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Maturation of the Cellular and Humoral Immune Responses to Persistent Infection in Horses by Equine Infectious Anemia Virus Is a Complex and Lengthy Process

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Equine infectious anemia virus (EIAV) provides a natural model system by which immunological control of lentivirus infections may be studied. To date, no detailed study addressing in parallel both the humoral and cellular immune responses induced in horses upon infection by EIAV has been conducted. Therefore, we initiated the first comprehensive characterization of the cellular and humoral immune responses during clinical progression from chronic disease to inapparent stages of EIAV infection. Using new analyses of antibody avidity and antibody epitope conformation dependence that had not been previously employed in this system, we observed that the humoral immune response to EIAV required a 6- to 8-month period in which to fully mature. During this time frame, EIAV-specific antibody evolved gradually from a population characterized by low-avidity, nonneutralizing, and predominantly linear epitope specificity to an antibody population with an avidity of moderate to high levels, neutralizing activity, and predominantly conformational epitope specificity. Analyses of the cell-mediated immune response to EIAV revealed $CD4^+$ and $CD8^+$ major histocompatibility complex-restricted, EIAV-specific cytotoxic T-lymphocyte (CTL) activity apparent within 3 to 4 weeks postinfection, temporally correlating with the resolution of the primary viremia. After resolution of the initial viremia, EIAV-specific CTL activity differed greatly among the experimentally infected ponies, with some animals having readily detectable CTL activity while others had little measurable CTL activity. Thus, in contrast to the initial viremia, it appeared that no single immune parameter correlated with the resolution of further viremic episodes. Instead, immune control of EIAV infection during the clinically inapparent stage of infection appears to rely on a complex combination of immune system mechanisms to suppress viral replication that effectively functions only after the immune system has evolved to a fully mature state 6 to 8 months postinfection. These findings strongly imply the necessity for candidate EIAV and other lentivirus vaccines to achieve this immune system maturation for efficacious immunological control of lentivirus challenge.

Equine infectious anemia virus (EIAV) causes a persistent infection in horses that is characterized by recurring cycles of viremia and of clinical episodes characterized by fever, anemia, edema, thrombocytopenia, and various wasting symptoms (62). Each viremic febrile episode is associated with the emergence of a predominant novel antigenic variant of the virus (31, 44). Among lentiviruses, EIAV infection is unique in that despite aggressive virus replication and rapid antigen variation, a large majority of the infected animals progress from a chronic disease state to an inapparent carrier stage that can be immunologically maintained for life (42). It is interesting that the horse survives the infection not by completely clearing the viral infection but by maintaining immunological control over virus replication.

The host immune response to EIAV begins upon infection mediated by blood-feeding insects or contaminated syringe needles (28, 67). EIAV differs from the human and primate lentiviruses, human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV), by a tropism restricted to monocytes and macrophage with no apparent infection of T lymphocytes (63). EIAV establishes a high-titer, cell-free, infectious plasma viremia generally within the first 3 to 4 weeks postinfection (62). Infectious virus levels in plasma during chronic EIA diminish only upon intervention by an EIAV-specific immune response mediated by both humoral and cellular host factors (52). Clearance of the primary infectious plasma viremia has been correlated with the emergence of EIAV-specific, major histocompatibility complex (MHC)-restricted, $CD8^+$ cytotoxic T lymphocytes (CTL) (40) and nonneutralizing EIAV-specific antibody (49, 58), although humoral and cellular responses have not been analyzed in parallel. Neutralizing antibody specific for the initial viral quasispecies emerges weeks after the primary viremia (42). Upon resolution of the primary viremia, most animals develop chronic EIA, characterized by irregular, recurring cycles of viremia and associated disease. Disease episodes become less frequent and severe with time and are typically resolved by 8 to 12 months postinfection. The inapparent stage of infection is generally maintained indefinitely. In contrast to the chronic stages of EIA, the afebrile period is typically distinguished by negligible infectious cell-free virus levels and readily detectable levels of cell-associated virus (50). Even during this asymptomatic stage of infection, the animals are still infectious, as demonstrated by the results of whole-blood transfer from inapparently infected to naive animals (27). Viremic febrile episodes can be induced by stress or immunosuppressants administered

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to clinically asymptomatic carriers (33). Thus, the ability to induce chronic EIA in carriers suggests an active immunologic management of virus replication that can be overcome by stressing or immunosuppressing the immune system.

Identifying the immune mechanism(s) involved in managing EIA is fundamental for the development of candidate vaccines for EIA and other lentivirus diseases. To this end, various studies of candidate vaccines involving live attenuated EIAV (32, 56), inactivated EIAV (29), and EIAV subunit proteins (41) have met with limited success in protecting immunized animals upon viral challenge. Detailed information regarding the immunological correlates of protection induced by these vaccines has not been clearly elucidated. Furthermore, although it is known that EIAV-specific CTL are induced coincident with the primary viremia and thus may be involved in the initial immunological management of the infection, it is not known what specific types of cellular or humoral immune responses are necessary to control the subsequent recurring viremic episodes.

To date, no detailed study addressing in parallel both the humoral and cellular immune responses induced in horses upon infection by EIAV has been conducted. Therefore, we initiated the first highly comprehensive longitudinal analysis of the EIAV-specific humoral and cell-mediated immune response parameters elicited by an experimental EIAV infection. Our findings indicate that EIAV induces a highly complex immune response that is constantly evolving during the course of the infection and requires a minimum of 6 to 8 months to mature. The results of this longitudinal examination reveal a number of immunological parameters with critical implications for the development and evaluation of candidate lentivirus vaccines.

TABLE 1. Viremic febrile episodes of experimentally infected ponies

Pony	Febrile episode[a]	Time (days) postinfection[b]	Temp (°C)	Log plasma viremia[c]	ELA-A haplotype[d]
518[e]	NA[f]	NA	NA	NA	A2, A6
561	1	17	39.2	4.0	A5 (weak)[g]
562	1	17	40.0	5.5	A6, A10
564	1	18	40.3	5.0	A2, A3
	2	34	41.0	5.5	
	3	80	39.9	4.5	
	4	106	41.1	4.5	
567	1	19	39.9	3.5	A5
	2	40	40.6	4.5	
	3	223	40.3	4.5	
	4	258	40.9	5.5	

[a] Numerical designation of viremic febrile episodes observed after experimental infection.
[b] The day postinfection at which the rectal temperature peaked during a viremic febrile episode. Febrile episodes occurred for up to four consecutive days.
[c] Log_{10} of the reciprocal dilution of plasma necessary for half of the cultures to be positive for reverse transcriptase activity as assessed in a microtiter reverse transcriptase assay (see Materials and Methods). Data presented refers to the maximum virus titer observed for each febrile episode, which also corresponded during this study to the day of peak rectal temperature.
[d] Serologically defined antigenic specificities determined by the ELA-A system.
[e] Noninfected control pony.
[f] NA, not applicable.
[g] The "weak" designation refers to a low-level, cross-reactive reaction with antiserum specific for A5 and thus implies an ELA-A haplotype other than A5 that could not be discerned from the analysis.

MATERIALS AND METHODS

EIAV strains. Three reference strains of EIAV were used in this study. $EIAV_{Pr}$ is the prototype, nonpathogenic, cell culture-adapted strain of EIAV initially derived by cell adaptation of the Wyoming strain of EIAV (59). $EIAV_{PV}$ is a pathogenic and antigenic variant derived from $EIAV_{Pr}$ (59). $EIAV_{WSU5}$ is a virulent strain of EIAV isolated by McGuire et al. by procedures described to produce $EIAV_{PV}$ (40). These strains of EIAV are very closely related, having <1% variability at the amino acid level.

Experimental subjects. Five outbred Shetland ponies were selected for this study. MHC class I phenotyping was conducted to confirm the heterogeneous status of the group. Serological typing was performed on lymphocytes for the 11 A-locus antigenic specificities of the equine lymphocyte alloantigen (ELA-A) system recognized by the Third International ELA Workshop (2, 3). Lymphocytes were ELA typed by the University of Kentucky Equine Blood Typing Laboratory, using microcytolytic testing procedures and serological reagents developed and described previously (5, 6). ELA typing substantiated the heterogeneity of the experimental group as an outbred population, at least for the A locus (Table 1). Four (ponies 561, 562, 564, and 567) of the five ponies were experimentally inoculated intravenously with 10^3 median equid infectious doses of $EIAV_{PV}$. One pony (pony 518) remained as an uninfected control for all studies. All ponies were clinically monitored daily and maintained as previously described (29).

Clinical evaluation and longitudinal sample collection. Rectal temperatures and clinical status were recorded daily. Samples of serum, plasma, and whole blood were collected from each $EIAV_{PV}$-infected pony at specified intervals (0, 7, 14, 21, 28, 35, 42, 49, 56, 70, 84, 119, 182, 238, and 301 days postinfection). Plasma samples were collected at the specified intervals and during each febrile episode (>39°C). The plasma samples were stored at −80°C until used to determine the quantity of circulating infectious EIAV. Serum samples were stored at −80°C until tested for antibody reactivity to the $EIAV_{Pr}$ viral capsid protein, p26, or $EIAV_{PV}$ envelope glycoprotein in an enzyme-linked immunosorbent assay (ELISA) or in a neutralization test by procedures that are described below. Whole-blood samples were appropriately fractionated for enumeration of platelets (Unopette microcollection system; Becton-Dickinson, Rutherford, N.J.) or experimentation with peripheral blood mononuclear cells (PBMC). PBMC were either used immediately for the evaluation of EIAV-specific proliferative responses (described below) and EIAV-specific cytolytic activity (described below) or stored in liquid nitrogen for future evaluation.

