each cell population were cultured in cRPMI-10 in 96-well tissue culture plates for a total of 6 days and 1 µCi [^{3}H]thymidine per well was added for the last 10 h. Thymidine uptake was measured using a microplate scintillation and luminescence counter system (Top Count, Packard, Meriden, CT, USA). Proliferation was expressed as a stimulation index (SI), which was calculated by dividing the mean thymidine incorporation of stimulated cells by the mean thymidine incorporation of unstimulated cells.

Statistical analysis. In order to test the hypothesis that EHV-1-specific killing stimulated by each EHV-1 gene product was restricted by a specific ELA-A haplotype, the data of all six ponies were transformed using the following formula: (% specific lysis

EHV-1 gene product−% specific lysis empty vector)/(% specific lysis virus positive control−% specific lysis empty vector). The resulting values in the six ponies for each EHV-1 gene product were compared using an unpaired *t*-test. These *t*-tests were performed for effector to target ratios of 100 : 1, 33 : 1 and 11 : 1. Significant differences were reported when $P < 0.05$.

RESULTS

ELA-A typing

The results of ELA-A typing were as follows (ponies 86 and 17 were used only as a source of heterologous targets): pony 10, A3.1/W11; pony 82, A1/A3.1; pony 84, A3.1/A6; pony 85, A3.1/W11; pony 95, A4/A5; pony 99, A9/W11; pony 86, A2/A3.2; and pony 17, A4/A5. The reagents for this test were polyclonal antisera; therefore there is a likely subtypic variation within each serotype that reflects greater genetic complexity among horses. Ponies 10, 82, 84 and 85 shared a common ELA-A3 allele, as characterized by the positive reactivity pattern within the A3 antisera set, in addition to unique cross-reactivity to other antisera. This allele was designated ELA-A3.1. The ELA-A3 allele seen in pony 86 was different from the allele shared by ponies 10, 82, 84 and 85 based on reactivity patterns to the ELA-A3 antisera set and differences in cross-reactivity to other antisera. This allele was designated ELA-A3.2.

Clinical responses following primary and secondary EHV-1 infection

All ponies showed clinical signs of disease after their first EHV-1 infection, starting on day 1 post-infection and ending by day 21 post-infection. Rectal temperatures were $> 101.5\ °F$ for 2–4 days and mucoid to mucopurulent nasal discharge was seen for 10–21 days. In addition, ponies were depressed and anorexic for 2–5 days following infection and one pony exhibited posterior ataxia on day 7 post-challenge infection. This pony had recovered by day 14 post-challenge infection. None of the ponies showed any clinical signs of EHV-1 disease following the second experimental infection, which was administered 6 months after the first experimental infection.

Virus-neutralizing antibodies

None of the ponies had EHV-1-neutralizing antibodies in their serum prior to the first experimental infection. After infection, titres increased to peak titres of 32 : to 64 : 1 and then declined two- to fourfold by the time of the second infection. Following the second infection, titres increased only minimally or not at all in four of the six ponies. Titres in ponies 82 and 99 increased to a titre of 1 : 128.

CTL responses to EHV-1 proteins

EHV-1-specific CTL responses are shown in Fig. 1. Assays for each pony were performed once, except for ponies 82, 84 and 85 where assays were performed twice (representative results are shown). Positive control stimulator cultures, including dendritic cells infected with EHV-1, as well as EHV-1 alone, were capable of stimulating strong CTL responses in all six ponies. In all experiments, killing of mock-infected targets was less than 5 %, independent of the source or treatment of these targets. Empty vector-transfected dendritic cells did not induce CTL responses and were used in each pony to determine background killing activity, which was below 12 % specific lysis in all ponies. Dendritic cells expressing the IE gene induced CTL activity in four out of six ponies (ponies 10, 82, 84 and 85). These four ponies shared the ELA-A3.1 haplotype. The association of IE-induced CTL responses with the ELA-A3.1 haplotype was statistically significant at effector to target ratios of 100 : 1, 33 : 1 and 11 : 1. In addition, dendritic cells expressing the gC, gD, gI and gL proteins each induced cytotoxic activity in two out of six ponies and pony 10 showed a response to several EHV-1 genes. It was not possible to identify any significant association of these glycoprotein-specific responses with an ELA-A allele in this small group of ponies. No effector cultures were capable of killing autologous mock-infected targets in any pony.

