Detection of equine arteritis virus (EAV)-specific cytotoxic CD8+ T lymphocyte precursors from EAV-infected ponies


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Equine arteritis virus (EAV) causes a systemic infection in equids with variable outcome, ranging from subclinical infections to severe disease, and also has the capacity to induce abortion in pregnant mares and persistent infections in stallions. The serum virus-neutralizing antibody response that invariably develops in the infected animal lasts for many months or years and is believed to play an important role in virus clearance. However, very little is known about cellular immunity against EAV because of a lack of methods for evaluating these immune responses. In the present study, we describe methods for detecting cytotoxic T lymphocyte (CTL) precursors in the peripheral blood of EAV-convalescent ponies using a 51Cr release cytolysis assay. Primary equine dermal cells, used as CTL targets, were shown to express MHC I but not MHC II and to retain 51Cr efficiently and support EAV replication. Peripheral blood mononuclear cells (PBMC) collected from EAV-convalescent ponies that had been incubated with or without live EAV were used as effectors. EAV-induced PBMC cultures showed evidence of expansion and activation of lymphoblasts, with an increase in the CD8+/CD4+ ratio in comparison with mock-induced PBMC. The cytotoxicity induced by EAV-stimulated PBMC was virus specific, showed genetic restriction, was mediated by CD8+ T lymphocytes and could be detected for periods of 4 months to more than 1 year post-infection. These findings and methods will hopefully contribute to an understanding of virus–host interactions in horses, in particular the mechanisms of virus clearance occurring during EAV infection.

INTRODUCTION

Equine arteritis virus (EAV), the prototype member of the family Arteriviridae, is the causative agent of equine viral arteritis. This group of viruses includes porcine reproductive and respiratory syndrome virus (PRRSV), simian haemorrhagic fever virus (SHFV) and lactate dehydrogenase elevating virus (LDV). EAV infects only equids, causing systemic infection with variable outcome ranging from asymptomatic infection to severe disease and death, depending on virus strain, age and immune status of the host and environmental factors. Infected animals can present any combination of the following clinical signs: pyrexia; depression; conjunctivitis; oculocardinal discharge; peri-orbital, limb or ventral oedema; ataxia; stiff gait; diarrhoea; urticarial skin rash; and lymph node swelling. Although fatal infections have been reported during experimental infections with 'velogenic' strains of EAV (McCollum & Timoney, 1998), most field infections occur asymptotically (Timoney & McCollum, 1988). Of significance is the capacity of EAV to cause abortion in the pregnant mare, which can happen in the absence of clinical signs, and persistent infections in stallions. The latter can excrete virus in the semen for months or years after infection.

Virus-neutralizing antibodies (VNAs) in equids are believed to play an important role in immunity against EAV (Fukunaga et al., 1981; McCollum, 1969). Their appearance in serum coincides with clinical recovery and reduction of virus excretion, and passive transfer of colostral antibodies from immune mares to foals was found to moderate or prevent equine viral arteritis (McCollum, 1976). Furthermore, protection in animals immunized with inactivated whole virus vaccines (Fukunaga et al., 1990) or a prototype subunit vaccine comprising the Gt (ORF5) ectodomain, an immunodominant virion component (Chirnside et al., 1995; Castillo-Olivares et al., 2001), correlated with VNA titres at the time of challenge. Currently, nothing is known about cell-mediated immunity to EAV, such as the role it plays in clearance of virus infection and whether it would be desirable to stimulate such immune responses by vaccination.

Studies of other arteriviruses have investigated cell-mediated immune responses. Virus-specific cytotoxic T
lymphocytes (CTL) have been detected in mice infected with LDV (Even et al., 1995; van den Broek et al., 1997).