Measurement of EIAV envelope glycoprotein-specific antibodies by ConA ELISA. Serum immunoglobulin G (IgG) antibody reactivities to EIAV native envelope glycoprotein were conducted by the concanavalin A (ConA) ELISA method developed by Robinson et al. (55). This method of capturing the viral glycoprotein minimizes the potential alterations in protein conformation and antigenic properties resulting from adsorption to plastic surfaces. Briefly, wells of Immunolon II assay plates (Dynatech Laboratories, Chantilly, Va.) were incubated with 50 μl of ConA (Vector Laboratories, Inc., Burlingame, Calif.) at 50 μg/ml in phosphate-buffered saline (PBS; pH 7.4) for 1 h at room temperature. All subsequent reactions conducted for this ELISA were carried out at room temperature. The wells were washed twice with PBS containing 0.025% Tween 20 (PBS-T). Gradient-purified $EIAV_{PV}$ was disrupted for 15 min in PBS containing 1% (vol/vol) Triton X-100. To each well, 1 μg of detergent disrupted virus in 50 μl of PBS–0.1% Triton X-100 was added, and the mixture was incubated for 1 h. The wells were washed four times with PBS-T. Unreacted ConA binding sites were blocked by incubating each well for 1 h with 50 μl of PBS-T containing 5% (wt/vol) nonfat dry milk and 5% (vol/vol) fetal calf serum (BLOTTO). Virus-negative control wells consisted of ConA-coated wells incubated only with BLOTTO. To test for antibody binding to the ConA-immobilized antigen, the blocking reagent was removed and serum samples, diluted appropriately in BLOTTO, were added to the wells at 50 μl/well for 1 h. The wells were washed four times with PBS-T and incubated for 1 h with 50 μl of affinity-purified, horseradish peroxidase-conjugated, goat anti-horse IgG (United States Biochemical Corp., Cleveland, Ohio) diluted 1:100,000 in BLOTTO. They were then washed four times with PBS-T. To each well, 200 μl of TM-Blue (Intergen, Milford, Mass.) substrate was added, and the mixtures were incubated for 20 min. The enzymatic color reaction was terminated by the addition of 50 μl of 1 N H_2SO_4. Antibody IgG reactivity to the ConA-captured viral glycoprotein was determined by measuring the absorbance at 450 (A_{450}) with the MR5000 microplate spectrophotometer (Dynatech Laboratories).

Measurement of serum antibodies to EIAV CA protein (p26) by ELISA. Capsid protein was isolated from gradient-purified EIAV in a one-step reverse-phase high-pressure liquid chromatography (HPLC) procedure as described previously (7). The p26 antigen was directly adsorbed to the Immunolon II plates in 50 μl of PBS at 2 μg/ml for 1 h. The wells were washed four times with PBS-T and blocked with BLOTTO for 1 h. Detection of serum IgG antibody reactivity to p26 was conducted as described above for the primary and secondary antibody incubation and for the color substrate addition and measurement.

Antibody avidity measurements. The avidity index values of polyclonal serum IgG antibodies specific for envelope glycoprotein and p26 were measured by

examining the stability of antigen-antibody complexes in the presence and absence of an 8 M urea treatment (25, 26). A range of dilutions for each test serum were selected which produced an A_{450} of approximately 1.0 to 1.5 in either the ConA envelope ELISA or the p26 ELISA. Quadruplicate wells were treated in parallel for 5 min with three washes of either 200 μl of PBS-T or 200 μl of 8 M urea–PBS-T. All the wells were washed an additional four times with PBS-T to remove all traces of the urea before addition of the secondary antibody and the color-reactive substrate, TM-Blue. For the calculation of the avidity index, no sets of values with an A_{450} above 1.5 (PBS wash) and below 0.15 (8 M urea wash) were used. The A_{450} values of 0.15 to 1.5 are within the linear measurable range of the microtiter plate reader. The avidity index was calculated from the ratio ($[A/B] \times 100\%$) of the absorbance value obtained with the urea treatment (A) to that observed with the PBS-T treatment (B). Serum antibodies with avidity index values of <30% are designated low-avidity antibodies, those with values of 30 to 50% are designated intermediate-avidity antibodies, and those with values of >50% are considered high-avidity antibodies (26).

EIAV envelope glycoprotein-specific antibody conformational epitope dependence measurements. Serum samples were analyzed for conformation dependence by comparing the serum IgG antibody reactivities with native and denatured viral envelope glycoprotein antigen in a ConA envelope ELISA, as described above. Density gradient-purified $EIAV_{PV}$ disrupted with 1% Triton X-100 was the source of the native viral envelope glycoprotein. To produce denatured viral envelope glycoprotein, density gradient-purified $EIAV_{PV}$ was treated with β-mercaptoethanol and urea to reduce disulfide bonds and then with iodoacetic acid to irreversibly carboxymethylate the reduced sulfhydryl groups (16). Briefly, density gradient-purified $EIAV_{PV}$ was denatured and reduced by incubation for 4 h at room temperature over a nitrogen gas barrier in a solution volume of 12 ml containing 8 M urea, 0.2% EDTA, 0.12 M β-mercaptoethanol, and 0.36 M Tris-HCl (pH 8.6). To irreversibly carboxymethylate the reduced sulfhydryl groups, a freshly prepared solution of 0.268 g of iodoacetic acid in 1 ml of 1 N NaOH was added to the reaction mixture, which was incubated for 15 min at room temperature over a nitrogen barrier excluding light. The entire reaction mixture was dialyzed for 12 to 16 h at 4°C with three 1,000-fold volume changes of PBS to terminate the reaction and to remove all traces of the reactants. Each serum sample was tested in a ConA envelope ELISA with, in parallel, native or denatured sources of viral envelope glycoprotein. A range of test serum dilutions were selected in the linear range of the end-point titer curve. A conformation index was then calculated from the ratio of antibody reactivities to native and denatured viral envelope glycoprotein antigens. The A_{450} values used to calculate the conformation index were always between 1.5 and 0.15, the linear measurable range of the microtiter plate reader. Thus, the conformation index is a direct measure of the conformation dependence of a particular antibody sample; i.e., the larger the conformation index, the greater the specificity for conformational viral envelope glycoprotein determinants compared to linear envelope determinants.

Neutralizing-antibody assay. The chronological development of serum neutralizing antibody during the course of an EIAV infection was assessed in an indirect cell ELISA-based infectious-center assay with a constant amount of infectious $EIAV_{PV}$ and various dilutions of serum. Propagation of $EIAV_{PV}$ in vitro in fetal equine kidney (FEK) cells has been described previously (9). Maintenance of FEK primary cell cultures has been described in detail previously (9). All sera were heat inactivated (56°C for 1 h) before use in the neutralization assay to inactivate infectious virus and labile serum proteins. Briefly, 10^5 FEK cells were added to each well of a 24-well tissue culture-treated plate and were cultured at 37°C for 18 to 24 h. After the incubation, twofold serial dilutions of each serum sample in duplicate were incubated with 100 infectious centers of $EIAV_{PV}$ for 1 h at 37°C. The serum-virus supernatant was added to the cell culture and incubated for 18 to 24 h at 37°C. An overlay of 0.8% carboxymethyl cellulose was added to the infected cell culture to prevent the spread of the virus throughout the entire culture. The infected culture was further incubated at 37°C for 9 days. Infected cells in the wells were detected by conducting a cell-based ELISA. The cells were fixed with 3.7% formaldehyde and permeabilized with 1% Triton X-100. The primary antibody used was a 1:200 dilution of a reference immune serum (Lady) from an EIAV-infected horse (43). The secondary antibody used was a 1:3,000 dilution of an affinity-purified, horseradish peroxidase-conjugated goat anti-horse IgG (United States Biochemical Corp.). The peroxidase substrate added to visualize EIAV infectious centers was 3-amino-9-ethyl-carbazole (Sigma) in a sodium acetate buffer (pH 5.5) supplemented with H_2O_2. The enzymatic reaction was terminated with water. All wells were air dried before the visually apparent foci were enumerated with a dissecting microscope. The 50th percentile reciprocal neutralization titers of each serum sample were determined by linear regression analysis of the $\log_{10}$ reciprocal dilution versus the number of apparent foci.

Lymphocyte proliferation assays. EIAV-specific proliferative responses were measured with autologous $EIAV_{Pr}$-infected monocytes as antigen-presenting cells. Briefly, 2×10^5 PBMC were added to appropriate wells of a 96-well U-bottom microtiter plate in 200 μl of culture medium (CM) alone or CM containing 10 μg of density gradient-purified $EIAV_{Pr}$ per ml. CM is composed of RPMI 1640 (GIBCO/BRL) supplemented with 10% heat-inactivated horse serum (Sigma), 50 U of penicillin (GIBCO/BRL) per ml, 50 μg of streptomycin (GIBCO/BRL) per ml, 4 mM L-glutamine (GIBCO/BRL), 50 μM β-mercaptoethanol (Sigma Cell Culture), 50 μM each of minimal essential medium nonessential amino acids (Sigma), and 0.5 mM sodium pyruvate (Sigma). The cultures were incubated at 37°C for 4 days. On day 4, 1 μCi of [^{3}H]thymidine (DuPont NEN, Boston, Mass.) was added to each well in 50 μl of CM. After 24 h of culture with [^{3}H]thymidine, the cells were harvested onto fiberglass filters with a cell harvester (Skatron Instruments, Inc., Sterling, Va.) for determination of [^{3}H]thymidine incorporation by scintillation counting. EIAV-specific lymphocyte proliferative responses were reported in the form of a stimulation index (SI) representing the average [^{3}H]thymidine incorporation of replicate EIAV-infected monocyte stimulated lymphocyte cultures (E) divided by the average [^{3}H]thymidine incorporation of replicate unstimulated lymphocyte cultures (M), or SI = E/M. Reporting EIAV-stimulation in the form of an SI for the longitudinal samples normalizes the observed [^{3}H]thymidine incorporation between each experiment. Mean incorporations of [^{3}H]thymidine by replicate unstimulated lymphocyte cultures were generally below 400 cpm.

