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Major Histocompatibility Complex-Restricted CD8$^+$ Cytotoxic T Lymphocytes from Horses with Equine Infectious Anemia Virus Recognize Env and Gag/PR Proteins

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Cytotoxic T lymphocytes (CTL) can control some viral infections and may be important in the control of lentiviruses, including human immunodeficiency virus type 1. Since there is limited evidence for an in vivo role of CTL in control of lentiviruses, dissection of immune mechanisms in animal lentiviral infections may provide needed information. Horses infected with equine infectious anemia virus (EIAV), a lentivirus, have acute plasma viremia which is terminated in immunocompetent horses. Viremic episodes may recur, but most horses ultimately control infection and become asymptomatic carriers. To begin dissection of the immune mechanisms involved in EIAV control, peripheral blood mononuclear cells (PBMC) from infected horses were evaluated for CTL to EIAV-infected cells. By using noninfected and EIAV-infected autologous equine kidney (EK) cells in ^{51}Cr-release assays, EIAV-specific cytotoxic activity was detected in unstimulated PBMC from three infected horses. The EIAV-specific cytotoxic activity was major histocompatibility complex (MHC) restricted, as determined by assaying EIAV-infected heterologous EK targets, and was mediated by CD8$^+$ T lymphocytes, as determined by depleting these cells by a panning procedure with an anti-CD8 monoclonal antibody. MHC-restricted CD8$^+$ CTL in unstimulated PBMC from infected horses caused significant specific lysis of autologous EK cells infected with recombinant vaccinia viruses expressing EIAV genes, either *env* or *gag* plus 5′ *pol*. The EIAV-specific MHC-restricted CD8$^+$ CTL were detected in two EIAV-infected horses within a few days after plasma viremia occurred and were present after viremia was terminated. The detection of these immune effector cells in EIAV-infected horses permits further studies to determine their in vivo role.

Among lentivirus infections, the disease caused by equine infectious anemia virus (EIAV) has distinctive features during both the acute clinical and the subsequent asymptomatic carrier stages. Horses with EIAV have an initial plasma viremia and associated fever, anemia, and thrombocytopenia occurring as early as 1 to 2 weeks after infection (4, 19, 38, 42). The initial viremia is usually terminated (19, 42), and viremic episodes and clinical disease of a duration of several days may recur. Occurrence of viremic episodes correlates with the appearance of viral antigenic variants which are resistant to existing neutralizing antibody (21, 35, 40). Despite antigenic variation of epitopes recognized by neutralizing antibody (21, 35) and other possible mechanisms of viral escape from immune responses, most horses control EIAV and become clinically quiescent virus carriers (5). These carriers have extremely small amounts of virus in their blood (6), indicating that the virus is effectively controlled.

The mechanisms by which horses eventually control EIAV are unknown, although evidence indicates that immune responses are involved. Foals with genetically transmitted severe combined immunodeficiency disorder and lacking only B- and T-lymphocyte function are unable to clear the initial viremia associated with EIAV infection (42). That EIAV carriers become viremic within 5 to 7 days following either corticosteroid or cyclophosphamide treatment further suggests virus control by immune responses (20). Several immune mechanisms directed against viral proteins could be involved in control of EIAV. Antibodies to Env protein epitopes cause either viral neutralization (9, 15) or possible destruction of cells expressing Env or other proteins by antibody-dependent cellular cytotoxicity (22) or complement lysis (12). In human immunodeficiency virus type 1 (HIV-1)-infected humans, cytotoxic T lymphocytes (CTL) recognize epitopes from several viral proteins, including Env, Gag, Pol, Nef, and Vif proteins (36, 39, 44, 50), and CTL recognizing epitopes on similar simian immunodeficiency virus (SIV) proteins are present in infected rhesus monkeys (2, 31, 52, 53).

Knowledge of the relative roles of the various immune responses in preventing lentivirus infections and in controlling infections once they occur is needed to design effective vaccines and immunologic interventions (13). Thus, CTL were investigated to begin dissecting immune responses involved in the termination of plasma viremia in acute EIAV infection and in EIAV control in asymptomatic carriers. Since major histocompatibility complex (MHC)-restricted CTL to EIAV-infected cells had not been clearly described, cell targets for demonstrating cytotoxicity were identified, and an assay was developed to detect CTL in unstimulated peripheral blood mononuclear cells (PBMC) from infected horses. MHC-restricted cytotoxic activity was found in unstimulated PBMC that caused significant specific lysis of EIAV-infected equine kidney (EK) cell cultures. Depletion of CD8$^+$ T lymphocytes removed the EIAV-specific MHC-restricted CTL activity. Viral proteins recognized by CTL were determined by using

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autologous EK cells infected with recombinant vaccinia viruses expressing EIAV genes as targets. MHC-restricted $CD8^+$ CTL caused lysis of targets infected with recombinant vaccinia viruses expressing EIAV genes, either *env* or *gag* plus 5′ *pol*.

MATERIALS AND METHODS

EIAV-infected horses. The horses used were mixed-breed ponies. One kidney was removed by nephrectomy from a 13-month-old male (H507), and 2 months later the horse was infected with 10^7 50% tissue culture infective doses ($TCID_{50}$) of $EIAV_{WSU5}$. $EIAV_{WSU5}$ is a pathogenic cell culture-adapted strain isolated after three sequential passages of prototype EIAV in horses, with the predominant virus type selected by three limiting dilutions on EK cell cultures from a newborn foal (34, 37). A percutaneous renal biopsy was taken from a 10-month-old female (H521), and 1 month later, the horse was infected with 10^8 $TCID_{50}$ of $EIAV_{WSU5}$. Similarly, a renal biopsy was taken from a 6-year-old male (H525), and 2 weeks later, the horse was infected with 10^8 $TCID_{50}$ of $EIAV_{WSU5}$. Rectal temperature was taken daily (three times per week), the blood packed-cell volume was measured, and plasma virus titer was determined by using EK cell cultures (37, 42). Blood thrombocyte counts of H525 were done three times a week.

