JOURNAL OF VIROLOGY, July 2000, p. 5968–5981 Vol. 74, No. 13
0022-538X/00/$04.00+0

Immune Responses and Viral Replication in Long-Term Inapparent Carrier Ponies Inoculated with Equine Infectious Anemia Virus

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Received 21 July 1999/Accepted 10 April 2000

Persistent infection of equids by equine infectious anemia virus (EIAV) is typically characterized by a progression during the first year postinfection from chronic disease with recurring disease cycles to a long-term asymptomatic infection that is maintained indefinitely. The goal of the current study was to perform a comprehensive longitudinal analysis of the course of virus infection and development of host immunity in experimentally infected horses as they progressed from chronic disease to long-term inapparent carriage. We previously described the evolution of EIAV genomic quasispecies (C. Leroux, C. J. Issel, and R. C. Montelaro, J. Virol. 71:9627–9639, 1997) and host immune responses (S. A. Hammond, S. J. Cook, D. L. Lichtenstein, C. J. Issel, and R. C. Montelaro, J. Virol. 71:3840–3852, 1997) in four experimentally infected ponies during sequential disease episodes associated with chronic disease during the first 10 months postinfection. In the current study, we extended the studies of these experimentally infected ponies to 3 years postinfection to characterize the levels of virus replication and development of host immune responses associated with the progression from chronic disease to long-term inapparent infection. The results of these studies revealed over a 10^3-fold difference in the steady-state levels of plasma viral RNA detected during long-term inapparent infection that correlated with the severity of chronic disease, indicating different levels of control of virus replication during long-term inapparent infections. Detailed analyses of antibody and cellular immune responses in all four ponies over the 3-year course of infection revealed a similar evolution during the first year postinfection of robust humoral and cellular immunity that then remained relatively constant during long-term inapparent infection. These observations indicate that immune parameters that have previously been correlated with EIAV vaccine protection fail to provide reliable immune correlates of control of virus replication or clinical outcome in experimental infections. Thus, these data emphasize the differences between immunity to virus exposure and immune control of an established viral infection and further emphasize the need to develop and evaluate novel immunoassays to define reliable immune correlates to vaccine and infection immunity, respectively.

Equine infectious anemia virus (EIAV) infection of horses provides a novel system in which to examine the natural immunological control of lentivirus replication and disease (reviewed in reference 27). Horses infected in the field or experimentally with EIAV typically develop within the first month postinfection acute disease (fever, diarrhea, lethargy, anemia, and thrombocytopenia) and an associated high level of infectious plasma viremia. Following this initial clinical episode that lasts 3 to 5 days, most infected horses experience recurring disease episodes and associated waves of viremia at irregular intervals. This cyclic disease is designated chronic EIA. The frequency of disease episodes and the severity of clinical symptoms typically decrease with time and are usually completely resolved by 1 year postinfection. At this time, persistently infected horses become clinically asymptomatic for EIA and negative for infectious plasma viremia, indicating a highly effective control of virus replication and disease. In fact, most horses infected by EIAV are inapparent carriers that will remain asymptomatic for the remainder of their life span of up to 20 years. Thus, the EIAV system offers a uniquely dynamic model in which to examine changes in viral replication and host immune responses during the clearly demarcated progression from chronic disease to a long-term inapparent infection.

A number of studies indicate that the eventual control of EIAV replication and disease in horses is mediated by host immune responses that control virus infection to subclinical levels and not by the attenuation of the virus during persistent infection. For example, transfer of whole blood from long-term inapparent carriers to naive horses reproducibly causes infection and disease (11), and experimental immune suppression of inapparent carriers can cause recrudescence of disease and associated viremia (19, 43). Recent analyses of EIAV infection in long-term apparent carriers by genetic (8, 40) and in situ (29) methods demonstrate persistent low levels of virus infection and replication predominantly in tissue macrophages, with negligible virus detectable in plasma or peripheral blood cells. These studies indicate that the progression from chronic EIA to inapparent infection is associated with the evolution of highly effective and enduring host immune responses that are able to suppress EIAV replication, despite the array of persis-

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tence and escape mechanisms employed by this virus. A major goal of EIAV research during the past decade has been to elucidate the specificity of the humoral and cellular immune responses that achieve control of virus replication in inapparent carriers. This information then can provide immunological goals for EIAV vaccine development and serve as a model to guide the design of vaccine strategies for other animal and human lentiviruses.

To date, there have been only limited analyses of the development of host immune responses to experimental EIAV infection, and most of these studies have focused on the development of antibody and cellular immune responses during chronic EIA, with only limited cross-sectional analyses of long-term inapparent carriers. In general, these studies indicate that chronic disease is associated with the rapid development of high-titer broadly neutralizing serum antibodies (28, 31, 32) and robust cellular immunity (6, 25, 45), but not with the presence of antibody-dependent cellular cytotoxicity (41, 42). While these analyses identify various immune responses to EIAV infection, they do not define specific immune mechanisms responsible for the resolution of individual disease cycles or for the progression to long-term asymptomatic infections. An additional limitation of these EIAV immunology studies is the lack of a comprehensive longitudinal characterization of the evolution of humoral and cellular immune responses in a single set of experimentally infected ponies during the progression from chronic EIA to long-term asymptomatic infection.