ELA-A restriction of CTL responses

Fig. 2 shows the ability of virus-stimulated effectors from each pony to kill homologous, but not heterologous, targets. Notably, effectors from horses with the A3.1 allele did not kill A3.2 targets. Both virus- and IE-stimulated CTL effectors from one of the ELA-A3.1 ponies killed targets from the other three ELA-A3.1 ponies (Fig. 3). When the same effectors were tested against targets from two ponies that did not share the ELA-A3.1 allele, no killing was observed for IE-stimulated CTLs, and limited killing was observed for virus-stimulated CTLs with the targets of one pony.

When an MHC I blocking antibody was used to block killing of homologous targets as well as killing of heterologous targets from two ELA-A3.1-matched ponies, killing was reduced (Fig. 4). This reduction was not seen when antibodies to MHC class II were used in the same assay.

Lymphoproliferative responses following stimulation with individual EHV-1 proteins

EHV-1-specific proliferative responses were increased in all ponies following stimulation of autologous PBMC with virus-infected dendritic cells or heat-inactivated virus. However, individual gene-transfected dendritic cells stimulated highly variable PBMC lymphoproliferative responses and no pattern was noted (data not shown).

DISCUSSION

This study demonstrates that transfection of equine dendritic cells with plasmids encoding EHV-1 gene products can be a valuable tool for screening the capacity of individual EHV-1 proteins to elicit CTL responses. Dendritic cells were chosen as targets for transfection because they are highly
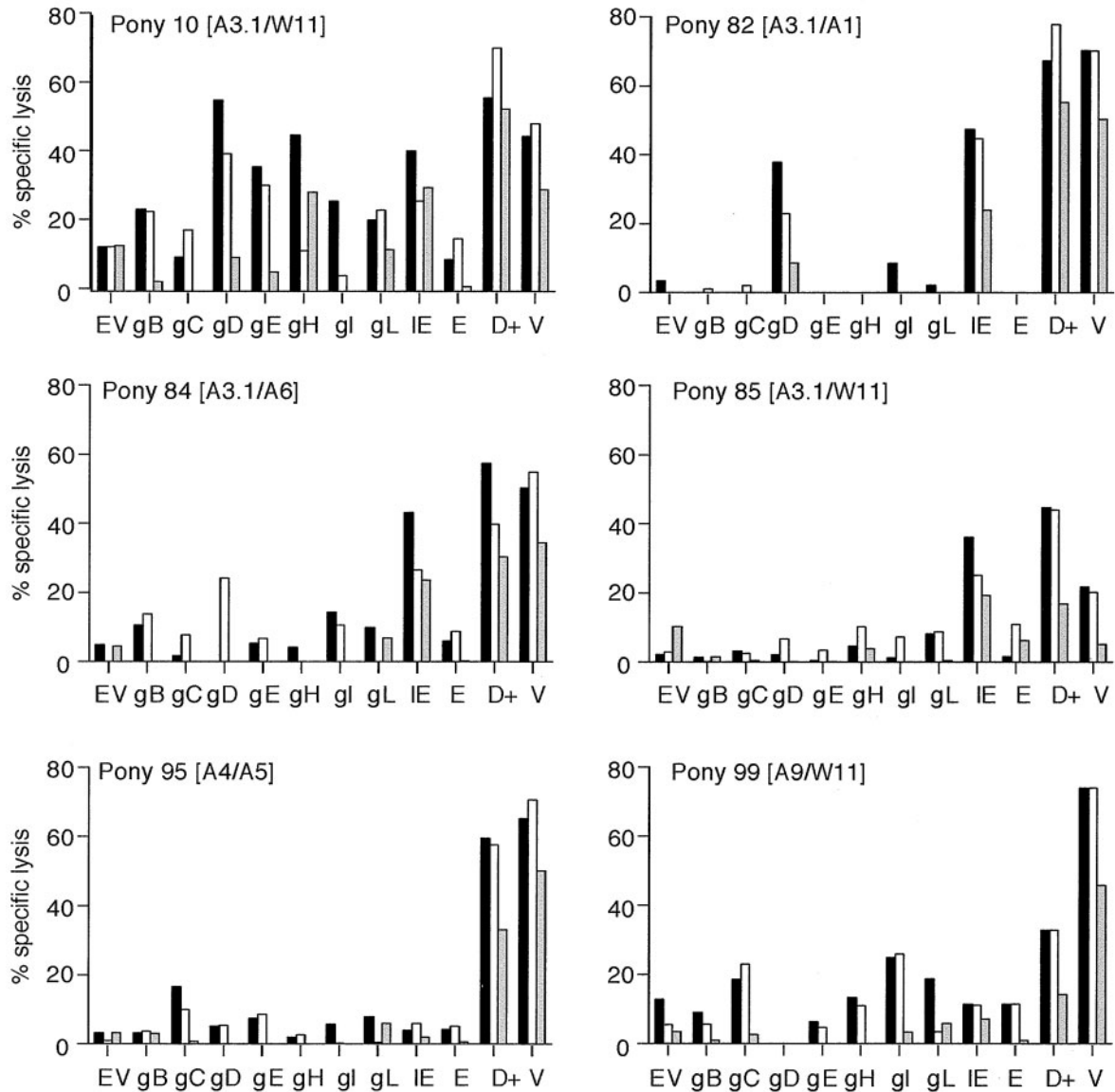
Fig. 1. CTL activity of cryopreserved PBMC effectors derived from ponies previously infected with EHV-1. Stimulators were autologous dendritic cells presenting different EHV-1 glycoproteins or the IE or early (E) proteins. The negative controls included empty vector-transfected dendritic cells (EV). Positive controls included dendritic cells exposed to virus (D+), or virus stimulation alone (V). The ELA-A type of each pony is indicated in square brackets in the graph titles. Effector to target ratios: black bars, 100 : 1; white bars, 33 : 1; grey bars, 11 : 1.

effective antigen-presenting cells (Banchereau & Steinman, 1998; Rouse *et al.*, 1994) and are extremely efficient at inducing and stimulating CTLs (Bhardwaj *et al.*, 1995; Ludewig *et al.*, 1998). CTL responses induced by dendritic cells that expressed individual gene products were lower than CTL responses induced by whole virus or dendritic cells exposed to whole virus. An explanation for this is that whole-virus stimulation is likely to result in presentation of multiple CTL epitopes. An additional explanation is that the efficiency with which dendritic cells are transfected *in vitro* is typically low: approximately 10 %. Despite this