Target cells for CTL assays. Attempts to demonstrate CTL in infected horses by using EIAV-infected autologous dermal fibroblasts as targets were unsuccessful. Subsequent studies with cultures of undefined EK cells established from renal tissue (34, 37) obtained by nephrectomy (H507) or biopsy (H521 and H525) and used in CTL assays at passages 1 to 6 were done. EK cells were infected with 10 $TCID_{50}$ of $EIAV_{WSU5}$ per cell for 2 h, washed, and maintained for 6 to 21 days before use. The percentage of EIAV-infected cells was determined by direct immunofluorescence (7, 37), and in this study, the mean was 61% (range, 55 to 72%).

CTL assay. Target cells (2×10^4) in collagen-coated wells of a 96-well plate were incubated for 24 h at 37°C with 5% CO_2 before ^{51}Cr labeling. Coating was done with water (25 μl per well) containing 1 mg of calf collagen (Sigma Chemical Co., St. Louis, Mo.) per ml, and then wells were air dried. To infect EK cell targets with vaccinia viruses, seeded cells were incubated for 7 h; infected with 10 PFU of VSC11, VEnv2, or VGag/PR per cell; and incubated for 17 h before ^{51}Cr labeling. Target cells were labeled for 90 min with 5 μCi of ^{51}Cr per well in 100 μl of Dulbecco modified Eagle medium with 5% calf serum. PBMC were isolated, and viability was determined (51). Effector/target (E:T) cell ratios of 10:1 and 50:1 were incubated for 6 or 17 h, and 100 μl of supernatant was removed to determine ^{51}Cr release. The formula percent specific lysis = $[(E - S)/(M - S)] \times 100$ (48), where E = the mean of six test wells, S = the mean spontaneous release from six target cell wells without effector cells, and M = the mean maximal release from six target cells wells with 3% Triton X-100, was used. The formula used to calculate standard error (SE) of the percent specific lysis accounts for the variability of E, S, and M (48).

Recombinant vaccinia viruses expressing $EIAV_{WSU5}$ genes. One recombinant vaccinia virus (VGag/PR) which contains EIAV *gag* and 5′ *pol* and expresses a 55-kDa Gag precursor, an 82-kDa Gag/Pol fusion protein including protease (PR), and subviral particles containing processed p26 (30) was used. Recombinants expressing *env* were made from overlapping cDNA clones containing the complete $EIAV_{WSU5}$ *env* gene (29). The *env* gene was assembled in the *Sma*I cloning site of pSC11 by using the 2,400-nucleotide *Sph*I-*Eco*RI fragment from one cDNA clone, the 400-nucleotide *Bam*HI-*Sph*I fragment from another clone, and an adaptor with a 3′ *Bam*HI site and a 5′ ATG translation initiation codon. The synthetic adaptor had *env* nucleotides 1262 to 1286 (45) in the 5′-to-3′ strand and 1262 to 1291 in the 3′-to-5′ strand. Fragment orientation in pEIA5E was verified by restriction endonuclease mapping, and the presence of the adaptor was verified by sequencing.

Recombinant vaccinia virus VEnv1 was made with pEIA5E to express the *env* gene. VEnv2 was the same as VEnv1 except that site-directed mutagenesis (49) was used to make a single-base mutation in pEIA5E to remove an early vaccinia virus transcription terminator signal, TTTTTNT (8). Sequencing confirmed that a synonymous C was substituted for T at position 2080 and that no other changes were introduced. VSC11 was a control recombinant made with pSC11. After recombination with WR strain vaccinia virus (26), recombinants were identified by β-galactosidase expression (3), and Env protein expression was verified by immunoblotting. Selected recombinants were plaque purified three times on BSC-1 cells.

Immunoblots to detect Env proteins. EK cell cultures in 150-cm^2 flasks with Dulbecco modified Eagle medium and 5% bovine serum were infected with 10 PFU of VEnv1, VEnv2, or VSC11 per cell. Ten minutes before infection, medium from one group of flasks was removed, and medium with 40 μg of cytosine arabinoside per ml was added (8). Cells were removed after incubation for 2, 4, or 24 h at 37°C; washed three times with phosphate-buffered saline (PBS); and lysed on ice for 30 min in 50 mM Tris hydrochloride (pH 8.0) with 5 mM EDTA, 5 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM *N*-tosyl-L-lysine chloromethyl ketone, and 0.5% Nonidet P-40. Lysates were cleared by centrifugation (9,000 × *g* for 5 min), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with equine monoclonal antibodies (MAbs) from equine-murine xenohybridomas (41). Equine MAb 30/8.12 recognizes EIAV SU (gp90), and MAb 30/249.2 recognizes TM (gp45) (41). Bound MAb was detected with antibodies to equine immunoglobulin conjugated to horseradish peroxidase and visualized by chemiluminescence (28).

Northern (RNA) blots of RNA from recombinant vaccinia virus-infected cells. After infection of EK cells with VEnv1 or VEnv2, 100 μg of cycloheximide per ml was added (8). Cultures were incubated at 37°C for 2 h and then rinsed, and cells were removed by scraping. Total RNA was extracted with guanidine thiocyanate and homogenization and then centrifuged through cesium chloride (47). Ten micrograms of RNA per lane was separated in a 1.2% formaldehyde gel, transferred to GeneScreen Plus nitrocellulose, baked at 80°C for 2 h, and hybridized with a ^{32}P-labeled *Bam*HI-*Xba*I *env* fragment (1).

Depletion of $CD8^+$ T lymphocytes from PBMC. Isolated PBMC were depleted of $CD8^+$ T lymphocytes by using an anti-equine CD8 MAb covalently bound to a surface-activated flask. Briefly, 250 μg of anti-CD8 MAb HT14A (23) in 5 ml of 0.01 M sodium phosphate–0.15 M NaCl (pH 7.4) (PBS) was added to MicroCELLector surface-activated cell culture flasks (Applied Immune Sciences, Santa Clara, Calif.) and incubated for 1 h at room temperature. After washing and blocking flasks with 0.2% bovine serum albumin (BSA) in PBS, 3×10^7 PBMC which had been blocked with 10% autologous serum in PBS were added to each flask and incubated at room temperature for 1 h. Nonadherent cells were collected for evaluation and use in CTL assays.

