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Major Histocompatibility Complex Class I-Restricted Cytotoxic T-Lymphocyte Responses in Horses Infected with Equine Herpesvirus 1†

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An experimental system that permits sensitive and reproducible detection of equine herpesvirus 1 (EHV-1)-specific cytotoxic T-lymphocyte (CTL) activity in the horse was developed. Peripheral blood mononuclear cells (PBMC) collected from immune horses were restimulated in vitro by culture with live EHV-1. Cytotoxic activity against virus-infected, pokeweed mitogen-stimulated lymphoblast targets was assessed in a 4-h ^{51}Cr release assay. The optimal conditions for in vitro stimulation of equine memory CTLs and for preparation of EHV-1-infected target cells expressing viral antigens were systematically identified by individually testing the effects of variations in responder cell concentration, culture medium composition, serum type, incubation time, antigen form, and exogenous mediator content. By using this optimized system for generation and assay of equine CTLs, the development of EHV-1-specific cytotoxic responses in 12 horses was evaluated after experimental viral infection. CTLs with the capacity for killing EHV-1-infected target cells were detected in equine PBMC as early as 1 week postinfection, reached maximal levels by 2 to 3 weeks, and remained detectable for a year after infection. Equine effector cells mediating lysis of EHV-1-infected targets were predominantly CD8$^+$ T lymphocytes, and the cytotoxicity was specific for virus and restricted by major histocompatibility complex class I molecules. The results define a reliable and convenient experimental system for generation and assay of EHV-1 CTLs which can now be used for more-detailed characterization of the equine CTL response to infection by this herpesvirus pathogen.

Equine herpesvirus 1 (EHV-1), a biologically typical alphaherpesvirus, is a worldwide cause of an acute and highly contagious infection of horses (11, 33, 39, 40). The economic losses caused by EHV-1 to equine-related industries are significant because of frequent epidemics of a spectrum of disease conditions in horses, such as respiratory disease, abortion, neonatal foal death, and myeloencephalopathy, that may result from EHV-1 infection (2, 10, 12, 18, 20, 28, 29). Devastating outbreaks of EHV-1 disease have historically plagued domestic horse populations for many decades (39, 40). The virus is transmitted from one susceptible horse to another by aerosol inhalation and replicates in the epithelium of the upper respiratory tract (2, 27). The pathogenesis of EHV-1-induced abortion and neurological dysfunction that may follow respiratory tract infection is complex and incompletely understood. A wave of virus-infected lymphocytes enters the peripheral blood circulation from the lymphoid tissue of the respiratory tract to disseminate virus systemically (8, 37, 42). The central pathological lesion of systemic EHV-1 infection that leads to subsequent abortion or myeloencephalopathy is infection, necrosis, and luminal sloughing of vascular endothelium with subsequent microthrombosis and infarction in the pregnant mare's endometrium or central nervous system, respectively (24, 25, 43, 46).

Vaccination of horses for EHV-1 with inactivated virus products is widespread (9, 13, 14, 33, 35). Although these vaccines elicit strong humoral immune responses and have been successful in reducing the overall economic burden of EHV-1-related clinical disease, antibody is not, in itself, fully protective, as breakthrough infections with consequent disease epidemics occur commonly in vaccinated horses (15, 16, 36, 39, 40). Much of the present research on EHV-1 is therefore directed at collection of information about host antiviral immunity that may be applied toward the design of improved vaccines for controlling infection by this herpesvirus pathogen (1).

Fully protective immunity to reinfection by EHV-1 exists in the horse for 4 to 8 months after primary infection (22). The host effector mechanisms operating during this narrow window of protection are unknown. Infection by EHV-1 elicits the production of antibody and of sensitized lymphocytes that proliferate in EHV-1 antigen-stimulated cultures (2, 8, 23, 26, 38, 41). However, preexposure levels of serum antibody or lymphocyte proliferative responses are not reliably predictive of the clinical outcome of challenge of pregnant mares with EHV-1 (37). The unique pathogenesis of EHV-1 abortion and neurological disease, involving infection of endothelial cells by virus-infected lymphocytes as they circulate through the vasculature of the endometrium and central nervous system, suggests an importance for the role of cellular immunity in defense against the virus. Immune mechanisms that destroy virus-infected cells may therefore be particularly relevant in control of the abortigenic and neurologic manifestations of EHV-1 infection, since the virus's ability for systemic spread via cell-to-cell contact renders it inaccessible to antibody neutralization.