To perform a comprehensive longitudinal analysis of host immune responses to EIAV infection, we initiated an experiment to characterize in detail virus infection and host immune responses in a group of four ponies experimentally infected with our reference $EIAV_{PV}$ strain (6, 20). Two of the experimentally infected ponies experienced multiple disease cycles characteristic of chronic EIA, while two ponies became asymptomatic after the initial acute disease episode. Thus, the four experimental infections fortuitously separated clinically into two distinct groups, providing a novel opportunity to assess the evolution of the virus infection and host immunity during very different time frames for the establishment of long-term asymptomatic infections. We previously reported on the evolution of EIAV quasispecies during sequential febrile episodes associated with chronic EIA in one of the experimentally infected ponies (20). In addition, we characterized in detail the development of humoral and cellular immune responses in all four experimentally infected ponies during the first 10 months postinfection to elucidate the changes in host immunity during chronic EIA (6). The results of these studies revealed for the first time a similar complex and lengthy maturation of humoral and cellular immune responses during the first 10 months postinfection in all four ponies, regardless of the clinical course of the infection. This maturation of immune responses to EIAV appears to be common to the early stages of lentivirus infections, including simian immunodeficiency virus (SIV) or simian-human immunodeficiency virus infection of monkeys and human immunodeficiency virus type 1 (HIV-1) infection of humans (2, 3). Moreover, we have demonstrated further that the serological parameters that define mature and immature immune responses can also be useful in distinguishing protective and nonprotective immune responses to experimental EIAV (7) and SIV (3) vaccines.

While these studies provide fundamental information on the development of EIAV-specific immune responses during the early stages of persistent infection and chronic EIA, they do not address the important issue of the nature of immune responses that are associated with the enduring suppression of virus replication and disease in long-term inapparent carriers. Thus, we have continued to monitor virus infection and to analyze antibody and cellular immune responses in the four experimentally infected ponies for up to 3 years postinfection. We describe here the first comprehensive longitudinal study of persistent EIAV infection in experimentally infected ponies during the progression from chronic disease to maintenance of long-term inapparent infections. The results of these longitudinal studies over a 3-year period reveal fundamental new information about the steady-state levels of EIAV replication in inapparent carriers, the kinetics of immune maturation to persistent infection, and the nature of the host immunity associated with long-term asymptomatic infections.

MATERIALS AND METHODS

Experimental subjects. All animals in this study were outbred, mixed-breed ponies and maintained as previously documented (12). The early chronic stages of infection by EIAV in these ponies have been detailed previously (6). Clinical EIA episodes were determined by clinical impressions (temperature and platelet count) in combination with the presence of infectious plasma viremia (6, 44).

Virus strains. Three reference strains of EIAV were utilized 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 (23). The $EIAV_{PV}$ biological clone is a pathogenic and antigenic variant derived from $EIAV_{Pr}$ (37) and used as a standard challenge in vaccine trials (5, 12, 33, 44). $EIAV_{WSU5}$ is a virulent strain of EIAV generated using procedures described elsewhere to produce $EIAV_{PV}$ (25). $EIAV_{PV}$, $EIAV_{Pr}$, and $EIAV_{WSU5}$ envelope glycoproteins are very closely related, having <1% divergence at the amino acid level.

Immunological analyses. Concanavalin A (ConA) enzyme-linked immunosorbent assay (ELISA) procedures for analyzing antibody titer, avidity, and conformational dependence have been described in detail previously (6). Assays for measuring EIAV-specific neutralizing antibody activity, lymphoproliferation, and cytolytic T lymphocytes (CTL) were conducted as previously detailed (6). Isotyping of the EIAV-specific serum antibodies were conducted as described for the ConA ELISA listed in reference 6, except that the secondary antibodies used were polyclonal goat serum coupled with horseradish peroxidase and having specificity for equine immunoglobulin Ga (IgGa), IgGb, IgGc, IgG(T), or IgM (Bethyl Laboratories, Montgomery, Tex.).

Immunoadsorption of isotypic antibodies. Removal of equine IgGa and/or IgGb from experimental serum samples was performed using Sepharose beads covalently coupled with sheep anti-horse IgGa (4.55 mg of IgG/ml of gel) or sheep anti-horse IgGb (3.8 mg of IgG/ml of gel) purchased from Bethyl Laboratories. Briefly, 250 μl of immunosorbents was added to 700 μl of each serum sample at room temperature for 30 min. Beads were pelleted, and the supernatant of serum was transferred to a fresh tube. The addition of immunosorbent to serum was performed seven times to effectively remove the isotypic IgG from each sample. ConA ELISAs were conducted to confirm the removal of each isotypic IgG and to measure the remaining EIAV-specific antibody levels for IgG, IgGa, and IgGb.

Quantitation of virus RNA levels in plasma. Semiquantitative measurements of viral genomic RNA molecules present per milliliter of plasma were conducted as described previously (21). Briefly, plasma virus pelleting by ultracentrifugation and viral RNA extraction using RNAzol were performed (22). A single-tube reaction for cDNA synthesis and PCR amplification using the Promega Access reverse transcription-PCR (RT-PCR) system (Promega) was utilized. The RT-PCRs were conducted using 4 μl of plasma viral RNA sample as directed by the manufacturer, using the EIAV *gag*-specific primer Gag 34 (GCTGACTCTTCT GTTGTATCG) for both RT and PCR and an EIAV *gag*-specific primer, Gag 11 (ATGTATGCTTGCAGAGACATTG), for PCR only. First-strand cDNA synthesis occurred for 45 min at 48°C, and denaturation occurred at 94°C for 2 min. Second-strand cDNA synthesis and DNA amplification used the following cycle conditions: 30 s at 94°C, 30 s at 60°C, and 30 s at 68°C for 40 cycles; 7 min at 68°C for 1 cycle; and holding at 4°C. RT-PCR products were separated by electrophoresis in a 2% agarose gel. Gels were stained in pH 8.0 Tris-acetate-EDTA buffer containing a dilution of 1:10,000 SYBR Green I stock solution (Molecular Probes, Eugene, Oreg.) for 45 min. The intensity of each band was quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) and the analytical software ImageQuant (Molecular Dynamics). Estimation of the number of viral genomic RNA molecules per milliliter of plasma was based on linear regression analysis of a standard curve on known amounts of synthetic RNA prepared by in vitro transcription with a T7 MEGAscript kit (Ambion, Austin, Tex.). The standard RNA curve was linear in the range of 100 molecules as a lower limit and 10^6 molecules as an upper limit.