Immunofluorescence flow cytometry. PBMC (10^7/ml) were suspended in PBS with 0.5% BSA and 0.02% sodium azide, both before and after depletion of $CD8^+$ T lymphocytes. Next, 50 μl of cell suspensions was added to 50 μl of 15-μg/ml MAb

HT14A to equine CD8 (23), 15-μg/ml MAb HB61A to equine CD4 (23), or 5-μg/ml fluorochrome-labeled anti-horse immunoglobulin (Chemicon International, Inc., Temecula, Calif.). After incubation at 4°C for 30 min, cells were washed three times and pelleted at 4°C for 3 min at 1,500 × *g*. Cells were resuspended in 100 μl of cold PBS–0.5% BSA–0.02% sodium azide and incubated at 4°C for 30 min with 100 μl of 5-μg/ml fluorescein-conjugated anti-mouse immunoglobulin serum (Caltag Laboratories, South San Francisco, Calif.) adsorbed with normal horse serum. After washing, cells were fixed with 2% formaldehyde in PBS and analyzed with a FACScan equipped with a Consort 32 computer and LYSYS II software (Becton Dickinson, San Jose, Calif.).

Nucleotide sequence accession number. The $EIAV_{WSU5}$ *env* gene sequence has been assigned EMBL accession number X16988.

RESULTS

EIAV-specific cytotoxic activity in unstimulated PBMC from infected horses. PBMC from H507, infected with 10^7 $TCID_{50}$ of $EIAV_{WSU5}$, were evaluated for cytotoxic activity on $EIAV_{WSU5}$-infected autologous EK cells over a 1-year period. During this period, the horse had antibodies to p26 but was afebrile (<39°C), and no plasma viremia or anemia was detected. However, PBMC taken 7 weeks after infection caused low-level, but significant, specific lysis of EIAV-infected cells. The percent specific lysis of EIAV-infected EK cells was defined as significant when it exceeded the percent specific lysis of noninfected EK cells by 2.5 SE. The maximum specific lysis (13.5%) occurred with PBMC taken at 27 weeks and used at an E:T cell ratio of 50:1 for 17 h (Fig. 1). Both 6- and 17-h assay times resulted in significant specific lysis of EIAV-infected cells by PBMC (Fig. 1). Since percent specific lysis was higher with 17-h assays, this time was used in subsequent assays. The range of ^{51}Cr spontaneous release in most 17-h assays was 15 to 31%. On the basis of this and a similar range in a CTL study using 18-h assays (14), assays with a spontaneous release exceeding 32% were not used. Mixtures with E:T cell ratios of 10:1 and 50:1 were assayed, and EIAV-specific lysis was always greater with a 50:1 ratio.

Unstimulated PBMC from H521 infected with 10^8 $TCID_{50}$ of $EIAV_{WSU5}$ caused remarkable specific lysis of $EIAV_{WSU5}$-infected autologous EK cells (Fig. 2). By using an E:T cell ratio of 50:1, the maximum percent specific lysis of noninfected EK target cells by PBMC was 5.4% over 100 days (Fig. 2). No cytotoxic activity was detected in PBMC taken before and 1 week after infection; however, PBMC taken 2 weeks after infection caused 29.2% specific lysis (Fig. 2). The maximum specific lysis (55.3%) of $EIAV_{WSU5}$-infected autologous EK cells occurred with PBMC taken 7 weeks after infection. The amount of specific lysis of infected EK cells by PBMC decreased to 13% at 9 weeks and increased again to 27% at 14 weeks after infection (Fig. 2). Plasma viremia was detected in H521 at 7 to 26 days after infection, with a peak virus titer of $10^{3.8}$ $TCID_{50}$/ml detected on day 9 (Fig. 2). Plasma viremia was terminated by day 28, and no viremia was detected between days 28 and 100; day 100 was the last day evaluated. No fever or anemia were detected in the 100-day observation period.

PBMC from H525 infected with 10^8 $TCID_{50}$ of $EIAV_{WSU5}$, the same dose as for H521, were examined for cytotoxic activity against $EIAV_{WSU5}$-infected autologous EK cells (Fig. 3). Unstimulated PBMC from H525, 21 days after infection and at an E:T cell ratio of 50:1, caused 48.5% specific lysis of EIAV-infected EK target cells and 2.4% specific lysis of uninfected EK target cells. Plasma viremia was detected at day 8, peaked on day 20 at $10^{2.1}$ $TCID_{50}$/ml, and was terminated by day 39 (Fig. 3). Thrombocytopenia occurred at day 18 and reached a minimum of 45,000 platelets per μl on day 22 (data not shown). PBMC collected at 42 and 56 days after infection, times when viremia was terminated and thrombocyte counts had returned to normal, also caused significant specific lysis of EIAV-infected EK cells. The cause of relatively high percent specific lysis of uninfected autologous EK cells on days 28 and 42 (17 and 13%, respectively) is unknown, as lysis of heterologous EK cells did not occur.

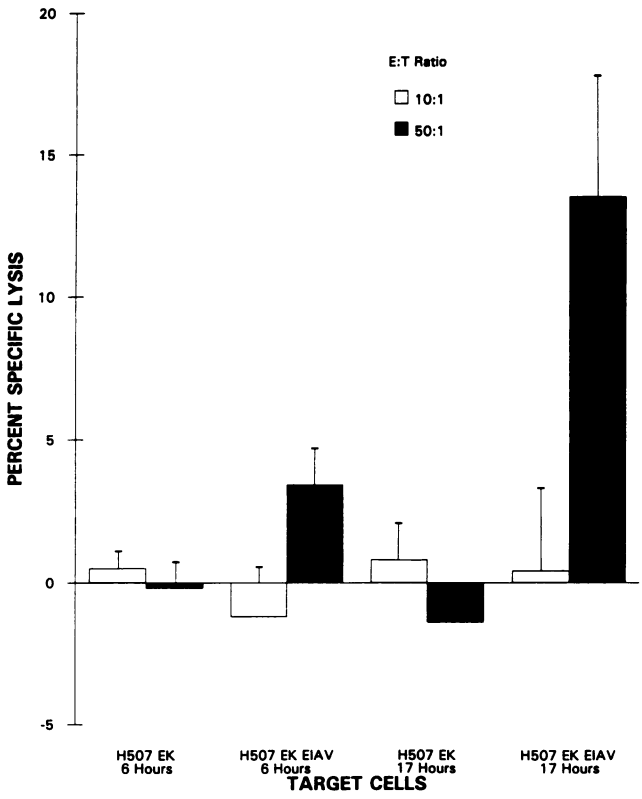

FIG. 1. EIAV-specific cytotoxicity by unstimulated PBMC from H507 taken 27 weeks after infection. Six- and 17-h ^{51}Cr-release assays were done with noninfected and $EIAV_{WSU5}$-infected autologous EK cells. Vertical lines on columns are SEs (48).