Research in our laboratory is currently focused on elucidating the host cytotoxic T-lymphocyte (CTL) responses that participate in protection against EHV-1 and also the immunogenic determinants that induce those cellular defense mecha-

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nisms. Because results obtained from laboratory animal model systems are not always easily or reliably extended to the natural host, we have chosen to use horses for investigation of such questions. As yet, there have been no recorded studies demonstrating EHV-1-specific, major histocompatibility complex (MHC)-restricted CTLs in immune horses. Attempts at development of a specific assay system for detection of MHC-restricted CTL activity against EHV-1 in the horse have presented formidable and long-standing challenges for EHV-1 investigators. Among such experimental obstructions have been the lack of a suitable equine target cell for assay of cytotoxic activity, an absence of information on the culture and antigen requirements for in vitro expansion of primed equine memory CTLs, and the heretofore nonavailability of specific serological reagents needed for identifying MHC haplotypes, lymphocyte subpopulations, and the expression of individual EHV-1 antigens in the equine experimental system.

We describe here the development and optimization of an experimental system that overcomes these obstacles to allow identification of CTL activity against EHV-1 in peripheral blood lymphocytes of the horse. We report, for the first time, the detection and characterization of $CD8^+$, MHC class I-restricted, virus-specific CTL responses in horses after experimental infection with EHV-1.

Experimental infection of horses with EHV-1. The study population of horses consisted of 14 nonpregnant mares with diverse MHC types and genetic backgrounds (six Thoroughbreds, six quarter horses, and two American Saddlebreds). Their ages ranged from 8 to 18 years, and their histories of previous exposure to EHV-1 or EHV-4 infection or vaccination were unknown. The mares, maintained at the Equine Research and Resources Facilities of the College of Agriculture, University of Kentucky, were kept isolated from other horses throughout the study period and were serologically monitored for evidence of recent natural infection by EHV-1 or EHV-4 for 6 months prior to experimental infection. Routine care and experimental utilization of the horses were done in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research, U.S. Department of Agriculture. The experimental animal procedures performed as part of this research project were approved by the Institutional Animal Care and Use Committee of the University of Kentucky (project 91-0044A).

The MHC class I phenotype was determined for each horse by serological typing of lymphocytes for the 11 A-locus antigenic specificities of the equine lymphocyte alloantigen (ELA-A) system recognized by the Third International ELA Workshop (4, 5). Lymphocytes were ELA typed by the University of Kentucky Equine Blood Typing Laboratory, using microcytotoxicity testing procedures and serological reagents developed and previously described by Bailey (6, 7). ELA typing confirmed that the horse population studied was heterogeneous with respect to MHC class I alleles and thus represented an unbiased sampling of experimental animals (Table 1). Eight of the 11 ELA-A specificities, in various heterozygotic permutations, were represented within the group of 14 experimental horses; only the low-frequency ELA-A alleles A4, A7, and A8 (5) were not present in the horses.

The humoral immune status of each animal prior to infection with EHV-1 was determined by enzyme-linked immunosorbent assay (ELISA) with purified EHV-1 used as the antigen (38). To estimate the preinfection status of cellular immunity to EHV-1 possessed by the mares, peripheral blood mononuclear cells (PBMC) were cultured in the presence of a range of concentrations (0.3 to 30 PFU per macrophage) of EHV-1 for 5 days, and [^{3}H]thymidine incorporation was used to quantitate virus-specific lymphocyte proliferation (30).

Twelve of the mares were inoculated with EHV-1 by intranasal instillation of 3 ml of cell-free, virus-infected tissue culture fluid containing 2.5×10^7 PFU of EHV-1 strain Army-183 per ml. Two uninoculated horses served as uninfected controls and also as sentinel animals to allow detection

TABLE 1. Pre- and postinoculation values of immune status indicators for experimental horses

Horse	Breed[a]	ELA-A haplotype[b]	Value of immune status indicator					
			Preinoculation			Postinoculation[c]		
			Ab[d]	CTL[e]	Prolif.[f]	Ab	CTL	Prolif.
110	QH	A2/A2	200	4 ± 0.7	ND[g]	7,680	68 ± 3	ND
113[h]	TB	A3/A5	400	12 ± 1	ND	ND	ND	ND
154	TB	A9/A10	1,280	12 ± 0.8	ND	15,360	73 ± 2	ND
314	QH	A5/W11	400	10 ± 0.9	1.4	12,800	90 ± 5	23.0
319	SB	A6/W11	1,280	6 ± 1	1.2	12,800	39 ± 2	5.1
324	SB	A1/W11	12,800	75 ± 3	ND	12,800	82 ± 3	ND
327	TB	A2/A5	400	15 ± 1	ND	7,680	79 ± 5	ND
337	QH	A6/A9	200	8 ± 2	2.4	6,400	51 ± 4	137.4
355	QH	A3/W11	200	23 ± 3	6.8	3,200	46 ± 3	93.4
358	TB	A5/A10	400	19 ± 4	12.7	6,400	36 ± 3	20.1
359	TB	A3/A3	400	50 ± 5	3.0	3,200	60 ± 3	13.6
531	TB	ND	1,280	13 ± 1	ND	2,560	52 ± 2	ND
533[i]	QH	ND	200	7 ± 1	ND	200	11 ± 0.2	ND
200[i]	QH	A1/W11	200	2 ± 0.4	ND	400	6 ± 0.6	ND