There are some indications that cellular immunity plays an important role in clearance of SHFV infections since persistently infected patas monkeys with low-titre VNAb can clear subsequent acute infections caused by a different strain (Gravell et al., 1986). It has also been shown that PRRSV infection of pigs stimulates a strong cellular immune response (Lopez Fuertes et al., 1999) and that there is an increase in cytolytic lymphocytes in the lungs during infection (Samsom et al., 2000). Animals that recover from EAV infection develop a long-lasting immunity against the disease (Gerber et al., 1978), although not always against re-infection (McCollum, 1969). However, EAV replication in chronically infected stallions, which is restricted to cells of the accessory sex glands, persists for several months or years, despite high levels of circulating VNAb. In addition, experimental infections with EAV can cause a cell-associated viremia that lasts several weeks after serum VNAb becomes detectable (Neu et al., 1987; J. Castillo-Olivares, unpublished observations). One step towards a better understanding of virus–host interactions in equine viral arteritis would be to determine whether virus-specific cytotoxic cell-mediated immune responses are elicited during infection in the natural host.

Various methods have been developed to study CTL responses against equine herpesvirus type 1 (EHV-1) (Allen et al., 1995; O’Neill et al., 1999), equine infectious anaemia virus (McGuire et al., 1994, 1997, 2000; Hammond et al., 1998; Zhang et al., 1999; Lonning et al., 1999) and equine influenza virus (Hannant & Mumford, 1989). The aim of this work was to determine whether EAV induces cytotoxic cell-mediated immune responses and to examine some of the features of this response in the natural host.

**METHODS**

**Viruses.** A derivative of EAV strain LP3A (Castillo-Olivares et al., 2001) was used for in vitro infections and for the experimental infection of ponies. This virus, designated EAV LP3A+, was obtained by a single passage of EAV LP3A in equine embryonic lung cells.

**Animals.** Five 2-year-old castrated male ponies (7378, 027a, 5d66, 5062 and 697b) infected intranasally with 10⁶ TCID₅₀ EAV LP3A+ strain were used to obtain peripheral blood mononuclear cells (PBMC) and equine dermal cells (EDC). Both types of cell were used in cytolytic assays to detect EAV-specific cell-mediated cytotoxic responses. The animals presented a moderate to severe acute EAV syndrome, characterized by pyrexia, anorexia, lethargy, weight loss, mild ataxia and convulsant. Virus was isolated from nasal secretions for the first week of infection and from blood up to day 21 post-infection. VNAb were first detected in serum by day 6 p.i., VNAb becomes detectable (Neu et al., 1987). VNAbs were first detected in serum by day 6 p.i., VNAb becomes detectable (Neu et al., 1987).

**Preparation of targets for ⁵¹Cr release cytolytic assay.** Twenty-four hours before the effectors were incubated with the targets, the EDC monolayers were washed twice with PBS and the cells trypsinized. Once detached, the cells were resuspended in MEM/10% FBS and centrifuged for 5 min at 1400 r.p.m., the supernatant discarded and the cells resuspended in MEM/20% FBS at a concentration of 4 × 10⁵ cells ml⁻¹. Half of the cells were inoculated with EAV LP3A+ at an m.o.i. of 0.3 TCID₅₀ the other half was left uninfected. Each set of cells was then inoculated with 15 μCi Na₂¹⁵CrO₄ (Amersham) ml⁻¹, and 100 μl per well of each suspension was added to half of the wells of a 96-well flat-bottomed plate and incubated at 37°C, 5% CO₂ for 24 h.

**Secondary stimulation of in vivo–primed PBMC (effectors).** Induction of cytotoxic cells from PBMC was done as described for the detection of EHV-1-specific CTL by Allen et al. (1995) with minor modifications. Briefly, whole venous blood was collected at various times after infection into vacuum tubes containing 1 IU sodium heparin in PBS ml⁻¹. whole blood and the mononuclear cell fraction was isolated by Ficoll–Hyphaque density gradient centrifugation. The interface was harvested and the PBMC were washed three times in PBS to reduce the number of platelets and resuspended in either freezing medium or induction medium [1:1, v/v, mixture of AIM-V/RPMI 1640 supplemented with 2 mM l-glutamine, minimal essential medium non-essential amino acids (0.05 M each), 0.5 mM sodium pyruvate, 2-mercaptoethanol (55 μM), gentamicin (50 μg ml⁻¹) and equine serum (7%, collected from the ponies before EAV experimental infection and inactivated at 56°C for 40 min)]. The PBMC were incubated in induction medium for 7 days in upright 75 cm² tissue culture flasks at 1:1–2:10 × 10⁶ cells per flask in 40 ml in the presence or absence of 10⁻⁷ TCID₅₀ EAV LP3A+.