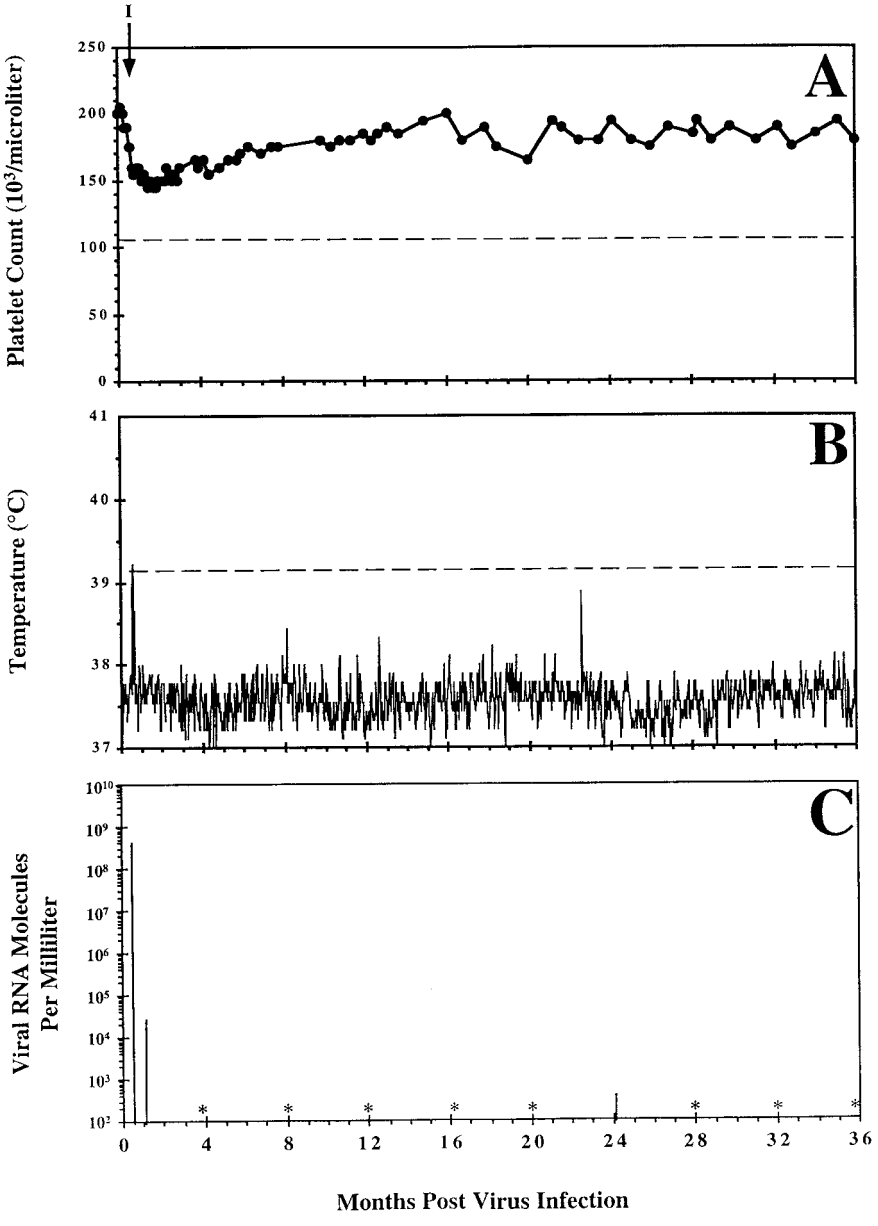

FIG. 1. Clinical course and dynamics of virus replication in pony 561 experimentally infected with EIAV. Shown are platelet counts per microliter of whole blood (A), daily rectal temperatures (B), and virus RNA molecules per milliliter of plasma (C) from pony 561 experimentally infected with EIAV. The pony was experimentally infected with 10^3 $TCID_{50}$ of $EIAV_{PV}$ on day 0 and observed for 3 years. Rectal temperatures in excess of 39.2°C, as denoted by the dashed line in panel B, were considered EIA episodes only in conjunction with a reduction in the number of circulating platelets and with the presence of infectious virus cultured from plasma collected during the febrile episode (Table 1). Infectious virus could be cultured only during EIA episodes and not during the asymptomatic periods of the infection. The dashed line in panel A marks the platelet count, 105,000/μl, at which thrombocytopenia has been clinically defined. The single EIA febrile episode is marked with an arrow in panel A and identified with roman numeral I. Viral RNA molecules per milliliter of plasma were quantitated for a single day every 4 months postinfection and during the EIA-related febrile episode. Viral RNA levels below 100 copies per ml are indicated with asterisks.