[a] QH, quarter horse; SB, American Saddlebred; TB, Thoroughbred.
[b] Serologically defined antigenic specificities determined by the ELA-A system.
[c] Maximal postinoculation values, usually at 3 to 4 weeks postinoculation.
[d] Ab, serum antibody ELISA titers to EHV-1 antigen.
[e] CTL, percent specific cytotoxicity values (± standard deviations) of EHV-1-stimulated PBMC cultures for virus-infected targets at an E/T ratio of 100:1.
[f] Prolif., lymphocyte proliferative responses of EHV-1-stimulated PBMC cultures, expressed as stimulation indices (cpm of [^{3}H]thymidine incorporated into virus-stimulated cells/cpm of [^{3}H]thymidine incorporated into nonstimulated cells).
[g] ND, not determined.
[h] Developed quadriplegia; euthanized at 2 weeks postinoculation.
[i] Noninoculated control horse.

of naturally acquired or recrudescent EHV-1 or EHV-4 infection within the experimental group. The animals were kept isolated after inoculation and observed daily for 3 weeks for clinical signs of EHV-1 infection. Rectal temperatures were monitored daily during the first 2 weeks following virus challenge. Samples of blood from each horse were collected by jugular venipuncture into sodium heparin for isolation of PBMC. Blood for preparation of serum was collected in tubes without anticoagulant and permitted to clot prior to harvesting of the serum fraction. Virus-specific humoral and cellular immune responses were examined weekly for 6 weeks postinfection.

Ten of the 12 mares inoculated intranasally with EHV-1 developed clinical signs typical of EHV-1 infection: eight animals developed signs of acute upper respiratory disease, and three developed ataxia. In one horse, the ataxia progressed to quadriplegia that necessitated euthanasia. Febrile responses occurred in eight infected mares and were monophasic and short-lived, lasting from 1 to 3 days. Two of the EHV-1-inoculated mares remained completely asymptomatic. With one exception, preinoculation titers of serum antibody to EHV-1 were low, ranging from 200 to 1,280, as determined by ELISA (Table 1). Nine of the 11 surviving inoculated horses showed serological evidence of infection by 3 weeks postinoculation, with increases in antibody titers after inoculation ranging from 8- to 38-fold. The two uninoculated control-sentinel mares remained clinically asymptomatic and had no increase in EHV-1 antibody titer or CTL activity over the duration of the project.

Pre- and postinoculation samples of PBMC from six of the infected horses were tested for lymphocyte proliferation when cultured in the presence of cell-free EHV-1. PBMC from all six selected animals demonstrated proliferative responses to in vitro stimulation with EHV-1 that increased by 1.7- to 16-fold after experimental viral infection (Table 1). Studies were thus performed to determine if PBMC from the mares could be stimulated in vitro with EHV-1 to generate effector lymphocytes capable of specific lysis of EHV-1-infected target cells.

Secondary stimulation of immune lymphocytes. PBMC were isolated from heparinized venous blood by Ficoll-Hypaque (Pharmacia-LKB, Piscataway, N.J.) density gradient centrifugation. The PBMC were washed three times in Hanks' balanced salt solution, counted, and suspended in CTL induction medium at 10^8 cells per ml. For long-term storage, PBMC were suspended at 2×10^7 to 5×10^7/ml in cryoprotective medium (50% RPMI 1640, 40% fetal bovine serum [FBS], and 10% dimethyl sulfoxide), cooled to −80°C in a controlled-rate liquid nitrogen freezer (Gordinier Electronics, Roseville, Mich.), and then transferred to a −135°C freezer.

For in vitro expansion of memory CTLs, primed responder PBMC were restimulated by secondary culture in a medium empirically formulated to result in maximal virus-specific cytotoxic activity after 6 days of antigenic stimulation. This medium, referred to herein as CTL induction medium, consisted of a 1:1 (vol/vol) mixture of AIM-V serum-free lymphocyte medium (no. 12055; Gibco-BRL, Grand Island, N.Y.) and RPMI 1640 medium (Gibco-BRL no. 21870) that was then supplemented with L-glutamine (2 mM), minimal essential medium nonessential amino acids (0.05 mM each), sodium pyruvate (0.5 mM), 2-mercaptoethanol (55 μM), gentamicin (50 μg/ml), and autologous equine serum (5%). The autologous serum was collected from each horse prior to experimental EHV-1 infection, heat inactivated at 56°C for 45 min, and stored in aliquots at −20°C.