**Measurement of cytolytic activity of ‘in vitro’-stimulated PBMC.** EAV-induced and mock-induced PBMC cultures were centrifuged at 800 g at 20°C for 10 min without the brake, the supernatant discarded and cell pellets resuspended in CTL medium (RPMI 1640 containing 10% heat-inactivated equine serum). Both cultures were adjusted to contain the same concentration of viable cells (determined by trypsin blue exclusion) and diluted appropriately in CTL medium to obtain different effector:target ratios when added to the overnight-grown fibroblasts. Target cells (24 h after addition of ⁵¹Cr, ± EAV infection) were washed three times with RPMI 1640 using 125 μl per well in each wash before the addition of either the effectors, a CTL medium control, or cell lysis solution (2% Triton X-100 in PBS). The plates were incubated for 4 h at 37°C, 5% CO₂, after which the supernatants were harvested (Supernatant Harvesting System) for quantification of ⁵¹Cr release by gamma counting. The lytic activity of each PBMC culture dilution was assessed against four to six replicates of EAV-infected and uninfected autologous or allogeneic radiolabelled targets. The percentage of specific ⁵¹Cr release was calculated according to the formula: [(c–sp)/(t–sp)]×100, where c is the experimental ⁵¹Cr release.
release in the presence of effectors, sp is the spontaneous $^{51}$Cr release in the presence of CTL medium and t is the total $^{51}$Cr release from targets incubated with cell lysis solution.

**Indirect immunofluorescence.** Equine dermal fibroblasts or cytopsins of PBMC were fixed in either acetone or 4% formaldehyde, 0-4% Triton X-100 in PBS for 15 min at room temperature. After washing in PBS, an anti-EAV nucleocapsid (N)-specific rabbit polyclonal antiserum (de Vries et al., 1992) diluted 1:100 in 2% BSA in PBS (PBSA) was applied to the cells and incubated for 1 h at 37°C. After washing in PBS, the samples were incubated for 1 h at 37°C with an anti-rabbit IgG FITC-conjugated antibody (Dako) diluted 1:40 in PBSA, washed again in PBS and observed using a fluorescence microscope.

**Flow cytometry.** Equine dermal cells were pretreated before immunostaining procedures in suspension were carried out. Confluent monolayers of EDC were trypsinized, washed in MEM/20% FBS, resuspended in MEM/20% FBS and incubated at 37°C, 5% CO₂ for 2 h. In vitro-induced effectors and pretreated targets were washed in PBSG (0-5% normal goat serum, 0-01% sodium azide in PBS) three times and 2 x 10⁶ cells resuspended in PBSG containing monoclonal antibodies (mAbs) (2 µg ml⁻¹) specific for equine lymphocyte antigens (Lunn et al., 1998). Cells were incubated with either mAb H58A, H42A, HT14A or HB61A (VMRD Inc.) specific for MHC I, MHC II, CD8 and CD4 antigens, respectively. Background staining was determined using an IgG1 isotype control mAb (Dako). After 1 h incubation on ice, the cells were washed with ice-cold PBSG and resuspended in goat anti-mouse IgG FITC-conjugated antibody (Dako) diluted 1:40 in PBSG and incubated for 1 h on ice. After a final wash in PBSG, the cells were fixed in 2% formaldehyde and analysed on a FACScalibur flow cytometer (Beckton Dickinson). Dead cells were identified using propidium iodide staining at a concentration of 20 µg ml⁻¹.

**Separation of CD4⁺ and CD8⁺ T cells from induced PBMC cultures.** Biomagnetic separation of effectors was performed using MACS goat anti-mouse IgG microbeads and MS Separation Columns (Miltenyi Biotec), essentially following the manufacturer’s recommendations. Briefly, after 7 days incubation, the PBMC cultures were centrifuged, the supernatant discarded and the cells washed twice in separation buffer (0-5% BSA, 2 mM EDTA in PBS). The stimulated PBMC were then incubated for 1 h at 4°C in separation buffer with or without 2 µg of either anti-equine CD4 mAb HB61A or anti-equine CD8 mAb HT14A ml⁻¹. The cells were washed twice in separation buffer and incubated with the anti-mouse IgG-conjugated microbeads for 1 h at 4°C before being washed again in separation buffer and applied to the separation columns. Both enriched and depleted cell fractions were collected, centrifuged and the cell pellets resuspended in CTL medium to obtain equivalent concentrations of effectors. The cells were then assayed for cytotoxic activity as described above.