Recombinant vaccinia virus vectors. The control recombinant vaccinia virus vector (vac) has been described in detail previously (vSC8 [12]). The recombinant vaccinia virus vector carrying the full-length $EIAV_{WSU5}$ *env* gene (vac-env) has been described in detail previously (vENV2 [40]). The recombinant vaccinia virus vector carrying the full-length $EIAV_{PR}$ *gag* and *pro* genes and part of the *rt* gene (vac-gag) was derived from a 2.1-kbp *Sma*I fragment of the $EIAV_{Pr}$ proviral clone (nucleotides 343 to 2526; accession number M16575). Target cells were infected with recombinant vaccinia viruses at a multiplicity of infection of 10 for 12 to 18 h at 37°C. More than 99% of the equine target cells infected with recombinant vaccinia viruses expressed antigen, as determined by indirect immunofluorescence (data not shown).

In vitro secondary stimulation of EIAV-specific T cells. PBMC were isolated from ponies at each designated interval after EIAV infection by discontinuous density gradient centrifugation and were stimulated with autologous monocytes infected with $EIAV_{Pr}$. Unfractionated PBMC were plated in 24-well tissue culture-treated plates at 2.5×10^6 cells/ml in CM supplemented with 200 U of recombinant human interleukin-2 (IL-2) (CM–IL-2; Chiron, Emeryville, Calif.) per ml. Autologous monocytes present in the plated PBMC were infected with $EIAV_{Pr}$ by the addition of gradient-purified EIAV to 10 μg/ml. The cultures were incubated for 4 days at 37°C. After the 4-day incubation, the cultures were split and fed as necessary with CM–IL-2. On day 8, the cultures were assayed for EIAV-gag- or EIAV-env-specific cytolytic activity.

CTL assay. Cytolytic activity was measured in a standard ^{51}Cr release assay (65). Briefly, pokeweed mitogen (PWM)-stimulated PBMC infected with various recombinant vaccinia virus vectors were used as target cells. Target cells were prepared by stimulating unfractionated PBMC plated in 24-well tissue culture-treated plates at 2.5×10^6 cells/ml in CM–IL-2 with PWM added to 250 μg/ml. After the 4-day incubation, the cultures were split and fed as necessary with CM–IL-2. At 7 to 21 days after lectin stimulation, the PWM-treated lymphoblasts were infected for 12 to 16 h with recombinant vaccinia virus vectors, as described above. The phenotype of equine lymphocytes in the PWM-stimulated cultures has been described previously (1). Infected cells were then concentrated to 10^7 cells/ml and were incubated for an additional 2 h with 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham, Arlington Heights, Ill.) in CM–IL-2. ^{51}Cr-labeled target cells were washed three times and were plated in 0.1 ml of CM–IL-2 at 5×10^4 cells/well in 96-well V-bottom microtitre plates. Ex vivo PBMC or responding cells from EIAV-infected monocyte-stimulated cultures were added to each well in 0.1 ml of CM–IL-2 at the indicated effector-to-target-cell ratios. Maximum ^{51}Cr release was determined by plating ^{51}Cr-labeled target cells with 0.1 ml of 1% Nonidet P-40. Background spontaneous lysis, or minimum ^{51}Cr release, was determined by plating ^{51}Cr-labeled target cells with 0.1 ml of medium alone. The plates were centrifuged at $200 \times g$ for 5 min at room temperature and then incubated at 37°C for 7 to 9 h. After the incubation, the plates were centrifuged at $200 \times g$ for 5 min at room temperature. Equal volumes of supernatant were removed from each well for γ-radiation measurement. All determinations were performed in quadruplicate. The percent specific lysis was calculated as follows:

$$\%\ \text{Specific lysis} = \frac{(E - C)}{(N - C)} \times 100$$

where E is the average counts per minute from experimental wells containing T cells and target cells, C is the average counts per minute from control wells containing target cells only (spontaneous lysis), and N is the average counts per minute from control wells containing target cells in 0.5% Nonidet P-40 (maximal lysis). Spontaneous lysis of the target cells was always below 30% of maximal lysis. The standard error of the mean percent specific lysis, determined as previously described (64), was <3%, except where indicated.

Depletion of CD4$^+$ and CD8$^+$ T cells. Enrichment of CD4$^+$ and CD8$^+$ T-cell subpopulations was accomplished by indirect panning as described previously (40). Briefly, petri dishes were coated for 1 h with 10 μg of affinity-purified goat anti-mouse Ig (Zymed Laboratories, Inc., South San Francisco, Calif.) per ml in 50 mM Tris-HCl (pH 9.5) at room temperature. The coated petri dishes were washed three times before the addition of cells. $EIAV_{Pr}$-infected-monocyte-stimulated PBMC responder cultures were divided into three groups for T-cell subset depletion: one responder group was incubated in PBS–5% horse serum, one responder group was incubated in PBS–5% horse serum with 10 μg of anti-equine CD8 monoclonal antibody HT14A (VMRD, Inc., Pullman, WA) per ml, and one responder group was incubated with 10 μg of anti-equine CD4

monoclonal antibody HB61A (VMRD, Inc.) per ml. After three washes, the treated groups of cells were plated separately onto the Ig-coated petri dishes and incubated for 1 h at 4°C. Nonadherent cells were removed by gently swirling the petri dishes and removing the supernatant. Petri dishes were washed once with PBS to further remove remaining nonadherent cells. Nonadherent cell populations were cultured in CM–IL-2 for 1 day at 37°C before T-cell subpopulation phenotype analysis was initiated by flow cytometry and EIAV-gag- and EIAV-env-specific cytolytic activity. Generally, >95% of the CD4 or CD8 T cells were depleted as determined by flow cytometry analysis (data not shown).

Statistical methods. The SI for the EIAV-specific proliferation assays was calculated as described above. The standard error of SI is approximately as follows:

$$\text{SE (SI)} = \left[\left(\frac{1}{M^2} \times \frac{\sigma_E{}^2}{N_E}\right) + \left(\frac{E^2}{M^4} \times \frac{\sigma_M{}^2}{N_M}\right)\right]^{1/2}$$

where σ^2 is the variance and N is the number of replicates for each variable. This formula takes into account the variability of the terms (E and M) that are used to calculate the SI (4).

RESULTS

Experimental infection of ponies with EIAV. Four outbred Shetland ponies were experimentally infected with 1,000 equid infectious doses of $EIAV_{PV}$. At 2 to 3 weeks postinfection, each pony demonstrated the EIA-specific clinical symptoms of fever (Fig. 1), thrombocytopenia (less than 150,000 platelets per μl of blood), anemia, and depression. Two ponies, ponies 561 and 562, experienced only a single clinical episode and remained free of clinical symptoms during the remainder of the study. Recurring febrile episodes and reduced platelet counts were observed for the other two ponies, ponies 564 and 567 (Fig. 1; Table 1). Pony 564 cycled through four febrile episode on days 18, 34, 80, and 106 postinfection before bringing the infection under control, and the infection remained clinically inapparent throughout the remainder of the study. Two initial febrile episodes on days 19 and 40 were observed for pony 567. After the two initial fevers, this pony entered a period in which the observable EIA symptoms became clinically inapparent. Interestingly, approximately 8 months after the second febrile episode, pony 567 cycled through two more febrile episodes. The two rectal temperature spikes, peaking on days 89 and 193, respectively, did correspond to a minor decline in platelet numbers in serum, a prognostic indicator of an EIAV-specific event. The development of the irregularly cyclical thrombocytopenia in which the nadir of the platelet counts accompanied each peak in fever was coincident in all ponies tested (Fig. 1).

Infectious EIAV circulating in the plasma peaked in titer during each febrile episode and declined to all but undetectable levels after the fevers had subsided (Table 1 and unpublished data). Levels of infectious EIAV were quantitated by incubating fetal equine kidney cells with serial half-log dilutions of collected plasma and by determining if reverse transcriptase a measure of virus production, could be detected in clarified tissue culture supernatants by using a micro-reverse transcriptase assay (38). Plasma viremia titers ranged from $10^{3.5}$ to $10^{5.5}$ 50% tissue culture infective doses (Table 1). The measured levels of circulating infectious EIAV varied nonproportionately to the observed peak febrile temperature levels.

Humoral response to EIAV. In the following four sections, we characterized in a longitudinal fashion the evolution of EIAV envelope-specific antibodies by using the described assays for antibody titers, antibody avidity, antibody conformation dependence, and antibody neutralization levels.