Induction cultures for in vitro expansion of in vivo-primed memory CTLs were initiated in upright 75-cm^2 flasks in 30 ml of CTL induction medium in an atmosphere of 4% CO_2 in air. Responder cells for CTL induction were PBMC isolated from control horses or from horses at various times following experimental EHV-1 infection. The stimulatory antigen was cell-free, live EHV-1 propagated in minimal essential medium containing fetal equine serum and was used at a concentration shown in preliminary experiments to induce maximal lymphocyte proliferative responses as measured by [^{3}H]thymidine uptake. Briefly, 1.1×10^8 responder PBMC were restimulated in vitro with antigen by culture for 6 days at 37°C in the presence of 3.75×10^7 PFU of EHV-1. Exogenous interleukin-2 (IL-2) or crude lymphocyte growth factor (TCGF) preparations were not added, nor was it necessary to replace culture medium, antigen, or antigen-presenting cells in the induction flasks during the 6-day incubation period. Parallel mock-induction cultures without virus were set up under otherwise identical conditions. After 6 days in culture, the cells were recovered from the stimulation flasks, washed, and used as effector lymphocytes in a 4-h ^{51}Cr release assay for cytotoxicity.

Experiments were performed to determine the surface phenotype of lymphocytes responding to these conditions of antigenic stimulation. The percentage of $CD4^+$ or $CD8^+$ lymphocytes in the PBMC from four immune horses was analyzed prior to in vitro stimulation and again on day 6 of the induction culture by using flow cytometry (FACScan instrument and Lysys II software; Becton Dickinson, San Jose, Calif.) (47) and monoclonal antibodies (MAbs) specific for equine CD4 and CD8 antigens (MAbs HB61A and HT14A, respectively; VMRD, Inc., Pullman, Wash.) (31). The results showed that although total viable-cell numbers in the cultures decreased during secondary stimulation, both $CD4^+$ and $CD8^+$ cells proliferated in the induction culture. In all animals examined, $CD4^+$ lymphocytes constituted the majority of cells present among blasts generated by stimulation with EHV-1, and the percentage of $CD8^+$ cells in cultures stimulated in vitro with EHV-1 was higher than that in unstimulated PBMC.

Two-color flow cytometric analysis of EHV-1-stimulated induction cultures, after staining with combinations of antibodies to EHV-1 and to equine T-lymphocyte subset markers, indicated that both $CD4^+$ and $CD8^+$ lymphocytes became infected with EHV-1 and expressed viral antigen. At the time of maximal expression of EHV-1 antigen (3 days following initiation of induction cultures), (17 ± 4)% of $CD4^+$ cells and (24 ± 2)% of $CD8^+$ cells were infected (data not shown).

Preparation of equine target cells for assay of cytotoxicity. In many other virus-host systems, mitogen-stimulated autologous lymphoblasts have been demonstrated to be useful as targets for assay of CTL activity (17, 44, 45). The suitability of pokeweed mitogen (PWM)-stimulated equine lymphoblasts (PWM-blasts) as target cells permissive for EHV-1 infection was therefore explored. The lymphoblasts were prepared by stimulation of freshly isolated PBMC with 2.5 μg of PWM per ml for 48 h at 37°C in a humidified atmosphere of 4% CO_2 in air. The cells were cultured at a concentration of 3×10^6/ml of CTL induction medium in upright 25-cm^2 flasks. After stimulation, the PWM-blasts were harvested by centrifugation onto Ficoll-Hypaque and then washed three times with Hanks' balanced salt solution.

To determine the phenotype of lymphocytes in the mitogen-stimulated cultures, MAbs specific for equine lymphocyte surface antigens CD2, CD4, and CD8 (MAbs HB88A, HB61A, and HT14A, respectively; VMRD, Inc.) (31) were used in conjunction with flow cytometry (47). Flow cytometric analysis for expression of MHC antigens on PWM-blast target cells was performed in a similar manner after incubation of the cells with MAbs that recognize monomorphic determinants on

either equine MHC class I (MAb H58A) or class II (MAb H42A) molecules (VMRD, Inc.). The percentage of immunoglobulin (Ig)-bearing cells was similarly determined by using fluorescein isothiocyanate-conjugated goat anti-horse Ig (Cappel, Durham, N.C.) directed against Ig μ, γ, and light chains. A mean of 96% (range, 93 to 98%) of the PWM-blasts expressed a pan-T lymphocyte marker (CD2), a mean of 63% (range, 57 to 71%) expressed CD4, and a mean of 24% (range, 20 to 28%) expressed CD8. Less than 5% of the PWM-blasts expressed surface equine Ig. Both equine MHC class I and class II molecules were expressed on all the PWM-blasts.

After inoculation of PWM-blasts with 10 PFU of EHV-1 per cell, methanol-fixed cytocentrifuge preparations were made and examined for viral antigen by indirect immunofluorescence using a pool of MAbs to EHV-1 gB, gC, and gD (3). By 12 h postinoculation, at least 80% of the cells stained positive for EHV-1 antigen. EHV-1 replication in PWM-blasts was confirmed by demonstration of a >10-fold increase in plaque titer in an infectious-center assay performed 72 h after a low-multiplicity (0.1 PFU of cell) inoculation with EHV-1.