MHC restriction of EIAV-specific, PBMC-mediated cytotoxicity. PBMC from H525 at 6 weeks after infection, which caused 24% specific lysis of autologous EK cells infected with $EIAV_{WSU5}$, caused only 2.2% specific lysis of $EIAV_{WSU5}$-infected EK cells from an unrelated horse, H521 (Fig. 4). Similarly, PBMC from H521 at 4 weeks after infection, which caused 49.4% specific lysis of $EIAV_{WSU5}$-infected autologous EK cells, caused only 5.3% specific lysis of non-MHC-matched $EIAV_{WSU5}$-infected EK cells from an unrelated horse, H507 (Fig. 5). These data indicated that over 90% of the EIAV-specific cytotoxicity was MHC restricted and not attributable to antibody-dependent cellular cytotoxicity mechanisms including $CD16^+$ lymphocytes armed with EIAV-specific antibody (44, 52).

Effect of depletion of $CD8^+$ T lymphocytes on EIAV-specific, MHC-restricted, PBMC-mediated cytotoxicity. $CD8^+$ T lymphocytes were removed from unstimulated PBMC of H525 and H521 at 10 and 20 weeks after infection, respectively, by a panning procedure with MAb HT14A to equine CD8. Specific

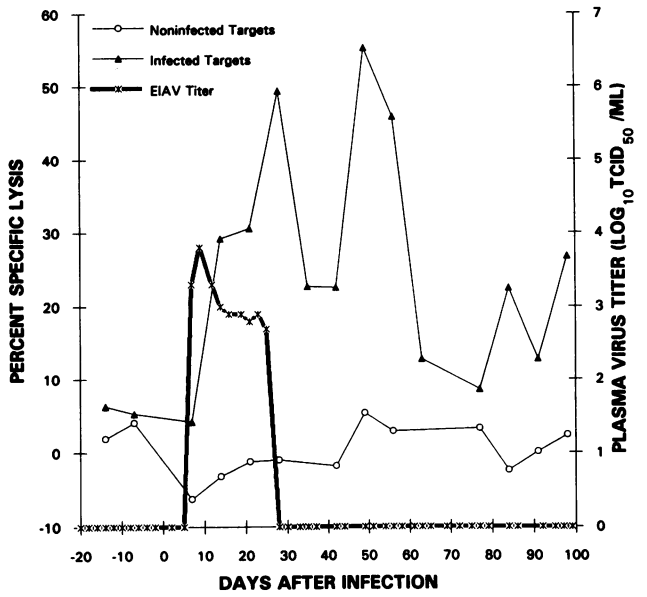

FIG. 2. EIAV-specific cytotoxicity by unstimulated PBMC from H521 over a 100-day observation period after infection. Targets were noninfected and $EIAV_{WSU5}$-infected autologous EK cells, E:T cell ratio was 50:1, and assay time was 17 h. The SE for percent specific lysis did not exceed 3.0 for either infected or noninfected EK cell targets. Plasma virus titers were determined by titration in EK cell cultures (42), and 0 titers are those in which no virus was detected in six wells inoculated with 50 μl of plasma per well (<1.3 $\log_{10}$ $TCID_{50}$/ml of plasma).

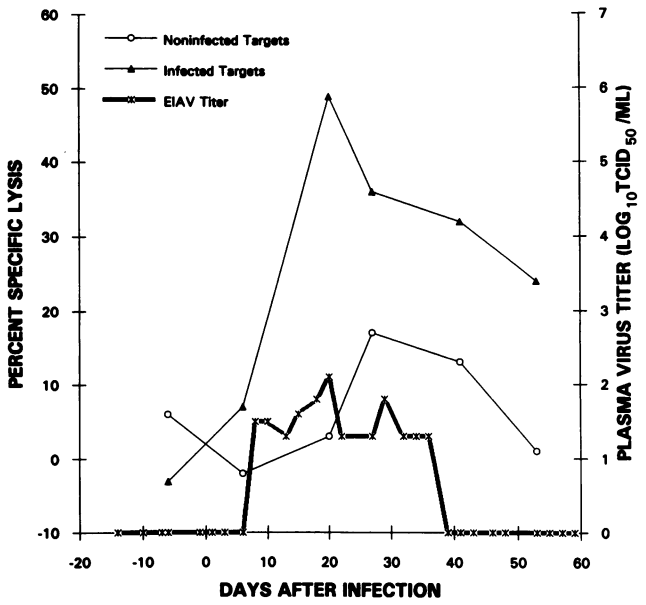

FIG. 3. EIAV-specific cytotoxicity by unstimulated PBMC from H525 over a 60-day observation period after infection. Targets were noninfected and $EIAV_{WSU5}$-infected autologous EK cells, E:T cell ratio was 50:1, and assay time was 17 h. The SE for percent specific lysis did not exceed 4 for either infected or noninfected EK cell targets, except that the SE was 5.5 with infected cells on day 28. Plasma virus titers were determined by titration in EK cell cultures (42), and 0 titers are those in which no virus was detected in six wells inoculated with 50 μl of plasma per well (<1.3 $\log_{10}$ $TCID_{50}$/ml of plasma).

removal of $CD8^+$ T lymphocytes without affecting other PBMC lymphocyte subsets was confirmed by fluorescent flow cytometry with MAb to $CD4^+$ T lymphocytes and antibodies to immunoglobulin M^+ B lymphocytes. PBMC from H525 containing 16% $CD8^+$ T lymphocytes caused 42.3% specific lysis of $EIAV_{WSU5}$-infected EK cells, while those PBMC depleted of $CD8^+$ T lymphocytes (<0.5% $CD8^+$ remaining) caused 10.3% specific lysis of the same target cells (Fig. 6). PBMC from H521 containing 11% $CD8^+$ T lymphocytes caused 23.8% specific lysis of EIAV-infected EK cells, while those PBMC depleted of $CD8^+$ T lymphocytes (<0.6% $CD8^+$ remaining) caused 6.6% specific lysis of the same target cells (Fig. 6). This demonstrated that 72 to 76% of the EIAV-specific, MHC-restricted CTL activity in these PBMC was mediated by $CD8^+$ T lymphocytes.