**RESULTS**

**Properties of primary equine dermal cells**

EDC cultures consistently displayed fibroblast-like morphology. Expression of cell-surface MHC I and MHC II was tested by indirect immunofluorescence microscopy and flow cytometry, using either the anti-equine MHC I mAb H58A or the anti-equine MHC II mAb H42A. Only MHC I was detectable in these cells by immunofluorescence microscopy (data not shown) or by flow cytometry (Fig. 1a and b). EDC were susceptible to infection with EAV, expressing the virus N protein in a high proportion of cells 24 h after infection (i.e. the time when effectors would be added to the targets) while retaining the original cell morphology (Fig. 1c). Preliminary studies demonstrated that EDC incorporated and retained $^{51}$Cr label appropriately. The mean $^{51}$Cr spontaneous release of all assays performed in this study for EAV-infected EDC [22-6% (10–33-7%)] and mock-infected EDC [20% (8–33%)] were within limits suitable for measuring cell lysis in a cytotoxicity assay.
Cytolytic activity of in vitro-stimulated PBMC

Cell-mediated cytotoxic responses against EAV were investigated using PBMC from convalescent EAV-infected ponies. These cells were incubated in vitro in the presence or absence of live EAV virus and the lytic activity of the effectors was tested against EAV-infected or mock-infected EDC. Various attempts to demonstrate EAV-specific cytotoxicity failed when the effectors were derived from cryopreserved stocks (data not shown). When freshly collected PBMC were employed, however, EAV-specific cytotoxicity was observed. Thus, PBMC collected 4 months p.i. (from pony 7378) and stimulated in vitro with EAV for 7 days lysed autologous EAV-infected targets (Fig. 2). EAV-induced effectors specifically lysed EAV-infected targets producing a percentage lysis at least 10% higher than those obtained from the other combinations of effectors and targets. Cytolysis of EAV-infected, autologous targets, using PBMC and target cells derived from four different ponies, was a consistent feature of eight experiments (unpublished data and results displayed herein), with specific lysis varying between 15 and 40%.

In a separate experiment, haematoxylin/eosin staining of

![Image](https://example.com/fig2a.png)

**Fig. 2.** EAV-specific cytotoxic activity displayed by in vitro-stimulated PBMC. PBMC collected from pony 7378 (4 months after infection with EAV) were either EAV-induced (a) or mock-induced (b), prior to testing for cytolysis of EAV-infected and mock-infected autologous EDC targets. Results show the percentage specific lysis of targets at different effector:target cell ratios: 50:1 (white bars), 25:1 (grey bars) and 12.5:1 (black bars). Error bars indicate the standard deviation between replicates for each effector/target combination (five replicates per data point).

![Image](https://example.com/fig2b.png)

7-day-old cultures of PBMC collected approximately 5 months p.i. from pony 7378 showed reduced numbers of monocytes in the EAV-stimulated effectors as opposed to the mock-stimulated effectors (Fig. 3a and b). Moreover, the lymphocytes of EAV-stimulated (but not

![Image](https://example.com/fig2c.png)

**Fig. 3.** Features of in vitro-stimulated PBMC cultures. (a, b) Haematoxylin/eosin staining of 7-day-old induction cultures using PBMC from pony 7378 collected at 5 months p.i. (a) Mock-induced cultures in which a significant proportion of monocytes can be seen (white arrows). (b) EAV-induced cultures in which monocytes are no longer apparent. (c) A 24 h culture of EAV-induced PBMC, stained for nucleocapsid (N) expression by immunofluorescence.
mock-stimulated) effector cultures had the enlarged appearance typical of activated lymphoblasts. Samples from these same cultures taken 24 h after inoculation with EAV revealed that approximately 5% of the cells with monocyte morphology expressed EAV N antigen (Fig. 3c). Furthermore EAV N protein-expressing cells in 24 h EAV-stimulated cultures could be detected when fresh PBMC were used, but not with PBMC derived from cryopreserved stocks (not shown), suggesting that lack of an EAV-susceptible cell type in cryopreserved PBMC was responsible for the lack of induction of cytolytic activity.