For use as target cells, washed PWM-blasts (2.5×10^6) were either mock infected or infected with 10 PFU of EHV-1 per cell for 90 min at 37°C and then incubated for 12 h in 1 ml of RPMI 1640 medium supplemented with heat-inactivated FBS (10%), L-glutamine (2 mM), 2-mercaptoethanol (55 μM), and gentamicin (50 μg/ml) (cRPMI-FBS) and containing 125 μCi of $Na^{51}CrO_4$ (ICN, Costa Mesa, Calif.) and 15 U of human recombinant IL-2 (Sigma Chemical Co., St. Louis, Mo.). Inocula for mock infection of target cells consisted of cell-free medium (minimal essential medium containing fetal equine serum) collected from equine dermis monolayer cultures after 2 days of incubation at 37°C. After three washes with Hanks' balanced salt solution, the labeled target cells were suspended at 10^5/ml in cRPMI-FBS and dispensed into microtiter plates for assay of cytotoxicity.

With the conditions used in these studies for labeling of target cells, efficient incorporation of ^{51}Cr isotope by PWM-blasts was routinely achieved. EHV-1-infected PWM-blasts incorporated a mean of 6×10^3 cpm of ^{51}Cr per 10^4 cells, and the spontaneous release of isotope was always <15% of maximal release after 4 h of incubation. Mock- or virus-infected PWM-blasts were resistant to killing by lymphokine-activated killer cell activity (<5% lysis) as determined by incubation for 4 h with lymphokine-activated killer effector cells (effector-to-target cell ratio [E/T ratio] = 100) generated by culture of PBMC for 10 days in the presence of 30 U of human recombinant IL-2 per ml (32).

Cytotoxicity assays. Killing of EHV-1-infected target cells by antigen-stimulated PBMC was detected by a standard ^{51}Cr release assay (34) performed with cRPMI-FBS. After being washed, 10^4 labeled target cells and serial dilutions of effector cells were incubated in 200 μl of cRPMI-FBS medium to achieve E/T ratios of 100:1, 33:1, 11:1, and 3.7:1. Suspensions of effector lymphocytes (100 μl) were added first to three replicate wells of 96-well round-bottomed assay plates (Costar, Cambridge, Mass.). A fixed number (10^4) of ^{51}Cr-labeled target cells in 100 μl was then added to each well. The plates were centrifuged at $200 \times g$ for 2 min, incubated at 37°C for 4 h in 5% CO_2, and then centrifuged at $300 \times g$ for 4 min. Supernatants (100 μl) were collected from each well, and the amount of ^{51}Cr released from target cells was measured with a gamma counter. Spontaneous release of isotope was determined by counting the radioactivity in supernatants from wells containing target cells incubated without effector cells. Maximal ^{51}Cr release was determined from wells containing target cells only, after cell lysis with 0.05% Nonidet P-40. The percent specific cytotoxicity was calculated as follows: [(cpm in experimental release − cpm in spontaneous release)/(cpm in maximal release − cpm in spontaneous release)] × 100. Spontaneous release of label from the target cells during the 4-h CTL assay did not exceed 15% of the maximal release in any of the experiments. Results are expressed as the mean percentages of specific target cell lysis ± standard deviations of triplicate determinations.

Development of CTL responses in horses after infection with EHV-1. Preliminary studies indicated that EHV-1-specific CTLs could be generated by in vitro culture of PBMC from immune horses with cell-free, live virus. The culture conditions for induction of virus-specific CTLs were optimized by individually testing the effect on mediating cytotoxicity of variations in composition of culture medium, species of origin and concentration of serum supplement, concentration of responder cells and antigen, requirement for exogenous mediators (IL-2 and crude TCGF preparations, etc.), and the duration of in vitro stimulation with antigen. PBMC used for experimental optimization of CTL induction were collected from mares at times ranging from 4 weeks to 4 months after experimental infection with EHV-1. Maximal specific target lysis values of 60 to 80% were routinely achieved by effector cell populations generated by use of the optimized conditions for in vitro expansion. The lytic ability of the CTLs was not reduced if cryopreserved PBMC were used. EHV-1 specificity of the observed cytotoxicity was demonstrated by the failure of mock-infected target cells to be lysed by EHV-1-stimulated PBMC and by the inability of mock-stimulated induction cultures to kill EHV-1-infected targets.

By using the method developed for induction and assay of CTLs in peripheral blood lymphocytes, the study population of 14 horses was evaluated for EHV-1-specific cytotoxic responses prior to experimental infection and at weekly intervals after infection. PBMC were purified from fresh whole blood or recovered from cryopreservation, stimulated with EHV-1 for 6 days, and then tested for cytotoxicity against virus-infected and -uninfected target cells. Preinoculation levels of EHV-1-specific cytotoxicity in stimulated cultures of PBMC from the 14 horses are shown in Table 1. In 10 of the mares, significant CTL activity (>15%) could not be induced by in vitro stimulation of PBMC collected prior to experimental inoculation with EHV-1. Low but significant preinoculation levels of virus-specific cytotoxicity, ranging from 19 to 50%, were present in three horses and increased only 1.2- to 2-fold following inoculation with EHV-1. In PBMC cultured from one horse (324), high levels of specific killing of EHV-1-infected target cells (75 to 80%) were present before inoculation with EHV-1 and did not increase after virus inoculation. This mare failed to show clinical signs or serological evidence of infection after intranasal inoculation of EHV-1.