Characterization of recombinant vaccinia viruses expressing $EIAV_{WSU5}$ *env*. Site-directed mutagenesis was used to alter a cryptic poxvirus early transcription termination signal in the $EIAV_{WSU5}$ *env* gene used to make VEnv2. Sequence analysis of the mutated gene in the insertion plasmid demonstrated that C was substituted for T at nucleotide position 2080, which should alter the termination signal without changing the encoded amino acid. Evidence for signal alteration was obtained by comparison of Northern blots of RNA isolated 2 h after infection of EK cells with VEnv2 or VEnv1 (made with nonmutated *env*) in the presence of cycloheximide. Cycloheximide was used to inhibit early protein synthesis by the vaccinia virus P7.5 promoter used to express EIAV *env* and, thereby, enhance mRNA accumulation. The major *env* transcript from VEnv1-infected EK cells was 0.9 kb (Fig. 7), representing mRNA truncated by the early termination signal. A minor transcript of 2.5 kb, the size of the complete *env* gene if signal read-through occurred, was also made. In contrast, EK cells infected with VEnv2 made only the complete 2.5-kb *env* transcript (Fig. 7), confirming alteration of the termination signal.

The Env proteins made by EK cells infected with VEnv1 and VEnv2 (mutated) were compared by immunoblotting with defined MAb to EIAV SU and TM (41). The amounts of Env proteins made at 2 and 4 h after infection were less in VEnv1- than in VEnv2-infected cells (Fig. 8); however, similar amounts of these proteins were present at 24 h. Four hours after infection, both lysates had proteins reactive with anti-SU MAb at 125 and 105 kDa (Fig. 8A), interpreted as Env precursor and SU (43, 46). At 24 h, lysates had an additional unidentified protein of 62 kDa reactive with anti-SU MAb. Major proteins reactive with anti-TM MAb appeared after 4 h infection with VEnv2 and were 125 and 55 kDa. After 24 h, lysates from VEnv1- and VEnv2-infected cells had major proteins of 125, 49, and 39 kDa reactive with anti-TM MAb (Fig. 8B). These were interpreted, respectively, as Env precursor, TM, and a proteolytic product of TM identified as gp35 in other studies (43). The minor bands reacting with anti-TM MAb in 24-h lysates and the 55-kDa protein in 4-h VEnv2-infected lysates were probably Env protein intermediates of proteolytic processing. Cytosine arabinoside was added to VEnv1- and VEnv2-infected EK cells to inhibit DNA synthesis and, thereby, late protein synthesis by the vaccinia virus P7.5 promoter that was used to express EIAV *env* (P7.5 has both early and late promoter activities [8]). This treatment decreased production of all Env proteins, but the decrease was more marked in cells infected with VEnv1. This demonstrated that mutation of the transcription termination signal enhanced P7.5-promoted early protein synthesis but had little effect on

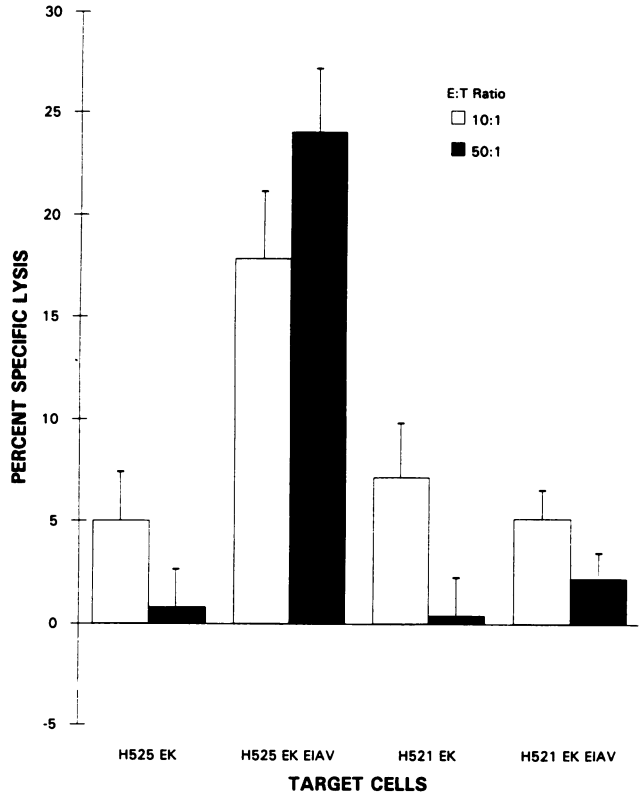

FIG. 4. EIAV-specific, MHC-restricted cytotoxicity by unstimulated PBMC from H525 at 6 weeks after infection. Assays were done for 17 h with autologous (H525) and heterologous (H521) EK cells targets that were noninfected and $EIAV_{WSU5}$ infected. Vertical lines on columns are SEs (48).