RESULTS

Experimental EIAV infection of outbred ponies. Four outbred ponies were experimentally infected with 10^3 50% tissue culture infective doses ($TCID_{50}$) of $EIAV_{PV}$. The clinical progression and immunological analyses of these experimental infections during the early acute and chronic stages of disease have been documented up to 10 months post-virus infection (6). We have continued to monitor these animals for up to 3 years after infection. The results of these long-term observations revealed that, after resolution of the initial febrile episode by 3 weeks postinfection, the ponies separated into two groups based on the clinical observations of temperature and platelet count. Two ponies, 561 and 562, were observed to have only one EIA-related clinical episode, at about 17 days postinfection, and then remained clinically asymptomatic throughout the rest of the study period (Fig. 1B and 2B and Table 1). The remaining two ponies, 564 and 567, had recurring fevers, typical of chronic EIA, with each pony experiencing a total of six fevers within 1 to 2 years postinfection (Fig. 3B and 4B and Table 1). Pony 564 cycled through six EIA episodes within a

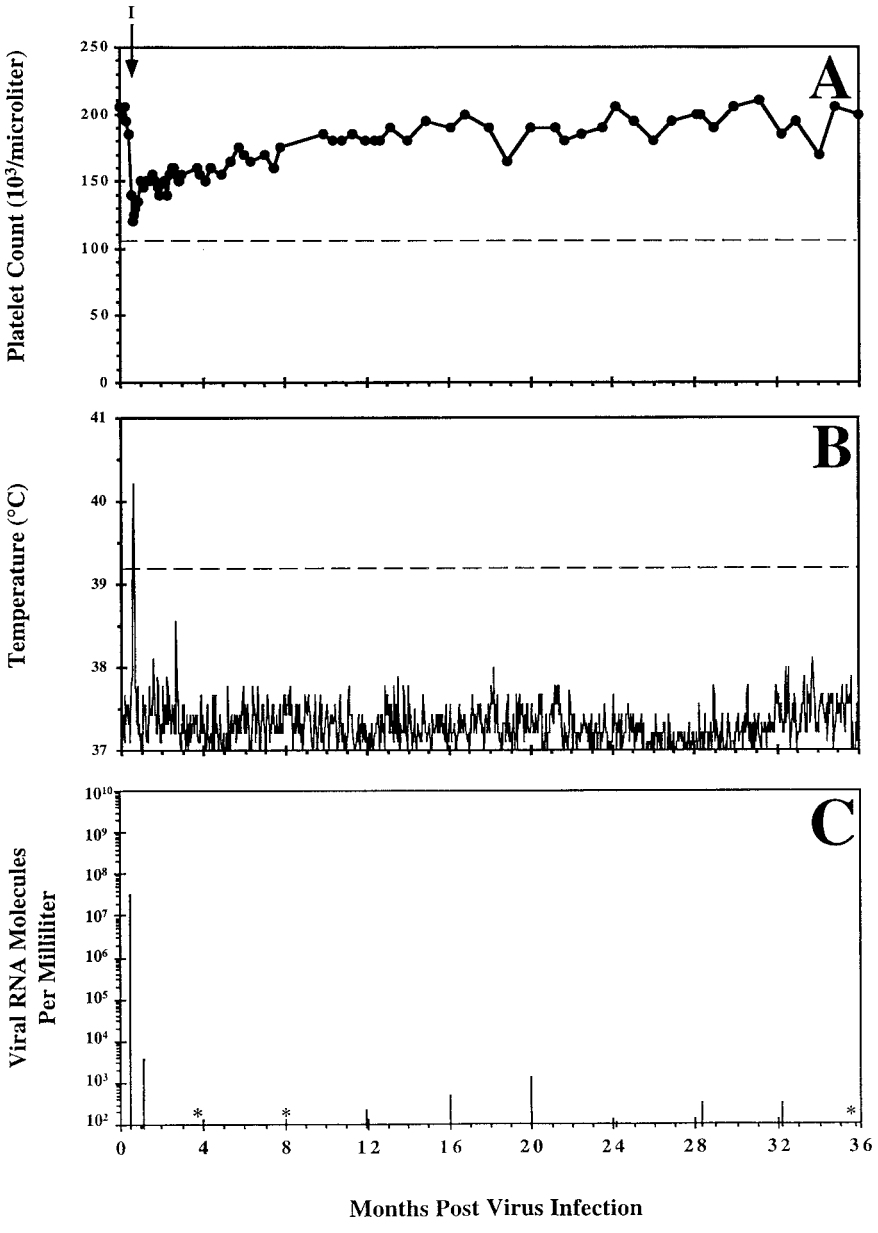

FIG. 2. Clinical course and dynamics of virus replication in pony 562 experimentally infected with EIAV. Shown are platelet counts per microliter of whole blood (A), daily rectal temperatures (B), and virus RNA molecules per milliliter of plasma (C) from pony 562 experimentally infected with EIAV. The single EIA febrile episode is marked with an arrow in panel A and identified with roman numeral I. See the Fig. 1 legend for criteria used to designate an EIA-related disease episode. Viral RNA molecules per milliliter of plasma were quantitated for a single day every 4 months postinfection and during the EIA-related febrile episode. Viral RNA levels below 100 copies per ml are indicated with asterisks.