low transfection efficiency, this report and others have shown the potential of *in vitro* transfection of dendritic cells for stimulating immune responses (Manickan *et al.*, 1997; Rouse *et al.*, 1994; Siedek *et al.*, 1999; Steinbach *et al.*, 1998). In the horse, we have previously demonstrated that dendritic cells outperform other autologous cells as antigen-presenting cells for the detection of memory lymphoproliferative responses (Soboll *et al.*, 2003). The role of CTL responses identified by this method in protecting horses from EHV-1 infection must be determined by the generation of antigen-specific CTL responses *in vivo*. However, this strategy for
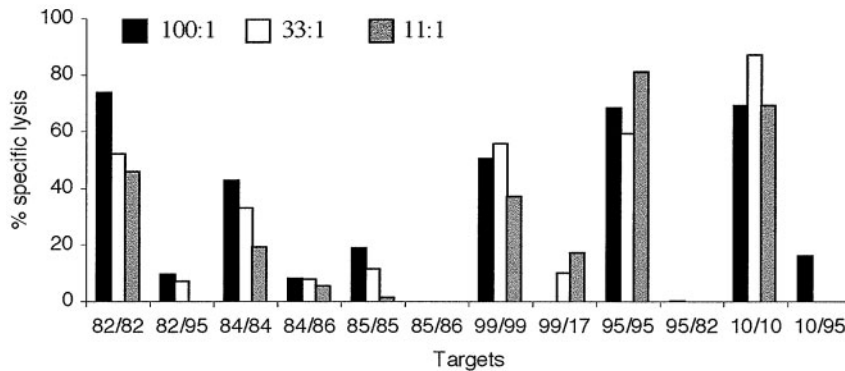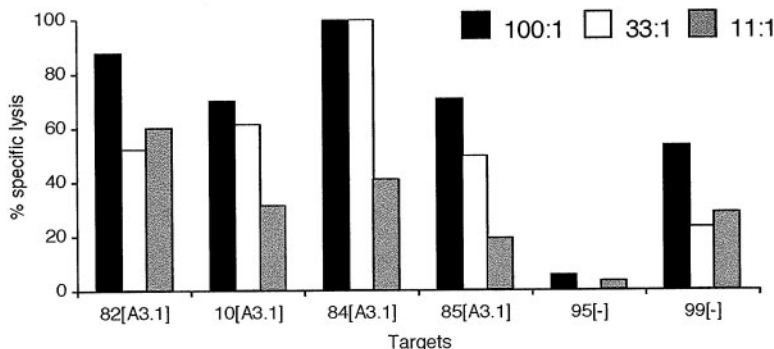
Fig. 2. CTL activity of effectors from each pony tested against homologous and heterologous (ELA-A mismatched) targets. Numbers on the *x*-axis represent the ponies used for effectors and targets, respectively. Effector to target ratios: black bars, 100 : 1; white bars, 33 : 1; grey bars, 11 : 1.

screening of EHV-1 proteins for the induction of CTL responses in horses of different genetic background will greatly facilitate such studies.

We have identified a number of EHV-1 proteins that may contain CTL epitopes. Most importantly, we have shown that the IE protein elicited CTL responses restricted by the ELA-A3.1 haplotype. A number of factors suggest that the IE protein may be an important target for protective immune responses. The single IE gene of EHV-1 is the first gene expressed in the replication cycle of the virus (Allen *et al.*, 1999; Caughman *et al.*, 1995; Smith *et al.*, 1995; Zhao *et al.*, 1995). CTLs directed against the IE protein may therefore be important for rapid clearance of virus-infected cells