(i) Reciprocal EIAV-specific serum IgG antibody end-point titer. We collected longitudinal serum samples from the experimentally EIAV-infected ponies to measure the development and maturation of the EIAV-specific humoral response. All EIAV-infected ponies seroconverted as measured by the presence of serum IgG (Fig. 2) and IgM (data not shown) to native EIAV envelope glycoprotein and EIAV p26 core protein 3 weeks postinfection. Envelope-specific serum IgG reciprocal endpoint titers increased gradually, reaching maximum values at 7 to 10 weeks postinfection. The levels of envelope-specific IgM in serum were initially detected 3 weeks postinfection, reached a maximum level at the same time interval postinfection, and decreased to all but undetectable levels at and beyond 5 weeks postinfection (data not shown). Serum IgG p26-specific antibody reciprocal end-point titers increased rapidly after seroconversion and reached maximum levels between months 1 and 2 postinfection (Fig. 2B). Antibodies specific for EIAV envelope were the predominant detectable population

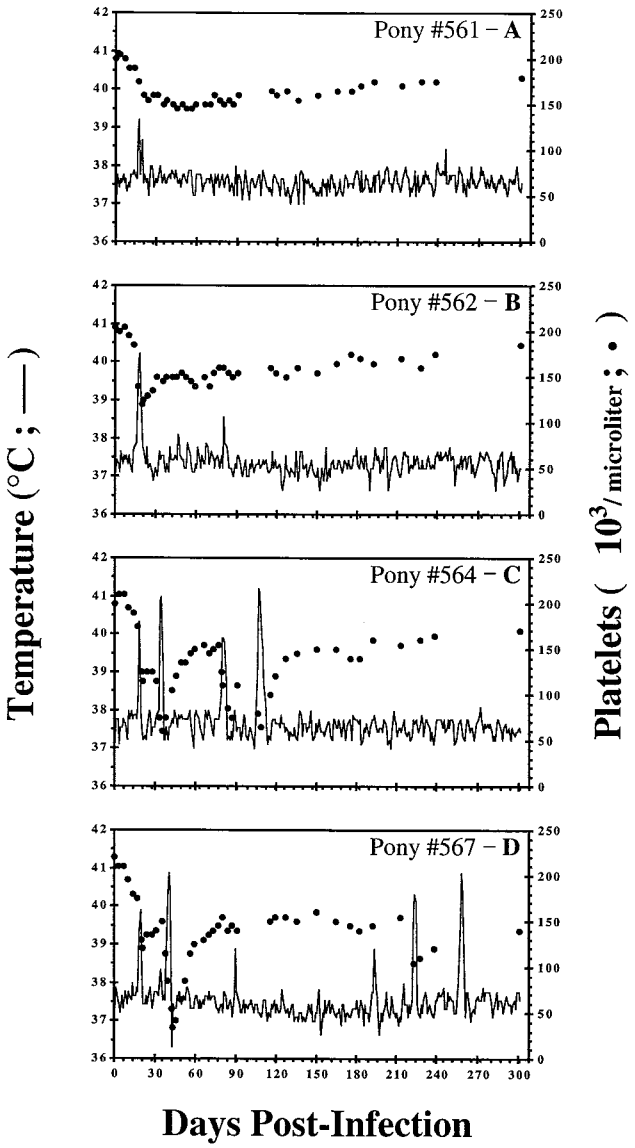

FIG. 1. Clinical course of experimental EIAV infections. The rectal temperature (solid line, primary *y* axis) and platelet counts per microliter of whole blood (solid circles, secondary *y* axis) of experimentally EIAV-infected Shetland ponies (ponies 561, 562, 564, and 567) are shown. The ponies were experimentally infected with 10^3 50% tissue culture infective doses of the PV strain of EIAV on day 0 and observed for 10 months (301 days). Rectal temperatures in excess of 39°C were considered EIA episodes only in conjunction with a reduction in the number of circulating platelets below 10^5 per μl of blood.

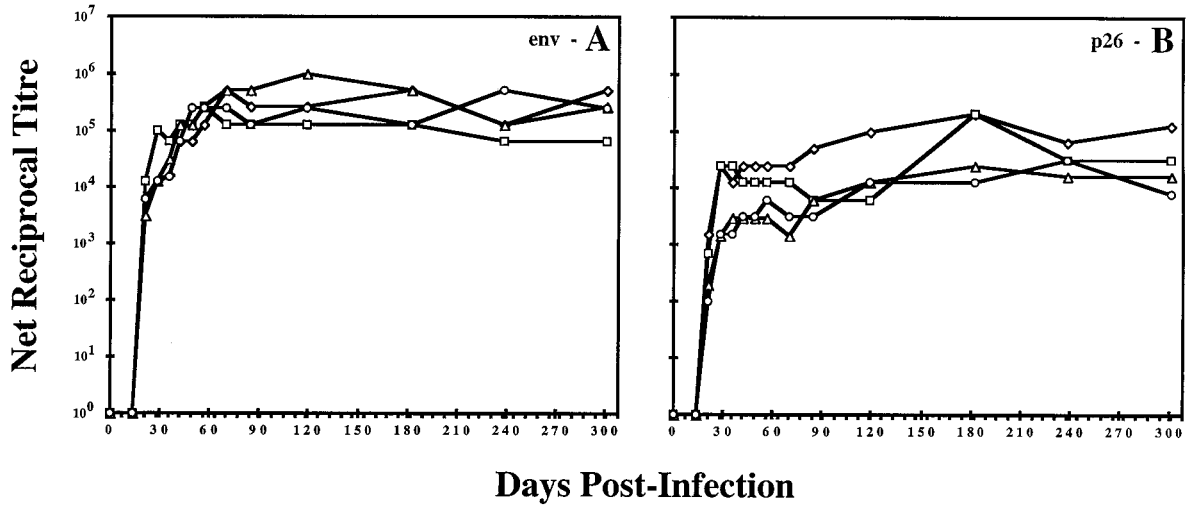

FIG. 2. Development of serum antibody to EIAV in experimentally infected ponies. Reciprocal end-point titers of EIAV envelope glycoproteins (A) and p26-specific IgG (B) in sera collected from experimentally EIAV-infected Shetland ponies. Reciprocal end-point titers of EIAV-specific IgG were determined by measuring in an ELISA the reciprocal dilution of test serum that produces reactivity at levels that are 2 standard deviations above background. Serum IgG reactivity to EIAV envelope glycoproteins was measured by a ConA ELISA as described in Materials and Methods. Serum IgG reactivity to EIAV p26 was measured by a standard ELISA as described in Materials and Methods. Longitudinal serum samples were collected from the following four Shetland ponies at various times after experimental infection with the PV strain of EIAV: 561 (◇), 562 (□), 564 (△), and 567 (○).

during the entire observation period of the infection. The kinetics of envelope- and p26-specific antibody responses were similar. Furthermore, the kinetics of the EIAV-specific antibody response did not distinguish infectious clinical courses, e.g., rapid control (ponies 561 and 562) versus recurring disease episodes (ponies 564 and 567). The observed differences between the apparent grouping of p26-specific antibody reciprocal end-point titers of the ponies which resolved the viral infection immediately following the primary viremic febrile episode (ponies 561 and 562) and the p26-specific antibody titers of the ponies which cycled through multiple viremic febrile episodes (ponies 564 and 567) was unclear regarding any correlation of immunological control of the primary viremia.

(ii) EIAV-specific serum IgG antibody avidity. The measured kinetics of appearance of EIAV-specific IgG in the infected ponies indicated a rapid response to the infection. We sought to examine the evolution of the EIAV envelope-specific antibody populations with respect to their avidity as measured by the stability of antigen-antibody complexes to treatment with 8 M urea. We have demonstrated that antibody avidity measurements provide an informative parameter for examining the evolution of antibody response to SIV in rhesus macaques and as an immune correlate of protection (14, 15).

IgG avidities in serum were determined by measuring the stability of antigen-antibody complexes after treatment with three stringent 8 M urea washes as compared to PBS washes (25, 26). In this functional assay, avidity index values below 30% were considered to indicate low-avidity antibodies, values between 30 and 50% were considered to indicate intermediate-avidity antibodies, and values above 50% were considered to indicate high-avidity antibodies (26). The data presented in Fig. 3A demonstrated that the envelope-specific antibody responses in all four EIAV-infected ponies increased gradually in avidity to the glycoprotein during the 10 months of examination and appeared to generally level off 8 to 10 months postinfection. Envelope-specific antibody avidity reached maximum levels in the intermediate index range of 40 to 50% (Fig. 3A). The measured slow increase in the avidity of antibodies to the envelope was in distinct contrast to the relatively rapid increase in avidities observed during other viral infections (30, 60, 69).

To determine if the relatively slow measured increase in antibody avidity to envelope glycoprotein was due to the complex nature of the highly glycosylated envelope antigen or to the nature of the lentivirus infection, the avidity of antibodies to the nonglycosylated viral p26 antigen was also determined (Fig. 3B). The data in Fig. 3 demonstrate that the measured kinetics of p26-specific antibody avidity maturation paralleled the measured avidity maturation kinetics of envelope-specific antibody. The p26-specific antibody avidity index values gradually increased postinfection for all animals tested and eventually leveled off at the 10-month postinfection interval with three of the four ponies in the high range of 50 to 60% (Fig. 3B). Only pony 567 maintained p26-specific antibody avidity index values generally below 30% during the period of observation. In addition, we observed no correlation between antibody reciprocal end-point titers and the measured avidity index values, in agreement with previously reported studies (37, 53). Taken together, it appeared that the slow EIAV-specific antibody avidity maturation occurring during the course of infection was not due to the complex nature of the envelope glycoprotein but was due to an undefined aspect of the persistent lentivirus infection. A similar 6- to 8-month evolution of envelope-specific antibody avidity has been previously described in SIV infection of rhesus macaques (14).