**Genetic restriction of cytotoxic responses**

The ponies used in this study came from an outbred population and it was not possible to obtain MHC I typing data. To determine whether the virus-specific cytolytic activity detected in EAV-stimulated PBMC collected from convalescent ponies was genetically restricted, we performed a series of cross-matching cytotoxicity assays. In these experiments, EAV-stimulated and mock-stimulated PBMC from one individual were incubated with EAV-infected target cells from the same individual or from a different pony (Fig. 4). PBMC were collected at different times p.i. for each of the ponies tested: 7378 (6 months p.i.), 5d66 (6–6 months p.i.) and 027a (8 months p.i.). Fig. 4(a) demonstrates that 7378-derived effectors specifically lysed EAV-infected autologous and 027a-derived target cells. In contrast, EAV-infected 5d66-derived target cells showed low levels of lysis similar to those of uninfected targets. In a second experiment, effectors derived from pony 5d66 were tested for cytolyis of EAV-infected or uninfected EDC from each of the three ponies (Fig. 4b). Only lysis of autologous EAV-infected targets was observed, supporting the hypothesis of genetic restriction of the effector cells. Lastly, the specificity of effectors derived from 027a was evaluated. Results of this experiment (Fig. 4c) demonstrated that the 027a-derived effector cells recognized EAV-infected target cells from ponies 027a and 7378, but not uninfected cells or EAV-infected cells from pony 5d66. Mock-induced effectors did not cause significant specific lysis of target cells in any of these experiments (<5%, data not shown). Assuming that cytolysis was mediated by MHC I-restricted CTL, these data are consistent with pony 7378 and pony 027a having at least one MHC I allele in common and pony 5d66 not sharing an MHC I allele with either of the other two ponies.

**Phenotype of cytotoxic effector cells**

The virus-specific, genetically restricted cytolytic activity of *in vitro* EAV-stimulated PBMC from EAV convalescent ponies, which could be detected for at least 1 year p.i. (data not shown), is consistent with a classical CD8<sup>+</sup> CTL-mediated immune response. Further indication of the phenotype of the cytotoxic cells was obtained via sorting of EAV-stimulated effectors, using PBMC from pony 697b (collected 8 months p.i.). The effectors were either assayed for cytolytic activity without subfractionation, or following enrichment or depletion of either CD8<sup>+</sup> or CD4<sup>+</sup> cells (using biomagnetic separation as described in the Methods). Immunoperoxidase staining of aceton-fixed PBMC following separation showed a complete absence of CD8<sup>+</sup> cells and an almost complete absence of CD4<sup>+</sup> cells in the CD8<sup>+</sup>- and CD4<sup>+</sup>-depleted populations, respectively (data not shown). Fig. 5(a) confirmed specific cytolysis of EAV-infected autologous targets by the unfractionated, EAV-induced effectors. Strikingly, despite the relatively low effector:target ratio (6:1), the CD8<sup>+</sup>-enriched fraction showed a high level of lysis of EAV-infected targets (60% specific lysis). In contrast, the CD8<sup>+</sup>-depleted and the CD4<sup>+</sup>-enriched subpopulations showed very low cytolytic activities. These results indicate that the EAV-specific cytotoxicity observed *in vitro* is mediated by CD8<sup>+</sup> T lymphocytes.
In the present study, generation of virus-specific CD8+ T lymphocytes was achieved by incubating the PBMC for a few days in the presence of live virus without adding exogenous cytokines such as IL-2. Although exogenous cytokines were likely to enhance the activation of lymphocytes during the in vitro induction process, previous studies in horses have shown that high concentrations of exogenous IL-2 stimulated cytotoxic effector cells of broad specificity, which killed in a genetically unrestricted manner (Hannant & Mumford, 1989). It is worth noting that, unlike EHV-1 CTL induction (O’Neill et al., 1999), the