a. Virus stimulated CTL effectors
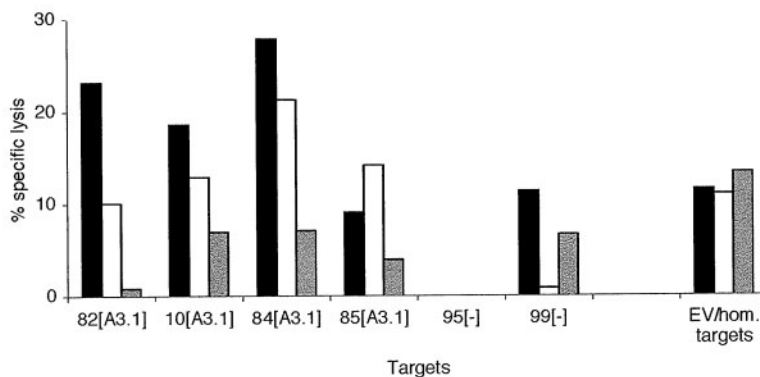

b. IE stimulated CTL effectors

Fig. 3. CTL activity of effectors from pony 82 against homologous targets and targets from the five other ponies (ponies 10, 84, 85, 95 and 99). When targets shared the ELA-A 3.1 haplotype, this is indicated in square brackets after the number of the respective pony. A − indicates that targets were ELA-A3 mismatched. (a) CTL effectors stimulated with EHV-1. (b) CTL effectors stimulated with dendritic cells expressing the IE protein; as a negative control the CTL activity of empty vector-stimulated effectors from pony 82 against homologous targets was included. Effector to target ratios: black bars, 100 : 1; white bars, 33 : 1; grey bars, 11 : 1.
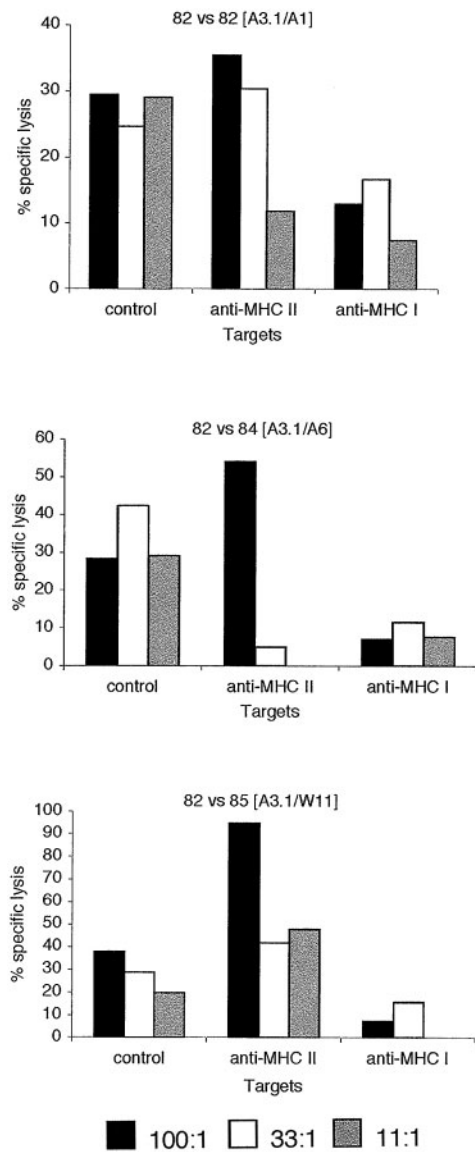
Fig. 4. CTL activity of EHV-1-stimulated effectors from pony 82 against homologous targets and targets from ponies 84 and 85. The haplotype of the targets is shown in square brackets. Prior to the addition of effectors to targets, target cells were incubated with media only, anti-MHC I or anti-MHC II antibodies. Effector to target ratios: black bars, 100:1; white bars, 33:1; grey bars, 11:1.

This study is also the first report of an ELA-A restriction of EHV-1 antigen-specific CTLs. The only other equine disease for which an ELA-A-restricted CTL response has been demonstrated is EIAV. CTL responses specific for the Gag p26 protein of EIAV are ELA-A5.1- and ELA-A9-restricted, and Env protein CTL responses are ELA-A1-restricted (Zhang *et al.*, 1998). While studies of MHC I restriction of CTL responses are still at an early stage in the horse, there are numerous examples of the importance of this phenomenon in herpesvirus infections in other species. In mice, several studies have shown correlation of the MHC haplotype with the clinical disease resulting from HSV infection (Foster *et al.*, 1986; Simmons, 1989; Sohn *et al.*, 2001). In addition, MHC-restricted murine CTL epitopes have been identified for the gB protein of HSV (Fu *et al.*, 1996; Jones *et al.*, 1997). Similarly, in humans an association with HLA type is seen for disease severity in HSV outbreaks (Lekstrom-Himes *et al.*, 1999).