The patterns of development of postinfection cytotoxicity for EHV-1 targets mediated by induction cultures of PBMC from horses without significant preinoculation CTL activity are illustrated in Fig. 1 for four of the animals. By 1 week postinfection, EHV-1-specific cytotoxicity values ranging from 35 to 60% (for an E/T ratio of 100) could be induced in cultures of PBMC from each of the infected horses. Maximal levels of specific lysis of virus-infected targets were observed by 2 to 3 weeks postinfection, were 4- to 18-fold higher than specific lysis values prior to virus infection, and were generally maintained for the remainder of the 6-week sampling period. Significant but diminished levels of EHV-1-specific CTL activity were still demonstrable in PBMC from most infected horses 1 year after exposure to the virus.

The demonstration of circulating EHV-1-specific memory

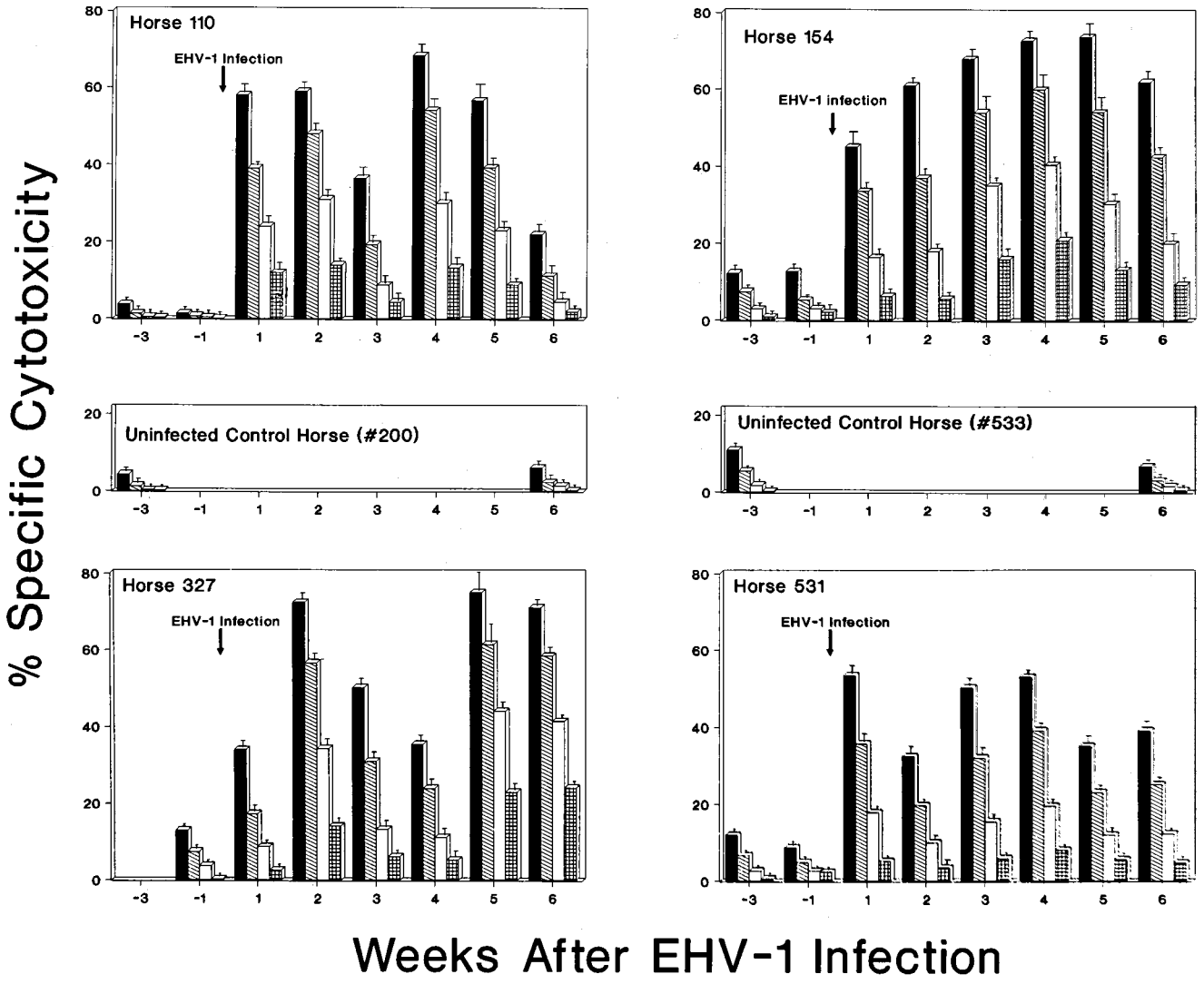

FIG. 1. Induction of EHV-1-specific cytotoxicity in PBMC from six horses with low preinoculation levels of CTL activity. PBMC from four horses were collected before and at weekly intervals after intranasal inoculation with EHV-1. PBMC from the two noninoculated control horses (200 and 533) were obtained and tested prior to beginning the animal infection studies and again at the end of the 6-week sampling period. After 6 days of culture in the presence of EHV-1, the stimulated PBMC were tested for cytotoxic activity against EHV-1-infected target cells. E/T ratios: ■, 100:1; ▨, 33:1; □, 11:1; and ▦, 3.7:1.

CTLs in convalescent horses required in vitro antigenic stimulation and expansion; no significant virus-specific cytotoxicity was present in PBMC induction cultures after 6 days of mock stimulation. In all cases, the CTL activity was specific for EHV-1-infected targets, as levels of specific cytotoxicity for mock-infected autologous target cells were less than 5%. Induction cultures of PBMC from the two uninfected control horses failed to exhibit significant CTL activity for EHV-1 target cells over the duration of the study (Fig. 1).

Characterization of CTL response by EHV-1-infected horses. After establishing that PBMC from EHV-1-immune horses were capable of virus-specific CTL responses following in vitro restimulation with EHV-1 antigen, a series of experiments were performed to characterize the observed cytotoxicity with regard to MHC restriction, phenotype of cytotoxic effector cells, and virus type specificity of the CTL response.