(iii) Conformational dependence of EIAV envelope-specific antibodies. We sought to measure the conformational dependence of the envelope-specific antibody responses in the longitudinal panel of serum samples collected from the EIAV-infected ponies. The reactivity of the serum antibodies at predetermined dilutions was measured in parallel against ConA-anchored native viral glycoprotein and against denatured viral glycoprotein produced by urea denaturation and reductive carboxymethylation of protein cysteine sulfhydryl groups (data summarized in Fig. 4). This assay compared the reactivity of serum antibodies with a native viral glycoprotein complex with that of viral glycoproteins in which all disulfide

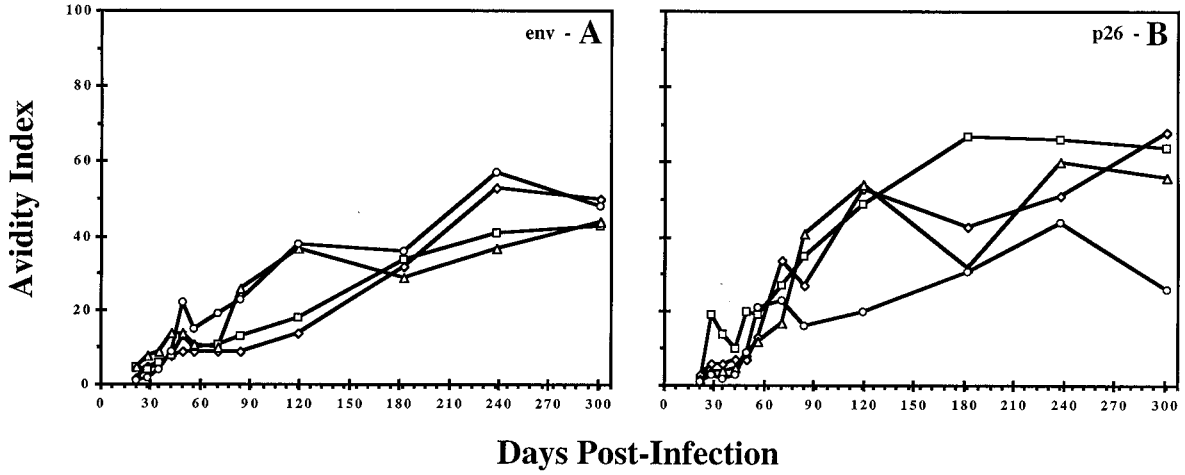

FIG. 3. Evolution of EIAV-specific serum antibody avidity in experimentally infected ponies. The avidity of serum IgG specific for EIAV envelope glycoproteins (A) and p26 core protein (B) is shown. The avidity index of the EIAV-specific IgG was determined by measuring in an ELISA the resistance of test serum IgG-envelope glycoprotein or IgG-p26 immune complexes to disruption by treatment with 8 M urea, as described in Materials and Methods. Longitudinal serum samples were collected from the following four Shetland ponies at various times after experimental infection with the PV strain of EIAV: 561 (◇), 562 (□), 564 (△), and 567 (○).

bonds had been irreversibly reduced to alter the tertiary structure of the protein.

Conformation index values less than 1.0 connoted a serum antibody reactivity preference for linear viral glycoprotein antigen rather than native viral glycoprotein substrate, while the converse was indicated by a conformation index value greater than 1.0. The initial antibody responses displayed conformational index values less than 1.0, indicating predominant reactivity to linear determinants on the envelope. The progressive change in the conformational dependence of envelope-specific antibody during the first 4 to 7 weeks postinfection indicated an evolution of antibody response from an early dominance of linearly dependent antibodies with a gradual maturation to a more conformationally dependent antibody population. At 7 to 14 weeks postinfection, the antibody response generated to the viral infection evolved to become up to twofold more reactive to the native viral glycoprotein substrate than to denatured viral glycoprotein antigen. Antibody conformation index values in general increased during the first 6 to 8 months postinfection, after which the conformation dependence was maintained at levels of 1.5 to 2.0 for the remainder of the observation period.

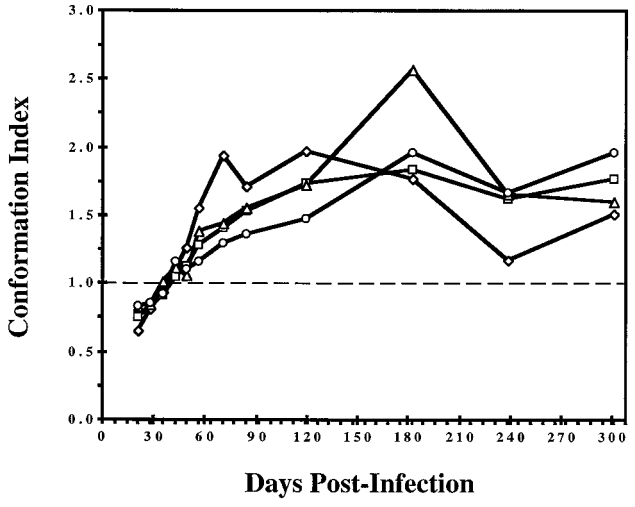

FIG. 4. Evolution of EIAV-specific antibody conformation dependence in experimentally infected ponies. The conformation dependence of viral envelope glycoprotein-specific serum antibodies elicited by infection with $EIAV_{PV}$ was determined by measuring in a ConA ELISA the antibody reactivities against native $EIAV_{PV}$ envelope glycoproteins and against denatured envelope glycoproteins prepared by an initial urea denaturation followed by a reduction and carboxymethylation of protein sulfhydryl groups, as described in Materials and Methods. Longitudinal serum samples were collected from the following four Shetland ponies at various times after experimental infection with the PV strain of EIAV: 561 (◇), 562 (□), 564 (△), and 567 (○).

(iv) EIAV-specific serum neutralizing antibody. To complement our studies of antibody titer, avidity, and conformational dependence, we sought to measure the levels of EIAV-specific neutralizing antibody in the longitudinal serum samples from the experimentally infected ponies. For this assay, we conducted an infectious-center assay based on a cell ELISA (see Materials and Methods) to quantify in vitro the levels of neutralizing serum antibody. Reciprocal serum dilutions that produced virus neutralization of 50%, as determined by linear regression analysis, were recorded (Fig. 5).

Neutralizing antibody was not detectable until 2 to 3 months postinfection in all ponies examined. By this time postinfection, each pony had resolved the initial viremic febrile episode weeks before this neutralizing antibody was detected. The levels of neutralizing antibody increased in all infected ponies until approximately 4 months postinfection. Interestingly, after 4 months postinfection, the observed neutralizing-antibody levels differed markedly among the infected ponies. Pony 561 exhibited a rapid increase in neutralizing-antibody titers (1/4,000); pony 562 maintained a relatively constant low titer (1/50); ponies 562 and 567 displayed net increases in neutralizing-antibody titer, although fluctuations were observed at the various time points examined; and pony 564 displayed a rapid decline in neutralizing-antibody titers from 1/300 to 1/50 at 6 months postinfection. Taken together, no correlation was observed between neutralizing-antibody titers to $EIAV_{PV}$ and the clinical course of disease in the experimentally infected ponies.

Cell-mediated response to EIAV. In the following four sections, we characterized in a longitudinal fashion cell-mediated immune response parameters elicited upon experimental infection with EIAV. These parameters included the kinetics of

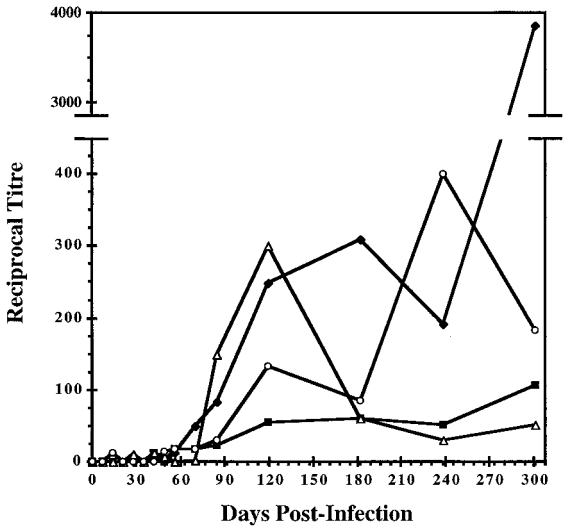

FIG. 5. Reciprocal titers of EIAV-specific neutralizing activity detectable in vitro in longitudinal serum samples collected from experimentally infected ponies. Neutralizing assays were conducted, as described in Materials and Methods, testing the effectiveness of serum to prevent in vitro the formation of infectious centers among fetal equine kidney cells by $EIAV_{PV}$. The results are the means of at least two independent assays. Longitudinal serum samples were collected from the following four Shetland ponies at various times after experimental infection with the PV strain of EIAV: 561 (◆), 562 (■), 564 (△), and 567 (○).

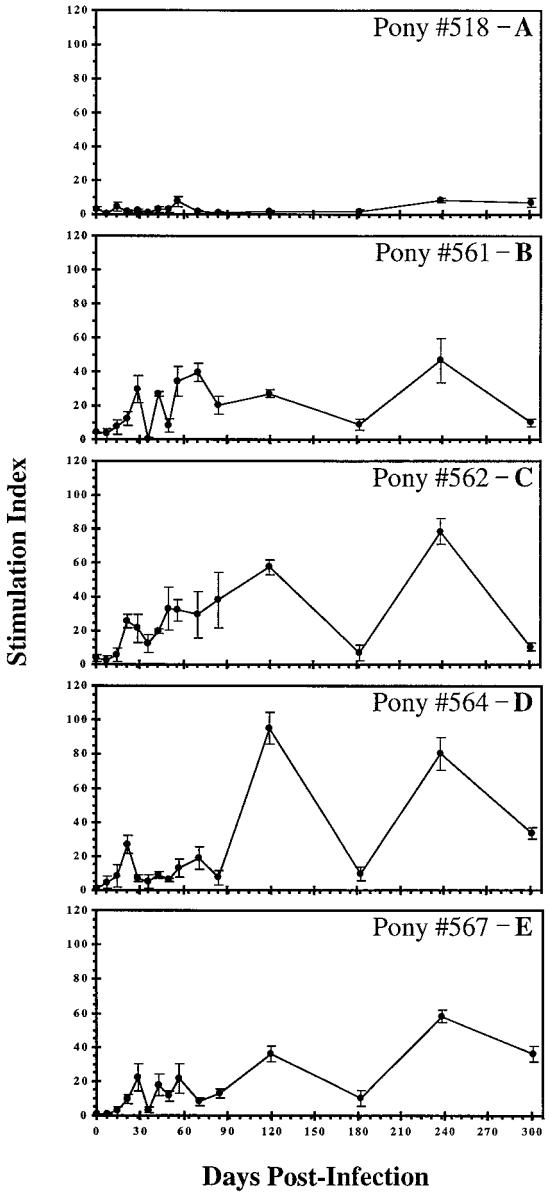

FIG. 6. In vitro lymphoproliferative reactivities of longitudinally collected samples of PBMC to EIAV-infected autologous macrophages. PBMC samples collected longitudinally from a control uninfected Shetland pony (Pony 518) (A) and from the four experimentally $EIAV_{PV}$-infected Shetland ponies (Ponies 561, 562, 564, and Pony 567) (B to E) were assayed for their proliferative responses to autologous macrophages infected with $EIAV_{Pr}$, as described in Materials and Methods. The proliferative reactivities were presented in the form of an SI representing [^{3}H]thymidine incorporation into antigen-stimulated cultures divided by [^{3}H]thymidine incorporation into unstimulated cultures. The mean incorporation of [^{3}H]thymidine into replicate unstimulated lymphocyte cultures was generally below 400 cpm. All experiments were conducted in quadruplicate. *y*-axis error bars represent the standard error of the mean SI as derived and calculated in Materials and Methods.