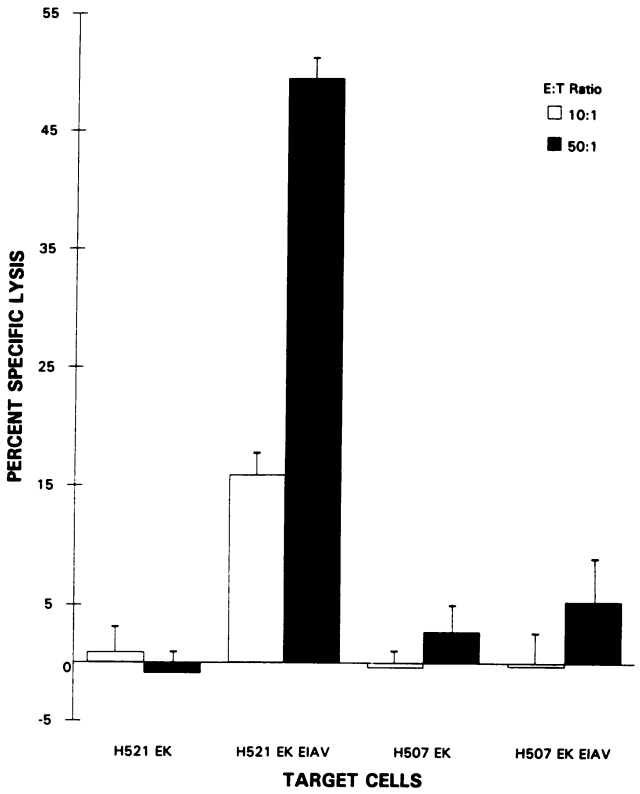

FIG. 5. EIAV-specific, MHC-restricted cytotoxicity by unstimulated PBMC from H521 at 4 weeks after infection. Assays were done for 17 h with autologous (H521) and heterologous (H507) EK cells targets that were noninfected and $EIAV_{WSU5}$ infected. Vertical lines on columns are SEs (48).

late protein synthesis. VEnv2 was used to infect EK target cells because of these data and because (i) transcription termination signal removal from HIV-1 *env* enhanced antibody in mice immunized with vaccinia virus expressing this gene (8) and (ii) levels of endogenously processed antigen may determine whether an epitope and class I MHC complex is efficiently presented to and recognized by class I-restricted CTL (33).

MHC-restricted $CD8^+$ CTL of vaccinia virus-infected target cells expressing Env or Gag/PR proteins. PBMC containing MHC-restricted $CD8^+$ CTL to $EIAV_{WSU5}$-infected EK cells were tested for CTL activity on EK cell targets infected with VEnv2-expressing EIAV *env*, VGag/PR-expressing *gag* and 5′ *pol* genes, or VSC11 control recombinant vaccinia virus. PBMC from H521 at 8 weeks after infection and used at an E:T cell ratio of 50:1 caused 18.2% specific lysis of VEnv2-infected autologous EK cells compared with 9.8% for VSC11-infected cells (Fig. 9). When the VSC11- and VEnv2-infected cells were treated with UV light for 2 min 17 h after infection to inhibit vaccinia virus replication during the CTL assay, there was 1.2 and 10.3% specific lysis, respectively (Fig. 9). Studies with PBMC from H525 taken 6 weeks after infection and used at an E:T cell ratio of 50:1 caused 32.0 and 13.3% specific lysis of $EIAV_{WSU5}$-infected and noninfected autologous EK cells, respectively (Fig. 10). These PBMC also caused 29.6, 22.6, and 15.7% specific lysis of VEnv2-, VGag/PR-, and VSC11-infected autologous EK cells, respectively (Fig. 10). When the same H525 PBMC were evaluated on noninfected and $EIAV_{WSU5}$-, VEnv2-, VGag/PR-, and VSC11-infected heterologous H521 EK cells, the highest specific lysis was 5.5%, demonstrating that lysis of targets expressing Env and Gag/PR proteins was also MHC restricted (Fig. 10).

DISCUSSION

EIAV-specific, MHC-restricted $CD8^+$ CTL were demonstrated in unstimulated PBMC from $EIAV_{WSU5}$-infected horses. The specificity of CTL for EIAV was demonstrated by significant specific lysis of $EIAV_{WSU5}$-infected autologous EK cells compared with that in noninfected cells. Features of the described assay that resulted in consistent detection of CTL were use of EIAV-infected autologous EK cells and incubation for 17 h with a 50:1 E:T cell ratio. Preliminary experiments using EIAV-infected autologous dermal fibroblasts were unsuccessful, and this may have resulted from fewer EIAV-infected cells in these cultures. The maximum number of fibroblasts infected as determined by immunofluorescence in this and other studies (18) was approximately 30%, while the mean number of infected EK cells was 61%. The highest percent specific lysis obtained with autologous EK targets was 55.2%. These targets had 67% EIAV-infected cells and a spontaneous release of 27.3%. Under these conditions, 55.2% specific lysis may be approaching maximum for this system. The observation that CTL activity was higher in the two horses given the highest dose of 10^8 $TCID_{50}$ of $EIAV_{WSU5}$ is consistent with correlations between amount of EIAV inoculum and clinical disease (17).

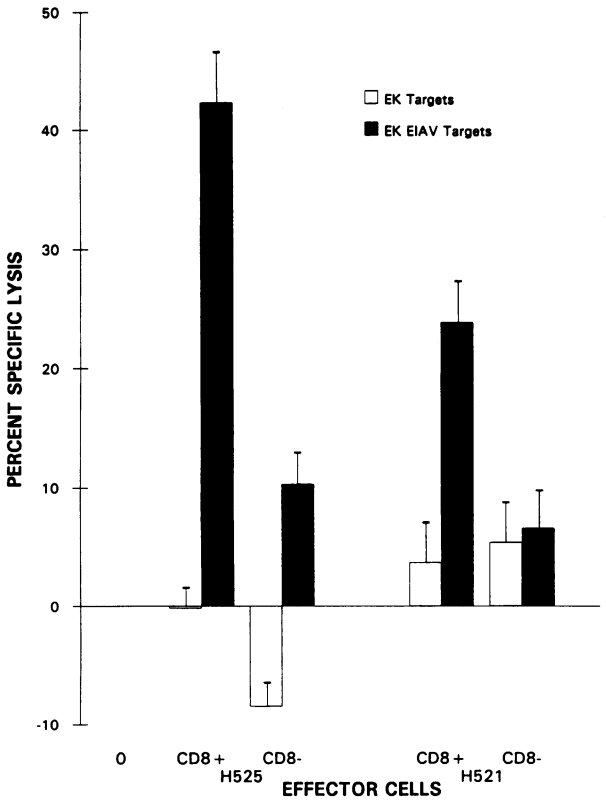

FIG. 6. $CD8^+$ T lymphocyte depletion of EIAV-specific, MHC-restricted cytotoxic activity from unstimulated PBMC from H525 and H521 at 10 and 20 weeks after infection, respectively. Target cells were noninfected and $EIAV_{WSU5}$-infected autologous EK cells, E:T ratio was 10:1 for H525 PBMC and 50:1 for H521 PBMC, and assay time was 17 h. $CD8^+$ T lymphocytes were removed by a panning procedure resulting in <0.6% $CD8^+$ in the remaining PBMC. Vertical lines on columns are SEs (48).