To investigate MHC restriction, PBMC from infected horses were expanded by in vitro induction with EHV-1 antigen and then used as effectors in a ^{51}Cr release assay either with targets that shared MHC class I alleles with the effector cells or with targets that had no class I alleles in common with the effectors. Figure 2 illustrates the efficiency of target cell lysis when effectors and targets were fully matched, partially matched, or nonmatched at the ELA-A class I locus. Only EHV-1-infected targets that shared at least one class I A-locus allele with the effectors were lysed to a significant degree. The results established that in the horse, targets and effectors must have common MHC class I alleles to participate in specific killing of virus-infected target cells.

To identify the phenotype(s) of lymphocytes from infected horses that mediate EHV-1-specific cytotoxicity, in vitro induction cultures initiated from cells from two animals were tested for CTL activity after separation into $CD4^+$ and $CD8^+$ cell fractions. Methods used for separation of the two lymphocyte subpopulations included both negative depletion by magnetic-bead cell sorting and positive selection by immunofluorescent cell sorting. In each case, the results indicated that EHV-1-specific cytolytic activity present in the in vitro-expanded

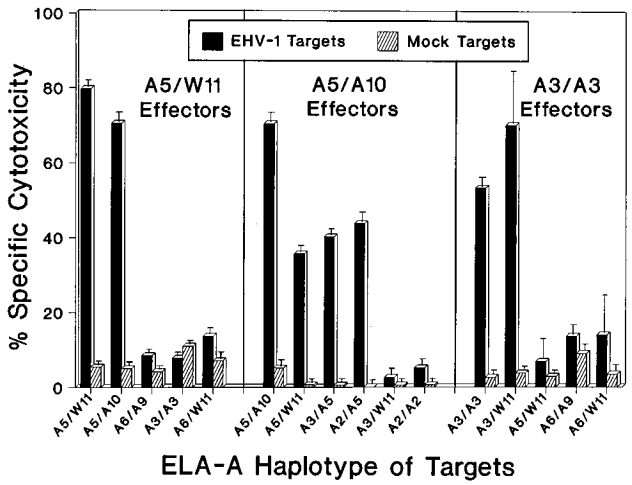

FIG. 2. Demonstration of MHC class I restriction of EHV-1-specific CTLs present in antigen-stimulated PBMC from horses convalescing from experimental viral infection. Effector PBMC from three horses with different ELA-A haplotypes (A5/W11, A5/A10, or A3/A3) were tested for cytotoxicity against target cells that were ELA-A matched, partially matched, or mismatched. Data are presented for an E/T ratio of 100:1.

effector populations was mediated predominantly by $CD8^+$ CTLs (Fig. 3). However, low levels of specific killing of EHV-1-infected target cells were also observed in $CD4^+$ cell fractions. This $CD4^+$ cell-mediated cytotoxic activity was not characterized for MHC class II restriction but appeared to be virus specific, as mock-infected autologous targets were not lysed.