EIAV-specific T-cell proliferative responses and EIAV-specific T-cell cytolytic activity. The T-cell cytolytic activity was further characterized to determine specifically which subset, $CD4^+$ and/or $CD8^+$, was mediating the lysis of antigen-expressing target cells as well as if the effector T cells were functioning in an MHC-restricted manner.

(i) EIAV-specific T-cell proliferative response. We evaluated the ability of an EIAV infection to generate an effective ex vivo antigen-specific T-cell proliferative response. PBMC isolated from infected and control ponies were combined with autologous EIAV-infected macrophages to measure the levels of antigen-specific lymphoproliferation present at various times postinfection. Relative levels of lymphoproliferation between the multiple time points for each pony were compared in the form of a stimulation index representing [^{3}H]thymidine incorporation of antigen-stimulated cultures divided by [^{3}H]thymidine incorporation of unstimulated cultures. A vigorous EIAV-specific lymphoproliferative response was observed 3 weeks postinfection in PBMC from each infected pony (Fig. 6B to E). No detectable EIAV-specific lymphoproliferation was observed in the PBMC of the control uninfected pony at any time during the study (Fig. 6A). Detectable EIAV-specific lymphoproliferative responses were maintained ex vivo from the infected ponies throughout the entire period of observation, which demonstrated a continuous stimulation of the cell-mediated arm of the immune system by the viral infection. The responding T cells which proliferated upon stimulation with EIAV-infected macrophage were of both the $CD4^+$ and $CD8^+$ subsets (data not shown). However, a majority (>85%) of the responding T cells were of the $CD4^+$ subset as determined by indirect immunofluorescence of the responding cultures (data not shown).

(ii) EIAV-specific CTL response. We analyzed the EIAV-specific CTL response induced in the host after infection, with an emphasis on the potential role that EIAV-specific CTL may have performed as an immune correlate of protection. EIAV-specific cytolytic activity was analyzed in a standard 8-h ^{51}Cr release assay against target cells expressing both MHC classes I and II to measure contributions of both $CD4^+$ and $CD8^+$ CTL, as described in Materials and Methods. Effector T cells were derived from PBMC collected from EIAV-infected ponies and a control uninfected pony. Target cells used in the ^{51}Cr-release assays were autologous PWM blasts infected with

a control recombinant vaccinia virus vector (vac) or recombinant vaccinia virus vectors carrying the EIAV *gag/pro* gene (vac-gag) or the full-length EIAV *env* gene (vac-env). Minor amounts of direct ex vivo EIAV-specific cytolytic activity were observed in our system but only during the days immediately preceding and immediately following a viremic febrile episode (data not shown). This observation was in marked contrast to that of McGuire et al. (40) and Fujimiya et al. (18), who each readily demonstrated, using 17- or 34-h ^{51}Cr release assays, respectively, ex vivo EIAV-specific cytolytic activity preceding and several weeks following the primary viremic febrile episode.

To analyze the CTL response induced by EIAV, an in vitro stimulation mediated by EIAV-infected autologous macrophages was used to activate and expand the antigen-specific memory T cells isolated from the peripheral blood of infected ponies. Responding T cells were tested for lytic activity against autologous target cells expressing control or EIAV antigens. EIAV gag- and/or envelope-specific cytolytic activity was observed for each pony immediately following the initial clinically defined viremic febrile episode (Fig. 7B to E). No EIAV-specific cytolytic activity was measured at any time during the study by using in vitro antigen-stimulated PBMC from the noninfected control pony (Fig. 7A). After the initial appearance of measurable cytolytic activity, EIAV gag- and/or envelope-specific cytolytic activity differed considerably between the ponies. Pony 561 was able to bring the EIAV infection under control after the initial viremic febrile episode with little, if any, EIAV-specific CTL activity detectable after 4 weeks postinfection (Fig. 7B). Pony 562 was also able to resolve the initial viremic febrile episode, but in marked contrast to pony 561, maintained a vigorous EIAV gag- and envelope-specific CTL response throughout the entire period of study (Fig. 7C). Pony 564, which cycled through four viremic febrile episodes during the study period, intermittently maintained a strong EIAV gag- and envelope-specific CTL response, increasing for the first 6 months postinfection and declining rapidly until no EIAV-specific CTL activity could be detected after 8 months postinfection (Fig. 7D). Interestingly, the loss of EIAV-specific CTL in the PBMC of pony 564 corresponded to the reduction in EIAV-specific neutralizing-antibody reciprocal titer at 6 months postinfection (Fig. 5). Pony 567, which cycled multiple times throughout the study period, never resolving the viral infection, had only intermittent levels of envelope-specific CTL activity (Fig. 7E). Only a minimal EIAV gag-specific CTL response was detected 8 to 10 months postinfection by antigen-stimulated PBMC from pony 567.

(iii) CD4 and CD8 subset characterization of the EIAV-specific effector CTL. Lentivirus-infected cells present gag antigens predominantly in an MHC class I-restricted manner to CD8$^+$ T cells (20, 70). Furthermore, the characterized antigen-processing pathways for presentation of enveloped viral antigens to T cells provide mechanisms by which both MHC class I-restricted CD8$^+$ and MHC class II-restricted CD4$^+$ CTL may be induced upon EIAV infection (17, 20, 23, 24, 66, 70). It is not surprising, then, that the induction of both equine CD4$^+$ and CD8$^+$ MHC-restricted CTL to viral antigens has been documented (1, 40). However, it is still unclear which individual antigens are responsible for the recognition and lysis by each T-cell subset in the equine herpesvirus and EIAV systems. Ex vivo EIAV-specific, MHC-restricted CD8$^+$ CTL have been documented (40), but the presence of both EIAV-specific memory CD8$^+$ and CD4$^+$ CTL has yet to be demonstrated.

We sought to determine the phenotype of the EIAV gag- and envelope-specific CTL by depleting the CD8$^+$ or CD4$^+$ T cells from the effector population used in standard ^{51}Cr release assays. In vitro EIAV-infected, macrophage-stimulated PBMC from an infected pony were undepleted, CD4$^+$ T-cell depleted, or CD8$^+$ T-cell depleted by the method of indirect panning as described in Materials and Methods. These effector T-cell pop-

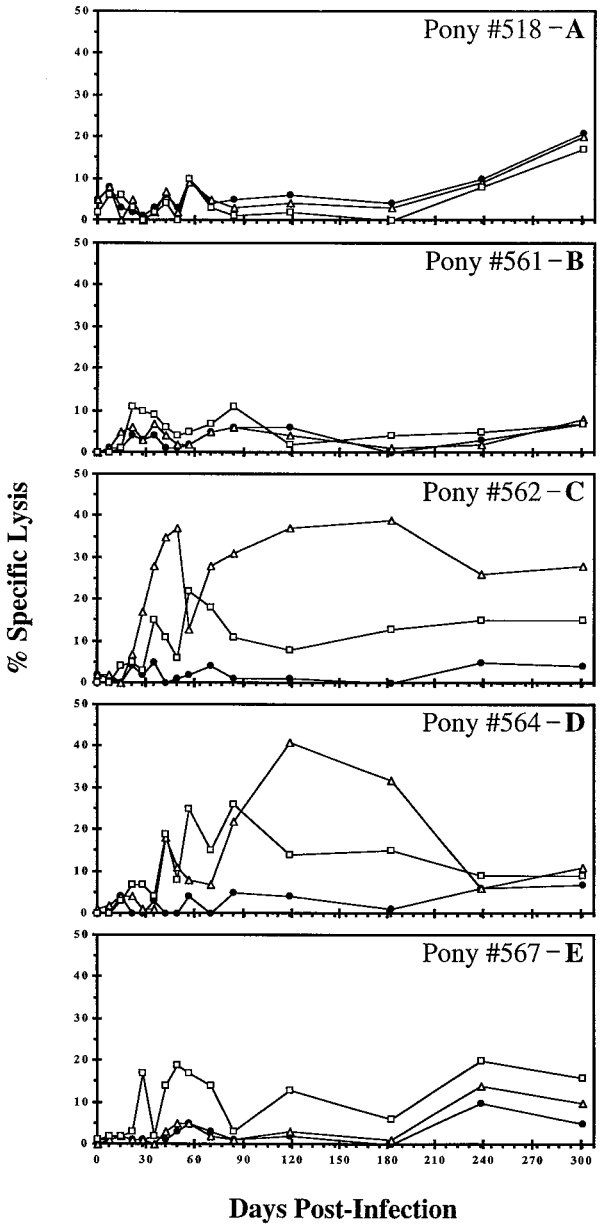

FIG. 7. EIAV envelope glycoprotein- and EIAV gag protein-specific cytolytic activity mediated by in vitro-stimulated PBMC isolated from uninfected and infected ponies. At 8 days after an initial in vitro stimulation of PBMC from uninfected and infected ponies with EIAV$_{Pr}$-infected autologous macrophages, responding cultures were assayed for cytolytic activity against autologous PWM lymphoblasts that had been infected with a control vaccinia virus vector (vac) (●), infected with a vaccinia virus vector carrying the EIAV$_{Pr}$ gag/pro sequences (vac-gag) (△), or infected with a vaccinia virus vector carrying the EIAV$_{WSU5}$ envelope glycoprotein sequences (vac-env) (□). The effector cells were stimulated PBMC from a control uninfected pony 518 (A), stimulated PBMC from infected pony 561 (B), stimulated PBMC from infected pony 562 (C), stimulated PBMC from infected pony 564 (D), and stimulated PBMC from infected pony 567 (E). Stimulated PBMC were tested in quadruplicate at effector-to-target-cell ratios of 30:1 giving optimal lysis. The standard error of the mean percent specific lysis was always <3%.