**DISCUSSION**

Understanding virus–host interactions during equine viral arteritis has been hampered by the absence of methods for measuring cellular immune responses to EAV. The work presented here describes for the first time methods to assess EAV-specific CTL responses of ponies. The methods employed were based upon those described by Allen et al. (1995) and O’Neill et al. (1999) for measuring EHV-1 CTL, with some modifications. The first of these modifications was the use of primary EDC as targets instead of in vitro-stimulated lymphoblasts, since preliminary studies indicated that lymphoblasts were non-permissive for EAV infection (unpublished data). The use of dermal cells as targets for cytotoxicity assays has been reported for several non-equine species (Ohishi et al., 1999; Sharpe et al., 2001; Koksoy et al., 2001; Flomenberg et al., 1996). In equine immunology studies, detection of cell-mediated cytotoxicity has been achieved using either mitogen-stimulated lymphoblasts (Allen et al., 1995; Hammond et al., 1998; O’Neill et al., 1999), primary kidney fibroblasts (McGuire et al., 1997) or primary equine dermal cells (Romito et al., 1999; D. Hannant, unpublished observations) as targets. We have found the use of EDC easy and convenient. They are more easily accessible than equine kidney cells, do not require previous incubation under special conditions (in contrast to mitogen-stimulated lymphoblasts), retain chromium efficiently and support EAV replication. However, they have the disadvantage of not constitutively expressing MHC II and therefore CD4+ -mediated cytotoxicity is unlikely to be measurable with these targets.

Flow cytometry analysis of effector cultures

Characterization of the effector cultures was performed by flow cytometry analysis of EAV-induced and mock-induced cultures prepared with PBMC collected from pony 5062 1 year p.i. (Fig. 6). Propidium iodide staining was used to exclude dead cells (data not shown) and hence the R1 gate (live cells) was chosen for the subsequent analyses. Incubation of PBMC in the presence of EAV resulted in a lower percentage of live cells in comparison with the mock-induced cultures. However, EAV-induced effectors showed a higher proportion of cells with increased size and granularity (region R2), indicative of activated lymphoblasts. In addition, EAV-induced PBMC displayed an upregulation of either CD4 or CD8 on a subpopulation of cells, as evident by a second peak of higher fluorescent intensity for CD4+ and CD8+ cells. Overall, there was an increase in the CD8/CD4 ratio (from 0:43 to 0:77) when comparing the mock- and EAV-induced cultures, resulting from an increase in the proportion of CD8+ cells and a decrease in the proportion of CD4+ cells in the R1 gate. Comparison of the number of cells within the R2 gate with the R1 gate demonstrated an increase in the proportion of both CD4+ and CD8+ cells within the activated lymphoblast population, when comparing mock-induced and EAV-induced cultures. Thus, the proportion of CD4+ cells in the R2 gate increased from 9 to 30% and the proportion of CD8+ cells from 18 to 42% (relative to the total number of viable CD4+ or CD8+ cells). Furthermore, the majority of CD4+ and CD8+ cells present within the R2 gate for the EAV-induced cultures displayed upregulated expression of CD4 or CD8, respectively. These data indicate activation of both CD8 and CD4+ lymphocytes in response to EAV induction.

**Fig. 5.** Determination of EAV-specific cytolytic activity for different subpopulations of effectors. PBMC collected from pony 5062 1 year p.i. were tested for cytolytic activity against either EAV-infected or mock-infected autologous EDC targets. (a) Activity of non-fractionated effectors, either EAV-induced (black bars) or mock-induced (white bars), used at an effector:target ratio of 6:1. Effectors were either enriched for (grey bars) or depleted of (hatched bars) specific cell-types, using mAb against CD8 or CD4.