The cytotoxic activity observed in this study was shown to be ELA-A-restricted, as has been reported previously for EHV-1 CTL responses (Allen *et al.*, 1995). Our data demonstrated that virus-stimulated effectors killed homologous but not heterologous ELA-A mismatched targets. However, heterologous targets sharing the ELA-A3.1 haplotype were susceptible to killing by ELA-A3.1-matched CTLs after stimulation with IE protein or whole virus. A similar observation was made in a study of EIAV (McGuire *et al.*, 1997). The importance of ELA-A restriction was further demonstrated in this study by the fact that ponies with the ELA-A3.1 haplotype (ponies 84 and 85) did not kill ELA-A3.2 targets (pony 86). This indicates the importance of subtypic variation within a given serotype. This type of ELA-A subtypic variation was also observed for CTL activity against EIAV peptides among a group of horses serotyped as ELA-A5 (Zhang *et al.*, 1998). While IE-stimulated CTL effectors from pony 82 killed targets from two out of three other ELA-A3.1 ponies (ponies 10 and 84), they did not kill targets from the third ELA-A3.1 pony (pony 85). This result could reflect further subtypic variation within the A3 serotype not defined by the antisera currently available for ELA-A typing. Similarly, some cytotoxic activity was seen when virus-stimulated CTL effectors from pony 82 were used with targets from the ELA-A mismatched pony 99 targets. This result could reflect differences in other MHC class I or class II molecules not defined by our antisera that are capable of presenting the IE protein. Experiments using a mAb previously shown to block MHC I-restricted CTL activity (O'Brien *et al.*, 1991) have further confirmed that the cytotoxicity reported in this study was MHC I (ELA-A)-restricted. Relative frequencies of the different ELA-A antigens differ considerably between horse breeds (Antczak *et al.*, 1986). This implies that in order to generate vaccines that protect the entire equine population, EHV-1 epitopes that can stimulate CTL responses in outbred horses expressing a broad range of ELA-A complexes need to be identified. The results of our study have already highlighted four additional EHV-1

(Caughman *et al.*, 1995; Smith *et al.*, 1995; Zhao *et al.*, 1995). Furthermore, studies in mice have demonstrated that the EHV-1 IE protein elicits protective CTL immunity (Koen *et al.*, 2000; Ruitenberg *et al.*, 1999b; Siedek *et al.*, 1999; Smith *et al.*, 1998). In another herpesvirus, herpes simplex virus (HSV), several reports indicate that the IE protein also contains CTL epitopes (Krisky *et al.*, 1998; Nugent *et al.*, 1995; Rouse *et al.*, 1994). This study is the first demonstration of the ability of the IE protein of EHV-1 to stimulate CTLs in the horse, the natural host of this pathogen.

glycoproteins that induced CTL responses in individual ponies (gC, gD, gI and gL). No association with an ELA-A allele could be made for these proteins. However, this study only looked at a total of six animals and further studies investigating horses with different genetic backgrounds are needed to identify additional associations.

Because CTL assays are difficult and tedious to do in the horse, we investigated whether the stimulation of lympho-proliferative responses with dendritic cells expressing individual EHV-1 proteins would correlate with the CTL responses observed. While lymphoproliferative responses increased consistently when stimulated with heat-inactivated virus or dendritic cells infected with virus, the proliferative data from PBMC stimulated with individual gene products showed great variability. Therefore, this assay is not a useful correlate for CTL responses. In the future, reagents capable of detecting equine IFN-γ may become available (Pedersen *et al.*, 2002) that could facilitate rapid screening of gene products using an adaptation of the strategy described here, with intracellular staining for IFN-γ replacing the CTL assay as an endpoint (Murali-Krishna *et al.*, 1998).

In conclusion, the results of this study have considerably advanced our understanding of the antigen specificity of CTL activity against EHV-1 in the horse. Using the DNA-based technology employed in this study, we can now proceed to identify a panel of EHV-1 antigens that are targeted by equine CTL responses. This information will be critical for developing vaccines against this important equine disease.

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