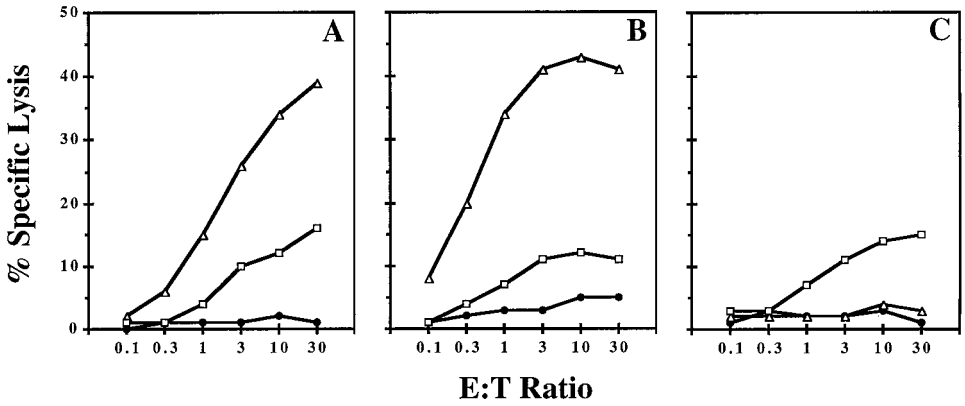

FIG. 8. EIAV-specific cytolytic activity is mediated by both $CD4^+$ and $CD8^+$ subsets of T lymphocytes. At 8 days after an initial in vitro stimulation of infected pony 562 PBMC with $EIAV_{Pr}$-infected autologous macrophages, the responding culture was fractionated as described in Materials and Methods by the method of indirect panning into three subsets: unfractionated antigen-stimulated PBMC (A), CD4-depleted antigen-stimulated PBMC (B), and CD8-depleted antigen-stimulated PBMC (C). The antigen-stimulated PBMC were used as effector cells and tested for lytic activity against target cells over a wide range of effector-to-target-cell (E:T) ratios. Target cells were autologous PWM lymphoblasts that had been infected with a control vaccinia virus vector (vac) (●), infected with a vaccinia virus vector carrying the $EIAV_{Pr}$ gag/pro sequences (vac-gag) (△), or infected with a vaccinia virus vector carrying the $EIAV_{WSU5}$ envelope glycoprotein sequences (vac-env) (□). The standard error of the mean percent specific lysis was always <3%.

ulations were assessed for cytolytic activity over a wide range of effector-to-target-cell ratios against autologous target cells expressing gag or envelope from recombinant vaccinia virus vectors (Fig. 8). Cytolytic activity mediated by undepleted antigen-stimulated PBMC was specific for both gag- and envelope-expressing autologous target cells (Fig. 8A). Antigen-stimulated $CD4^+$ T-cell-depleted PBMC also recognized and lysed both gag- and envelope-expressing autologous target cells (Fig. 8B). In contrast, antigen-stimulated $CD8^+$ T-cell-depleted PBMC recognized and lysed only autologous target cells expressing envelope glycoprotein (Fig. 8C). These results clearly demonstrated that both $CD4^+$ and $CD8^+$ memory CTL specific for EIAV antigens were induced during the viral infection in ponies. More specifically, $CD8^+$ CTL recognized and lysed both gag- and envelope-expressing target cells whereas $CD4^+$ CTL recognized and lysed only envelope-expressing target cells.

(iv) MHC restriction analysis of the EIAV-specific effector CTL. MHC restriction of EIAV-specific $CD4^+$ and $CD8^+$ memory CTL was examined in a standard ^{51}Cr release assay with autologous and nonautologous target cells expressing EIAV antigens via recombinant vaccinia virus vectors. The target cells were typed by serological methods as described in the Materials and Methods for the ELA-A locus, the equine equivalent of the human MHC class I A locus (Table 1). ELA-A serological typing demonstrated that at least at this one locus, the nonautologous target cells were MHC mismatched. Unfractionated PBMC activated and expanded with autologous EIAV-infected macrophages, recognized and lysed in an antigen-specific manner only autologous and not nonautologous target cells expressing gag and envelope (Fig. 9A). Similarly, $CD4^+$ T cell-depleted PBMC activated in vitro and expanded with autologous EIAV-infected macrophages demonstrated similar recognition and lysis of autologous and nonautologous antigen-expressing target cells as the aforementioned unfractioned PBMC (Fig. 9B). $CD8^+$ T-cell-depleted PBMC activated in vitro and expanded with autologous EIAV-infected macrophages specifically recognized and lysed only autologous target cells expressing envelope protein but not gag protein (Fig. 9C). Although there was significant lysis of nonautologous target cells by all effector cell populations, this lysis was alloreactive and not antigen specific. Thus, MHC restriction was demonstrated by the specific recognition and lysis of only autologous target cells by the $CD4^+$- and $CD8^+$-enriched CTL in an antigen-specific manner, in agreement with the above data in Fig. 8 and the known mechanisms of antigen processing and presentation for viral gag protein and envelope glycoprotein. Therefore, the EIAV-specific recognition and lysis observed by the effector T cells was mediated by MHC class I-restricted $CD8^+$ CTL and MHC class II-restricted $CD4^+$ CTL.

DISCUSSION

EIAV provides a unique natural model for immunological management of lentivirus replication. Using this system, we present for the first time a comprehensive analysis of both the humoral and cellular immune responses generated to EIAV, including new analyses of antibody avidity, antibody epitope conformational dependence, and CTL activity that have not been previously examined. The data presented here provide a framework for describing the evolution of protective immune responses to EIAV and for identifying potential immune correlates of protection to this lentivirus that could facilitate candidate vaccine design and evaluations.

The serological assays described here reveal a complex and lengthy evolution of antibody responses to EIAV infection that was not previously evident from studies of neutralizing antibodies (8, 11, 22, 49, 58). The relatively gradual evolution of EIAV envelope-specific antibody avidity and conformational dependence observed over the first 6 to 8 months postinfection is remarkably similar to the time required for antibody avidity and conformation dependence to achieve a steady state in SIV-infected rhesus macaques (14). The kinetics of antibody avidity maturation in EIAV and SIV infections are similar in nature. Both EIAV- and SIV-specific antibodies gradually mature over the first 6 to 8 months from low avidity to reach a steady-state level of intermediate to high avidity. Furthermore, while the kinetics of conformational dependence in EIAV and SIV infection are similar, the nature of this maturation in each system is distinct. In the case of EIAV, the antibody specificity progresses from linear to conformational envelope determinants. In contrast, SIV envelope-specific antibodies are initially directed overwhelmingly to conformational determinants. Antibodies to linear determinants become more prevalent during