13-month period and had fevers occurring on days 18, 34, 80, 106, 336, and 377 post-virus infection. Pony 564 was asymptomatic the last 23 months of observation. Pony 567 had six clinical episodes associated with EIA occurring over a 2-year span and on days 19, 40, 223, 258, 640, and 729 post-virus infection. Pony 567 was asymptomatic for the last 12 months during this study. All EIA febrile episodes listed above coincided with characteristic thrombocytopenia (panels A in Fig. 1 to 4) and the ability to isolate infectious virus from plasma ($10^{3.5}$ to $10^{5.5}$ $TCID_{50}$ per ml) (Table 1). Attempts to isolate infectious virus from plasma samples taken during periods of asymptomatic infections, either between disease cycles or during long-term inapparent infections, were uniformly negative (data not shown), in agreement with previous reports of stringent control of EIAV replication during afebrile periods (16, 18, 29, 38).

Dynamics of virus replication measured by RT-PCR assays of plasma viral RNA. The levels of virus replication during experimental EIAV infections have typically been measured using assays of plasma virus infectivity in cell culture, as noted above. However, these measurements of viral replication levels are complicated by the presence of neutralizing antibodies that may mask virus particles in plasma samples. With the recent development in our lab of a nonradioactive semiquantitative RT-PCR assay for EIAV genomic RNA (21, 26), we were able for the first time to monitor the dynamics of virus replication

TABLE 1. Viremic febrile episodes of experimentally infected ponies

Pony no.	Febrile episode[a]	Day postinfection[b]	Temp (°C)	Log plasma viremia[c] ($TCID_{50}$/ml)
561	I	17	39.2	4.0
562	I	17	40.0	5.5
564	I	18	40.3	5.0
	II	34	41.0	5.5
	III	80	39.9	4.5
	IV	106	41.1	4.5
	V	336	40.6	5.0
	VI	377	40.2	5.5
567	I	19	39.9	3.5
	II	40	40.6	4.5
	III	223	40.3	4.5
	IV	258	40.9	5.5
	V	640	40.1	4.5
	VI	729	40.0	5.0

[a] Viremic febrile episodes observed after experimental infection were designated by consecutive roman numerals.

[b] Day postinfection in which the rectal temperature peaked during a viremic febrile episode. Febrile episodes occurred for up to 4 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 refer to the maximum virus titer ($TCID_{50}$ per milliliter) observed for each febrile episode which also corresponded to the day of peak rectal temperature. Infectious virus was detected only during EIAV-related febrile episodes and not during subclinical periods of infection.

by quantifying viral genomic RNA in the plasma of the experimentally infected ponies as they progressed from chronic disease to long-term inapparent infections. Thus, EIAV genomic RNA levels in the plasma of each pony were measured during febrile episodes associated with chronic disease and then every 4 months during long-term asymptomatic infections over the 3-year observation period (panels C in Fig. 1 to 4). The results of these assays typically detected 10^8 to 10^9 copies of viral genomic RNA per ml of plasma during each disease episode, consistent with the high levels of infectious virus detected in the plasma during febrile episodes (Table 1). However, the plasma RNA analyses revealed for the first time a marked difference in the levels of virus replication associated with asymptomatic infections. The separation of infected ponies into two clinically distinct groups was further demonstrated by the quantitation of virus genomic RNA present in the plasma of each pony. Concurring with the lack of recurring clinical cycles in ponies 561 and 562, minimal (several hundred RNA copies per ml) to undetectable amounts of virus genomic RNA could be detected in these two ponies at all time points after the initial febrile episode (Fig. 1C and 2C). In distinct contrast, greater amounts ($>10^4$ RNA copies per ml) of virus RNA could be measured for almost every time point sampled for ponies 564 and 567. Interestingly, however, the levels of plasma RNA detected in ponies 564 and 567 at various times during long-term asymptomatic infection generally ranged from 10^4 to 10^5 copies per ml, indicating relatively high levels of EIAV replication in these inapparent carriers. Thus, these results demonstrate that long-term inapparent carriers of EIAV, while remaining asymptomatic, can differ substantially in the level of control of virus replication. These observations provide new insights into virus-host dynamics that have not been previously revealed by measurements of infectious virus in plasma of inapparent carriers, presumably due to the inhibitory effect of broadly neutralizing serum antibodies present in inapparent carriers.

Evolution of the humoral response to EIAV. While previous studies from our lab and others have focused on host immune responses associated with chronic EIA, there has been to date no systematic longitudinal analysis of changes in host immunity to EIAV during the progression to and maintenance of long-term asymptomatic infections. As described in the following three sections, the evolution of the humoral immune response specific for EIAV was characterized using quantitative assays including virus envelope-specific endpoint titer of total IgG, IgM, and subclasses of IgG; qualitative assays of avidity index and conformation dependence; and a functional assay which measured the levels of virus neutralizing activity. These analyses were initiated to characterize antibody responses associated with long-term clinical latency and to compare the evolution of antibody responses in ponies experiencing single or multiple EIA disease cycles.

(i) Quantitative analyses of the antibody response to EIAV envelope. Previous studies have documented the levels of total IgG specific for envelope glycoproteins during the initial 10 months postinfection (6). Infected ponies seroconverted with specificity for EIAV envelope proteins by 3 weeks postinfection and reached steady-state levels within 2 to 3 months. Thereafter, levels of envelope-specific IgG remained consistent up to 10 months postinfection. To extend and expand this initial analysis of antibody responses to persistent EIAV infection in the four experimentally infected ponies, we monitored over the 3-year observation period the production of EIAV envelope-specific IgM, total IgG, and the subclasses of IgG [IgGa, IgGb, IgGc, and IgG(T)] in a ConA ELISA (Fig. 5). Titers of envelope-specific IgM reached maximum values at the time of seroconversion 3 weeks postinfection in all ponies (Fig. 5A). Levels of IgM specific for envelope gradually declined during the first 2 to 3 months with a steady-state level of $1:10^2$ to $1:10^3$ that was maintained for the remainder of the study. Both IgG and IgM levels reached their respective set points at approximately the same time, 2 to 3 months postinfection, reflecting consistent levels of isotype switching occurring from IgM to IgG in the ponies for antibodies specific for EIAV envelope.

