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
It has been proposed that EHV-1 proteins expressed early in contrast to other herpesviruses, EHV-1 has only one IE gene. The genome of EHV-1 contains 76 different open reading frames encoding gene products (Allen 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, gl, gl, IE or UL5 genes of EHV-1, using the Pow德erject 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 lymphblast 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, gl, 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, gl 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 pPRCMV 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-amin-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 x 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 x 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.

In EHV-1 gene product—% 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...
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

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**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.
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.

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.

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.
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.

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 sero-typed 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 lymphoproliferative 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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