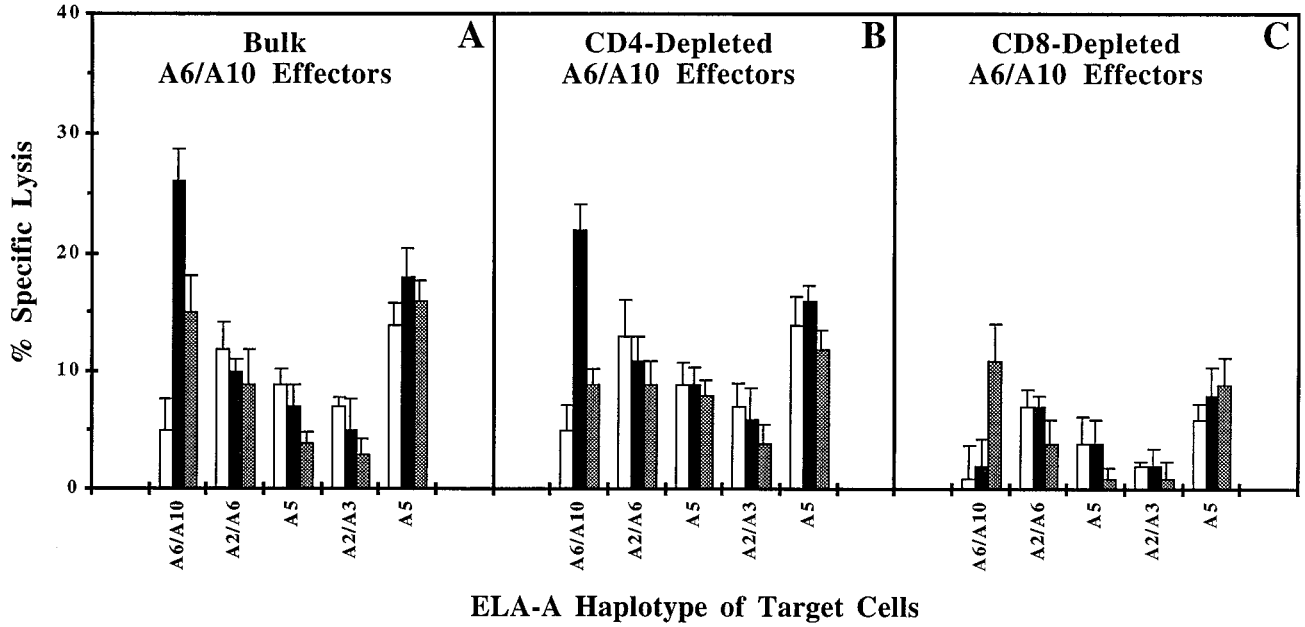

FIG. 9. EIAV-specific $CD4^+$ and $CD8^+$ T lymphocyte cytolytic activity is MHC restricted. At 8 days after an initial in vitro stimulation of infected pony 562 (ELA-A haplotype of A6/A10 [Table 1]) PBMC with $EIAV_{Pr}$-infected autologous macrophages, the responding culture was fractionated as described in Materials and Methods by the method of indirect panning into three subsets: unfractionated antigen-stimulated PBMC (Bulk) (A), CD4-depleted antigen-stimulated PBMC (CD4-depleted) (B), and CD8-depleted antigen-stimulated PBMC (CD8-depleted) (C). Stimulated PBMC were tested at effector-to-target-cell ratios giving optimal lysis (30:1 for bulk antigen-stimulated PBMC, 3:1 for CD4-depleted antigen-stimulated PBMC, and 30:1 for CD8-depleted antigen-stimulated PBMC). Target cells were nonautologous and autologous PWM lymphoblasts that had been infected with a control vaccinia virus vector (vac) (□), infected with a vaccinia virus vector carrying the $EIAV_{Pr}$ gag/pro sequences (vac-gag) (■), or infected with a vaccinia virus vector carrying the $EIAV_{WSU5}$ envelope glycoprotein sequences (vac-env) (▨). The ELA-A haplotypes of the autologous and nonautologous target cells are presented on the *x* axis for each group of effectors. *y*-axis error bars represent the standard error of the mean percent specific lysis.

the initial 6 to 8 months postinfection, as evidenced by steady decreases in the conformation index values. Interestingly, both infections reach steady-state conformation index values of about 2.0.

Efficacious EIAV-specific neutralizing antibody to the infecting strain of virus emerges only after the first 2 to 3 months postinfection, in agreement with previous observations in EIAV (8, 11, 22, 49, 58) and HIV-1 (34, 45). Lengthy neutralizing-antibody maturation kinetics are not observed in rhesus macaques experimentally infected with live attenuated SIV (14). Neutralizing antibody in the SIV system rapidly emerges 2 to 3 weeks postinfection. In SIV and HIV-1, neutralizing-antibody levels reach a steady-state level (dilutions of around 1/1,000). This is in contrast to EIAV, in which the neutralizing-antibody levels appear to fluctuate markedly during the course of infection. The reasons for the observed fluctuation are currently under investigation.

The results from this analysis of the immune response to infection by EIAV and the results documented by analyzing the immune response to infection by SIV (14, 15) suggest that maturation of the immune response may be common to infections by lentiviruses, including HIV-1. It may be important to characterize immune maturation in more detail, because other immunologic factors, in addition to avidity and conformation dependence, may be evolving. Elucidation of the maturation process may be important to define the immune status to be achieved by candidate vaccines and to design immunization regimens that adequately mature immune responses.

In contrast to the lengthy EIAV-specific antibody maturation kinetics, an effective EIAV-specific CTL response emerges just weeks postinfection. Previous studies have demonstrated the rapid development of EIAV-specific CTL in animals 2 to 4 weeks postinfection (18, 40). It has been postulated that the rapid production of virus-specific CTL early in the infection manages to impede virus replication, allowing the humoral arm of the immune system the time necessary to respond effectively (71). EIAV-specific T-cell evolution to the infection is observed by the fluctuations in measurable levels of in vitro EIAV-specific T-cell proliferation and cytolysis at the various time intervals. The fluctuations or even loss of EIAV-specific CTL activity may result from mutations in dominant viral epitopes, demonstrating the striking ability of the virus to alter antigenic sites. Indeed, even one amino acid alteration in a T-cell epitope can abate the association of the epitope with MHC molecules or recognition by a T cell (54). Mutations in T-cell epitopes can have a dramatic effect on the direction in which the effector and memory cell-mediated responses evolve. Evolution of memory T cells to viral antigens may be driven or maintained by persistent (21, 48) or cross-reactive (61) antigen. In contrast to these studies, some viral systems do not require a continuous supply of antigen to maintain an effective T-cell memory response (35, 46). From our observations with the loss of recognition of envelope and gag CTL epitopes (Fig. 7D and E), cell-mediated immune responses to EIAV appear to require a persistent supply of antigen to maintain recall ability. Interestingly, the loss of EIAV-specific T-cell proliferative or cytolytic activity in vitro did not correlate with the emergence of new viremic febrile episodes.

The mechanism(s) which prevents the host immune response from evolving rapidly in response to lentivirus infections is unknown. This is especially intriguing considering the following: (i) the high rate at which EIAV may replicate, most specifically during the viremic febrile episodes; (ii) the continuous presentation of lentivirus antigen to the immune system

by this persistent infection; and (iii) the viral replication in professional antigen-presenting cells, namely, macrophages (62), which can be found in intimate contact with B cells in the marginal zone of the spleen, where the B cells are most likely to first encounter any antigen (10). The prolonged maturation process of the humoral response may result from proposed immunosuppressive effects mediated by elements of EIAV (47). Indeed, elements of HIV have also been implicated to possess immunosuppressive effects (13, 57). In evidence against this hypothesis is the observation that all infected ponies rapidly seroconvert to EIAV antigens and produce maximal antibody levels to these antigens only a few weeks after the emergence of the initial EIAV-specific antibody. EIAV and other lentiviruses may also subvert the antibody maturation process through antigenic variation of recognized B-cell epitopes (43). It is conceivable that the immune system may not keep pace with the extreme rate of antigenic variation, thereby preventing rapid affinity maturation of virus-specific antibody. However, the observations that (i) maturation of SIV envelope-specific antibody occurs in the absence of envelope sequence variation (14) and (ii) antibody specific for EIAV p26, which does not mutate to the same extent as EIAV envelope (43), matures at the same rate as envelope-specific antibody argue against sequence variation prolonging the antibody maturation process in the lentivirus infections. Further complicating the maturation process are the high levels and complex nature of the N- and O-linked carbohydrate attached to envelope glycoprotein-producing immunorecessive antibody epitopes (36, 51). Most interestingly, p26 does not possess the high levels of covalently attached N- and O-linked carbohydrate, yet p26- and envelope glycoprotein-specific antibody avidity maturation rates are equivalent for all test subjects. This observation seems to potentially negate steric hindrance by carbohydrate as a major factor preventing the humoral immune response from maturing rapidly in lentivirus infections. Overall, a combination of effects produced by the complex nature of the EIAV infection process itself and/or the tropism of the virus may modulate antibody maturation to the lentivirus antigens.

Based on our previous studies of antibody responses to SIV infection, we have proposed that lentiviruses may have evolved "stealth antigens" that elude or delay effective immune surveillance during the early stages of infection (14, 15). The current studies of EIAV-specific antibody responses support this hypothesis, suggesting that a delayed maturation of antibody responses to viral antigens may be a previously unrecognized mechanism of lentivirus persistence. The delayed maturation of lentivirus antibody responses is in distinct contrast to the observed rapid maturation of antibody responses of only weeks postinfection in nonlentivirus infections such as hepatitis C virus and varicella-zoster virus infections (30, 60, 69). The presence of only low-avidity antibody during the first 6 months postinfection could also serve to amplify the initial virus infection via antibody-dependent enhancement of a relatively low virus exposure. In this regard, the highest levels of enhancing antibodies to HIV-1 and SIV have been identified during the early stages of infection (19, 39, 40), the period shown here to be associated with the presence of low-avidity antibody to lentivirus envelope proteins.

While the present studies elucidate the evolution of a variety of immune responses to EIAV infection, the data do not indicate any single correlate of cellular or humoral immune control of persistent EIAV infection and disease. The emergence of virus-specific CTL in the absence of high avidity and neutralizing antibody temporally correlated with the resolution of the initial viremia after EIAV infection, as observed in other lentivirus systems (9, 34, 40). However, the detection of CTL in the infected ponies did not reliably correlate with the ability of the animals to prevent subsequent viremic febrile episodes resulting from the emergence of novel antigenic variants. Likewise, analyses of the humoral immune response to EIAV failed to provide single correlates of protection or control among the serological parameters addressed in this study. All infected ponies seroconverted to EIAV in temporal conjunction with the clearance of the acute viremia and fever. The nature of the antibody population (low avidity, nonneutralizing, and linear epitope dominant) present in the infected ponies early in the infection process does not correlate with potentially advantageous parameters (high avidity and neutralizing) of protective humoral responses to viral infection. Indeed, the mere presence of EIAV-specific serum antibodies did not prevent further viremic febrile episodes from occurring. However, the early immature EIAV-specific antibody may increase the amount of virus cleared from the animal through opsonization of virus-antibody aggregates. Although not addressed in this study, antibody-dependent cell-mediated cytotoxicity has not been shown to correlate with resolution of viremia in EIAV (68). Increasing antibody avidity to EIAV envelope and gag antigens and maturation of EIAV envelope-specific antibody from predominantly linear-epitope specific to principally conformational-epitope specific did not necessarily relate to immune control of the viral infection. Furthermore, a lack of correlation between the levels of EIAV-specific neutralizing antibody and protection in experimentally infected and immunized horses has been reported previously (29, 43, 58), in agreement with our results. Taken together, humoral immune response maturation to EIAV does not necessarily ensure protection, and correlates for immune control are not obviously apparent by using the given parameters and limited number of test subjects. In this regard, a lack of reliable humoral correlates has also been reported for HIV-1 infection of humans (45). Thus, the need exists to examine additional aspects of immune responses to lentivirus infections to identify parameters that can be used alone or in combination as correlates of protection.

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