Quantitative analyses of the endpoint titer of IgG specific for EIAV envelope glycoproteins were performed for samples collected from each pony beyond the initial 10 months and up to 3 years postinfection. Levels of envelope-specific IgG as measured in a ConA ELISA remained at the steady-state levels established 2 to 3 months postinfection. The IgG endpoint titer was maintained in each pony within the range of $1:10^5$ to $1:10^6$ (Fig. 5A). Interestingly, the levels of envelope-specific IgG remained comparatively similar and constant among the four infected ponies throughout the entire 3-year period of study, even though they had dissimilar clinical responses and levels of virus replication. Thus, no association could be ascertained between the overall levels of antibody specific for envelope glycoprotein produced and the outward signs of clinical disease (febrile episodes and platelet reduction) or the levels of virus replication, as measured by levels of plasma viral RNA.

Serum samples were next analyzed to quantitate the levels of each IgG subclass with specificity for EIAV envelope glycoprotein. Polyclonal anti-IgG subclass antibodies were used to distinguish the relative levels of envelope-specific antibody. Endpoint titers of envelope-specific IgGa mirrored the levels of total IgG with maximum dilutions of $1:10^5$ to $1:10^6$ reached within 2 to 3 months postinfection and remained constant for the remainder of the study (Fig. 5C). Envelope-specific IgGb was observed by 3 weeks postinfection, reached maximum lev-

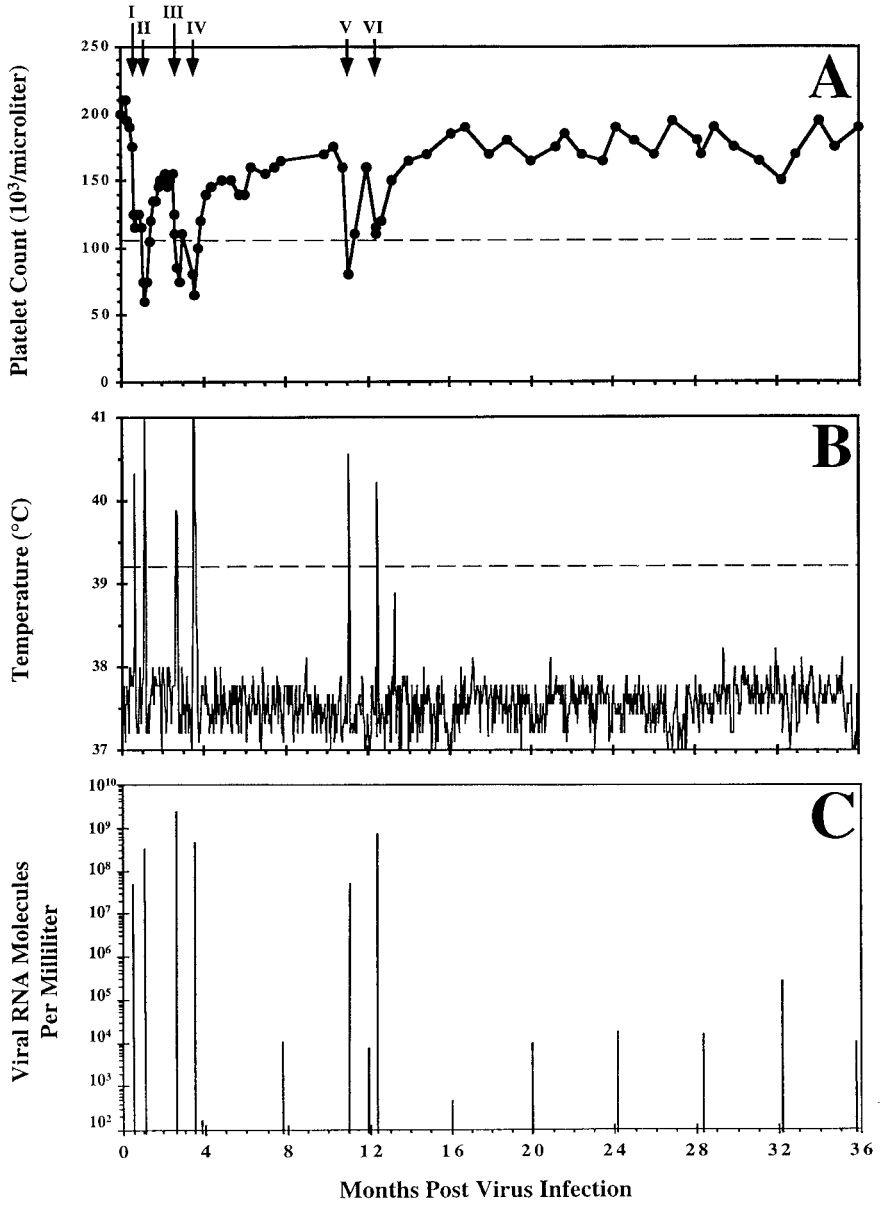

FIG. 3. Clinical course and dynamics of virus replication in pony 564 experimentally infected with EIAV. Shown are platelet counts per microliter of whole blood (A), daily rectal temperatures (B), and virus RNA molecules per milliliter of plasma (C) from pony 564 experimentally infected with EIAV. EIA febrile episodes for the pony are marked with an arrow in panel A and individually identified with consecutive roman numerals I through VI. See the Fig. 1 legend for criteria used to designate an EIA-related disease episode. Viral RNA molecules per milliliter of plasma were quantitated for a single day every 4 months postinfection and during each EIA-related febrile episode.