Although the MHC class II phenotypes for horses used in this study were not determined, the perfect correlation between target killing and MHC class I phenotype, along with the observation that most of the induced cytotoxic activity was present in $CD8^+$ cell fractions of effector populations, is consistent with the hypothesis that in vitro lysis of EHV-1-infected target cells by equine immune lymphocytes is mediated predominantly by MHC class I-restricted $CD8^+$ CTLs. However, an assessment of the quantitative significance of $CD4^+$ CTLs circulating within the horse and available for in vivo defense against EHV-1 must await the results of studies that employ limiting-dilution analysis of the frequencies of these two CTL phenotypes in nonstimulated PBMC.

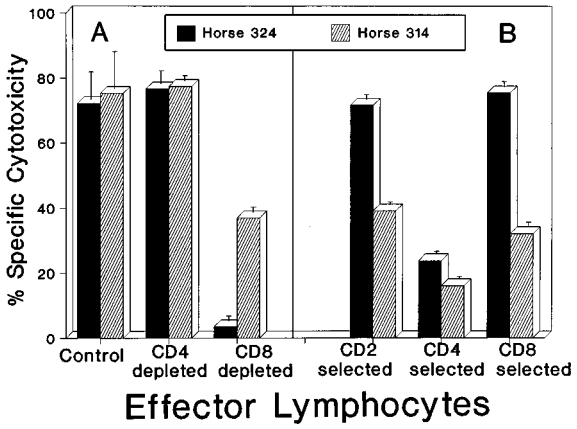

FIG. 3. Identification of lymphocyte subset(s) in PBMC induction cultures that mediate EHV-1-specific cytotoxicity. In vitro-stimulated effector cell populations from two horses were depleted of $CD4^+$ or $CD8^+$ lymphocytes by magnetic cell sorting (MACS; Advanced Magnetics, Cambridge, Mass.) or were enriched for $CD2^+$, $CD4^+$, or $CD8^+$ lymphocytes by fluorescence-activated cell sorting (FACS; FACStar cell sorter, Becton Dickinson). After lymphocyte subset negative depletion by MACS (A) or positive selection by FACS (B), the resulting cell fractions were assayed for cytolytic activity against EHV-1-infected target cells. The sorted cell populations were >95% pure as assessed by postsorting flow cytometry. Control effector cells for MACS were incubated with normal mouse serum prior to magnetic sorting. MACS results are shown for E/T ratios of 50:1 and 10:1 and FACS results are shown for E/T ratios of 4:1 and 20:1 for horses 314 and 324, respectively.

To characterize the virus type specificity of the cytotoxic response induced in horses by EHV-1 infection, immune PBMC from three horses were stimulated in vitro with EHV-4 strain T896 and then evaluated for cytolytic activity against EHV-1-infected targets. In vitro stimulation of PBMC with EHV-4 activated a memory CTL response that was cytolytic for EHV-1-infected target cells, indicating the presence of CTL epitopes shared by the two closely related equine herpesviruses (19). Because PWM-blasts are not permissive for EHV-4 infection, it was not possible to directly test the cytotoxicity of EHV-1-stimulated effector cells for EHV-4-infected targets.

In this report, we have described methods for the generation and detection of EHV-1-specific equine CTLs. Using the methods described above, we have demonstrated in these studies that antigen-primed PBMC collected from EHV-1-immune horses and restimulated in vitro with live EHV-1 are able to present viral antigenic peptides in association with MHC class I molecules to $CD8^+$ memory T lymphocytes and induce their conversion into effector CTLs during in vitro culture. No further exogenous mediators were required, indicating that all the signals necessary for conversion of $CD8^+$ memory T cells into cytotoxic effectors, including EHV-1-specific $CD4^+$ helper T cells with the ability for endogenous production of IL-2 and cells having the capacity to function as antigen-presenting cells for MHC class I presentation of EHV-1 antigen, are present in antigen-stimulated induction cultures of PBMC from immune horses. However, macrophages and nonactivated lymphocytes from mature horses have been shown to be resistant to EHV-1 replication (21). Collectively, these findings suggest a model in which $CD4^+$ lymphoblasts, induced to proliferate in PBMC induction cultures by the presence of EHV-1 antigen, play a major role in activation of antiviral $CD8^+$ memory T cells present in equine immune PBMC by serving both as a source of IL-2 and as virus-infected class I-positive antigen-presenting cells that present viral peptides to precursor $CD8^+$ T cells. This hypothesis is supported by the demonstration in these studies, using two-color immunofluorescent flow cytometry, of the presence of significant levels of EHV-1-infected $CD4^+$ T lymphoblasts in live virus-stimulated PBMC cultures.

The results of the basic studies reported here should now provide the groundwork for further and more quantitative investigations of CTL immunity to EHV-1 in the horse to (i) determine the frequency of virus-specific precursor CTLs in the horse before and after EHV-1 infection and the minimal frequency of precursor CTLs required for protection against infection, (ii) identify the viral antigens and their epitopes that serve as recognition structures for EHV-1-specific CTLs, and (iii) evaluate the effectiveness of experimental EHV-1 vaccines in stimulating CTL activity in the vaccinated horse.

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