EIAV-specific CTL from infected horses were MHC restricted, as demonstrated by the failure of PBMC with high CTL activity to cause significant specific lysis of $EIAV_{WSU5}$-infected heterologous EK cells from unrelated horses. This observation is in contrast to earlier results demonstrating that PBMC from EIAV-infected horses caused direct cytotoxicity of EIAV-infected heterologous (non-MHC-matched) dermal

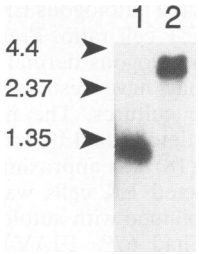

FIG. 7. Autoradiogram of a Northern blot of *env* transcripts made by EK cells infected with VEnv1 (lane 1) and VEnv2 (lane 2) in the presence of 100 μg of cycloheximide per ml. The blot was probed with a ^{32}P-labeled 827-bp DNA fragment removed from the *env* gene in pEIA5E. Migration of RNA standards (in kilobases) are shown on the left.

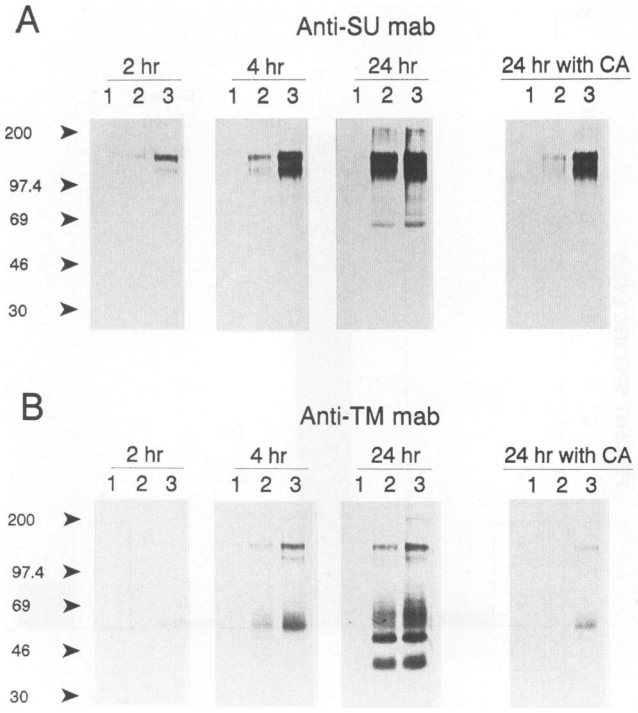

FIG. 8. Immunoblots of Env proteins expressed by recombinant vaccinia virus-infected EK cells. (A) Lysates from cells infected with VSC11 (lane 1), VEnv1 (lane 2), and VEnv2 (lane 3) for 2, 4, and 24 h, and for 24 h with cytosine arabinoside (CA) were reacted with equine MAbs derived from xenohybridomas (41) to EIAV SU (30/8.12). (B) The same lysates were reacted with equine MAb to EIAV TM (30/249.2). Migration of the protein (in kilodaltons) is indicated on the left side of each panel. Similar immunoblots reacted with an isotype control equine MAb were negative (not shown).

fibroblasts (10). The mechanism of the previously reported EIAV-specific, non-MHC-restricted equine PBMC cytotoxic activity is unknown. However, similar observations with PBMC from human patients seropositive to HIV-1 (27, 44) and SIV-infected rhesus monkeys (52) have been reported and attributed to $CD16^+$ MHC class I-unrestricted cells. There is another report of MHC-restricted specific lysis (6.5 and 8.4%) of EIAV-infected cells by PBMC from 2 of 11 horses with acute EIAV infection (11). The target cells used in this report were autologous PBMC from the EIAV-infected horses stimulated with phytohemagglutinin for 72 h (11). Since EIAV is not known to replicate in lymphocytes and the number of infected monocytes was not determined, it is difficult to interpret these findings. No other reports of PBMC-mediated cytotoxicity of EIAV-infected cells are known.

That the EIAV-specific, MHC-restricted CTL in PBMC from infected horses were mediated by $CD8^+$ T lymphocytes was determined by depletion studies. The $CD8^+$ T lymphocytes were removed from freshly isolated PBMC by a panning procedure with an anti-CD8 MAb, HT14A (23). Removal of almost all the detectable $CD8^+$ CTL from PBMC from two horses (<0.6% of remaining PBMC were $CD8^+$) resulted in 72 and 76% decreases in the MHC-restricted CTL activity. Both MHC class I-restricted $CD8^+$ CTL and MHC class II-restricted $CD4^+$ CTL clones have been isolated from PBMC from HIV-seropositive humans (24, 25). However, most MHC-restricted CTL from HIV-seropositive humans and SIV-infected rhesus monkeys are $CD8^+$ (14, 32, 52).