els by 1 to 2 months postinfection, and was consistently present during the course of the study at titers between $1:10^4$ and $1:10^6$ (Fig. 5D). Envelope-specific IgGc was detected in three out of the four ponies by 3 weeks postinfection and remained at lower titers (generally less than $1:10^4$) than did IgGa and IgGb (Fig. 5E). Levels of envelope-specific IgGc were more divergent among the four infected ponies. In fact, no significant amount of envelope-specific IgGc was ever detected in serum from pony 567, while IgGc levels in the other three ponies varied from $1:10^2$ to $1:10^4$ over the observation period. All ponies had detectable IgG(T) levels, first observed at 3 weeks postinfection and maintained at variable levels, but always less than $1:10^4$, throughout the entire study period (Fig. 5F). These data demonstrate that EIAV infection of outbred ponies induced an envelope-specific antibody response within 3 weeks postinfection characterized by high levels of IgM that rapidly switched isotype to predominantly IgGa and IgGb, with minor levels of IgGc and IgG(T). While the levels of IgGa and IgGb increased to steady-state levels within 1 month postinfection, the IgGc and IgG(T) levels tended to fluctuate over the 3-year observation period. The quantitative measurements of antibody responses to EIAV envelope proteins appeared similar in all four ponies, regardless of the number of disease episodes, indicating a lack of correlation between antibody levels and clinical progression.

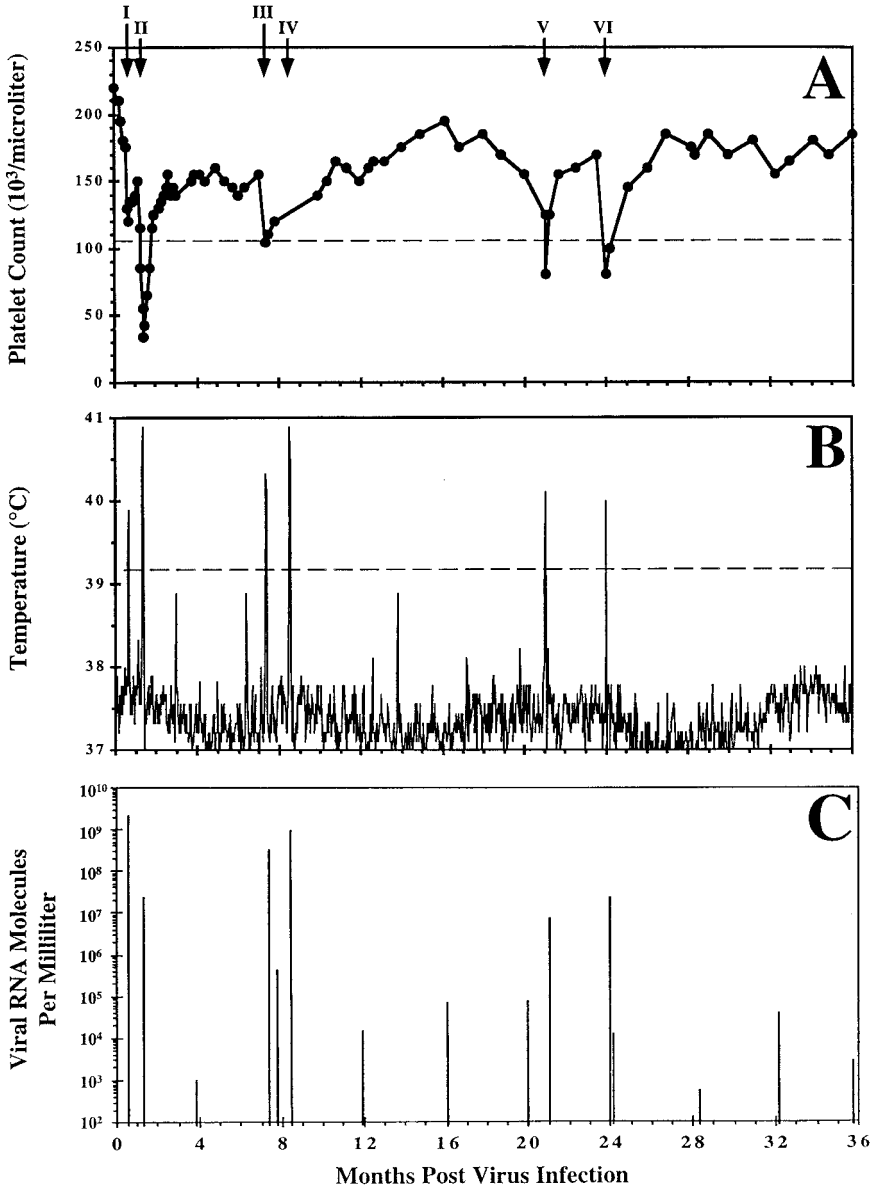

FIG. 4. Clinical course and dynamics of virus replication in pony 567 experimentally infected with EIAV. Shown are platelet counts per microliter of whole blood (A), daily rectal temperatures (B), and virus RNA molecules per milliliter of plasma (C) from pony 567 experimentally infected with EIAV. EIA febrile episodes for the pony are marked with an arrow in panel A and individually identified with consecutive roman numerals I through VI. See the Fig. 1 legend for criteria used to designate an EIA-related disease episode. Viral RNA molecules per milliliter of plasma were quantitated for a single day every 4 months postinfection and during each EIA-related febrile episode.