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cytotoxic activity against EAV-infected cells could not be induced using cryopreserved PBMC. This is probably due to damage of an EAV-permissive subpopulation of cells during the freezing and/or thawing processes. For EHV-1, it is known that T lymphocytes are the major population susceptible to EHV-1 infection and these cells survive the cryopreservation procedures well. In our studies, EAV N antigen expression was clearly detected in freshly collected PBMC but not in cryopreserved PBMC (24 h after infection with EAV) and infected cells had a monocyte-like morphology. Furthermore, we observed a significant reduction in the number of cells of monocyte morphology visible for EAV-induced compared with mock-induced PBMC (24 h p.i.), suggesting that monocyte-lineage cells may be the EAV permissive cell type acting as the principal antigen-presenting cell during the in vitro stimulation procedures. However, dual staining with an antibody specific for the human monocyte/granulocyte marker L1 (calprotectin) demonstrated that EAV-infected cells (24 h p.i. of PBMC) were L1 negative (data not shown). This may indicate that the EAV-infected cells are not monocyte/macrophage lineage, that EAV infection down-regulates expression of L1, or that only a subpopulation of differentiated monocytes/macrophages is susceptible to EAV infection, since it has been shown that a subset of blood monocytes do not express L1 and that expression is modulated during differentiation (Zwadlo et al., 1988). Further studies are required to identify the principal antigen-presenting cells required for in vitro induction of CTL against EAV.

Following EAV induction, we observed activation of both CD8+ and CD4+ cells (indicated by increased size and granularity and upregulation of CD8 and CD4 antigen expression), with an overall increase in the CD8+/CD4+ ratio. Significantly, subfractionation of effectors indicated that CD8+ cells were responsible for the lysis of EAV-infected target cells. We were not able to determine the equine leukocyte antigen phenotype of the ponies used in this study. However, our results suggested the cytolytic

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**Fig. 6.** Flow cytometry analysis of 7-day-old mock- or EAV-induced PBMC cultures derived from pony 5062 (PBMC collected 1 year p.i.). (a–d) Light scatter profiles, showing the regions gated for subsequent analyses (a and c; R1; b and d, R2). R1 corresponds to live cells as determined by propidium iodide exclusion (data not shown) and R2 corresponds to activated lymphocytes (indicated by increased size and granularity). (e–f) Two samples from each culture were immunostained for detection of CD4 (e–h) or CD8 (i–l). A third sample was immunostained with an IgG1 isotype control mAb (open histograms, overlaid on each of the CD4+ and CD8-specific plots). For each histogram plot, the M1 region was analysed to determine the proportion of live cells (R1 gate) expressing either CD4 or CD8. Histograms display analysis of cells within either the R1 gate (e, i, g, k) or the R2 gate (f, j, h, l). The number of cells counted within each region analysed is given in parentheses.
activity was genetically restricted. Although we have not definitively demonstrated MHC I-restricted killing, these results are consistent with stimulation of a classical, CD8+ CTL response.

We have demonstrated that EAV-specific CTL precursors persist for at least 1 year p.i. Clinical recovery from EAV and the reduction of virus excretion from nasal secretions coincides with the development of VNAbs in serum (usually after the first week of infection), as observed in the ponies used in this study and described in other reports (Fukunaga et al., 1981; McCollum et al., 1969). However, cell-associated viraemia persists for longer periods ranging from 2 or 3 weeks to around 3 months p.i. (Neu et al., 1987; J. Castillo-Olivares, unpublished data). The mechanisms responsible for the eventual disappearance of the virema are not known, but the detection of CD8+ CTL precursors from EAV-exposed ponies indicates that cell-mediated immunity might play an important role in the ultimate clearance of the infection. Following PRRSV infection of pigs, it appears that a combination of cell- and antibody-mediated immune mechanisms is necessary to clear infection. PRRSV induced an antibody response that was detectable by day 9 p.i. (Labarque et al., 2000), but lymphoproliferative responses and VNAbs were not detectable until 4 weeks p.i. (Lopez Fuertes et al., 1999), coinciding with clearance of virus from blood and lungs. Observations in mice infected with LDV, which exhibit a life-long persistent infection, indicate that CTL are stimulated but disappear within 30 days (Even et al., 1995). It is believed that the mechanism of viral persistence in LDV infections involves a process of clonal deletion of new LDV-specific CD8+ cells in the thymus, leading to a state of tolerance to LDV CTL epitopes. It will be interesting to determine the importance of CTL in the control and ultimate clearance of EAV infection, in particular, whether there are differences between the CTL responses of stallions persistently infected with EAV compared with those that clear the infection.

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