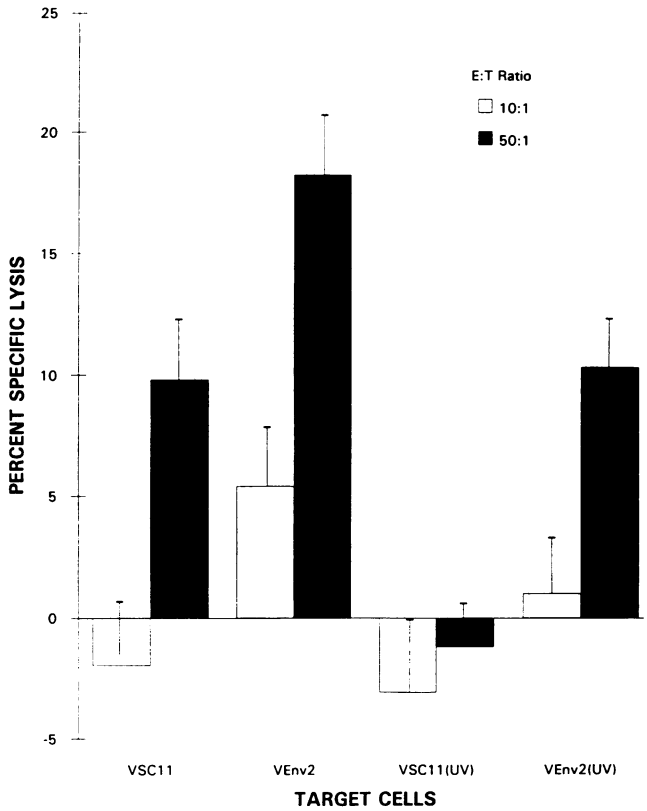

FIG. 9. MHC-specific CD8+ CTL in unstimulated PBMC from H521 at 8 weeks after infection were assayed for cytotoxicity of autologous EK cells infected with a control recombinant vaccinia virus (VSC11) or one expressing $EIAV_{WSU5}$ *env* (VEnv2). UV indicates that the vaccinia virus-infected cells were treated for 2 min with UV light 17 h after infection. The assay time after adding effector cells was 17 h, and the spontaneous release of ^{51}Cr from vaccinia virus-infected cells without effector cells was <32% of the total released by 3% Triton X-100. Vertical lines on columns are SEs (48).

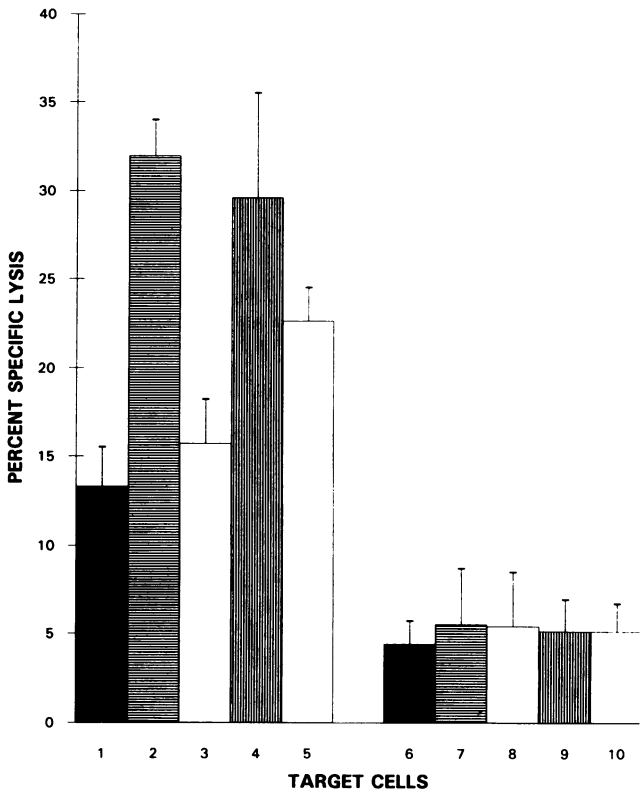

FIG. 10. MHC-specific CD8+ CTL in unstimulated PBMC from H525 at 6 weeks after infection were assayed for cytotoxicity of autologous EK cells (columns 1 to 5) or heterologous EK cells (columns 6 to 10) infected with EIAV or recombinant vaccinia viruses. Target cells in columns 1 and 6 were noninfected, those in 2 and 7 were $EIAV_{WSU5}$ infected, those in 3 and 8 were VSC11 infected, those 4 and 9 were VEnv2 infected, and those in 5 and 10 were VGag/PR infected. The E:T ratio was 50:1, assay time was 17 h, and spontaneous release did not exceed 32%. Vertical lines on columns are SEs (48).

To determine whether EIAV *env* or *gag*-5′ *pol*-encoded proteins were recognized by EIAV-specific, MHC-restricted CD8+ CTL from PBMC, recombinant vaccinia viruses expressing either $EIAV_{WSU5}$ *env* or *gag* plus 5′ *pol* genes were used to infect autologous EK cell targets. CTL from one horse caused significant percent specific lysis of target cells expressing Env, while CTL from a second horse caused significant percent specific lysis of cells expressing either Env or Gag/PR proteins. Since EK cell targets infected with recombinant vaccinia viruses were not evaluated for every assay in which EIAV-specific MHC-restricted CTL were demonstrated, it is not known whether both horses would have recognized Gag proteins. Furthermore, it is not known whether CTL detected by reactivity to EIAV-infected cells also recognize epitopes encoded by *pol* and other genes which encode regulatory proteins described for CTL from HIV-1-seropositive humans (36, 44) and SIV-infected rhesus monkeys (2, 36). In many HIV-1 and SIV studies, CTL have not been detected by using lentivirus-infected target cells, but the studies have used either recombinant vaccinia viruses expressing lentiviral genes or synthetic peptide-charged target cells. Therefore, it is possible that further identification of the epitopes recognized on EIAV-infected cells by primary CTL from infected horses that are successfully controlling EIAV will yield new information.

In conclusion, the demonstration of EIAV-specific, MHC-restricted CD8+ CTL in unstimulated PBMC of EIAV-infected horses as early as 2 weeks after infection and persisting for several months provides an important system to dissect unstimulated CTL responses to lentivirus-infected target cells. The CTL assay, particularly the use of EIAV-infected autologous EK cells, should also be useful in dissecting the mechanism of protective immunity induced by experimental vaccines to EIAV (16). In fact, the protective immunity induced by immunization with inactivated EIAV was correlated with activated T lymphocytes and thought to be mediated in some horses by CTL (16). Identification of the epitopes recognized on EIAV-infected cells by CD8+ CTL in unstimulated PBMC from infected horses in successful remission will facilitate experiments to induce such responses and to evaluate their role in protection against EIAV infection and disease.

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