(ii) Avidity and conformational dependence of EIAV envelope-specific antibodies. Using qualitative assays of antibody avidity and conformational dependence, we previously demonstrated a maturation envelope-specific antibody response to EIAV infection during the first 10 months postinfection (6) and a correlation of these antibody parameters with the efficacy of experimental EIAV vaccines (7). Therefore, we next sought to utilize these measurements of antibody avidity (Fig. 6A) and conformational dependence (Fig. 6B) to define the evolution of EIAV-specific antibody populations during the progression from chronic EIA to long-term asymptomatic infections. As reported previously, the initial envelope-specific antibody responses within the first 2 months postinfection displayed avidity values of less than 5%, despite reaching antibody endpoint titers of about $1{:}10^5$ (Fig. 6A). Thereafter, avidity values gradually increased from low avidity (<30%) to high avidity (>50%), until reaching an average value of 65% by 13 months postinfection that was maintained for the remainder of the observation period. The longitudinal analyses of antibody conformational dependence in the total IgG population (Fig. 6B) revealed a progression of antibody properties that paralleled the changes observed in antibody avidity. As reported previously, the conformational dependence of envelope-specific antibodies was determined to be less than 1.0 for the first 2 months postinfection, indicating a predominance of antibody specific for linear envelope determinants. The conformational dependence values gradually increased over the first 10 months postinfection to a value of about 1.5, reflecting a gradual in-

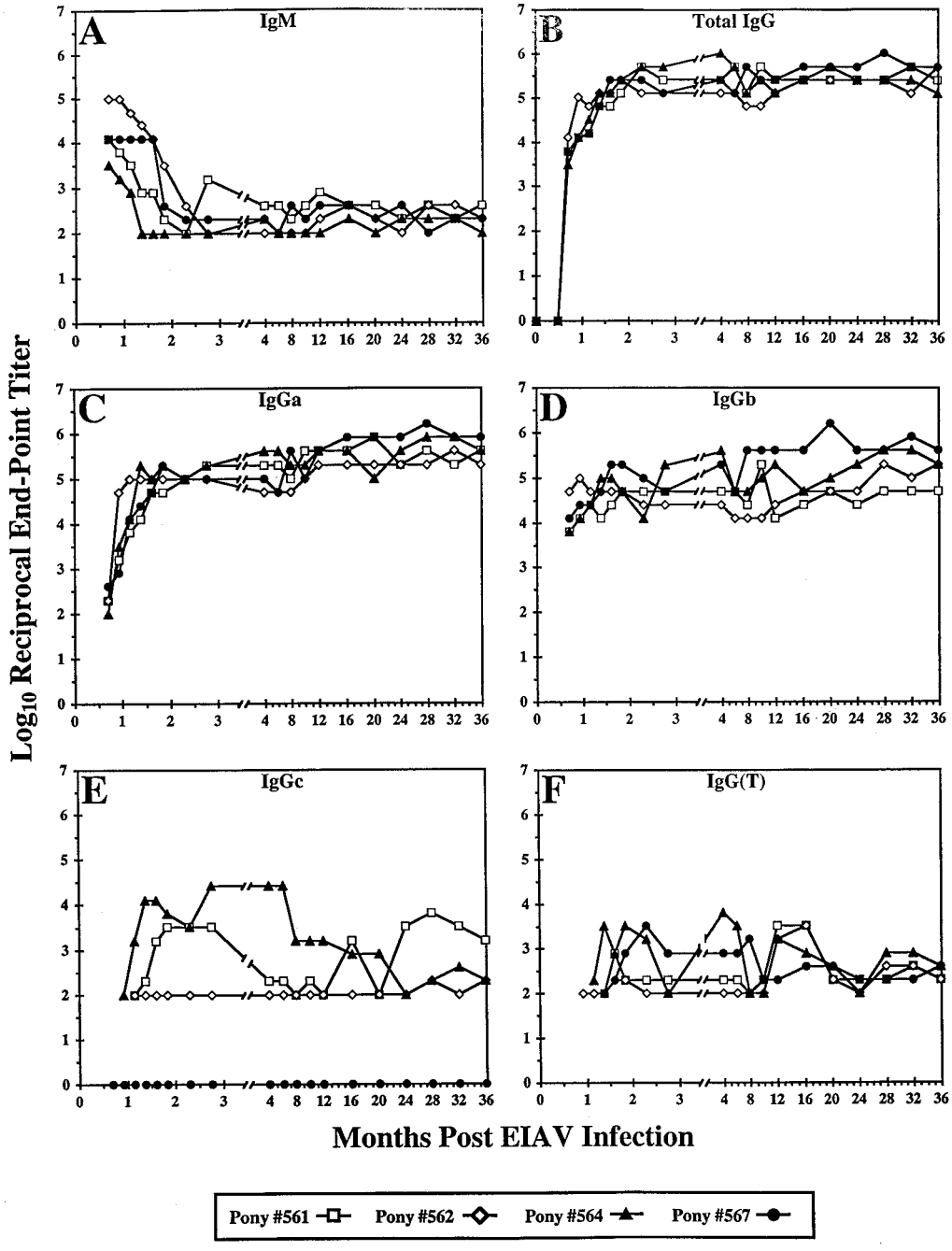

FIG. 5. EIAV-specific endpoint titers of IgG, IgM, and isotypic IgG in EIAV-infected ponies. By longitudinal analyses, the Env-specific antibodies in ponies 561, 562, 564, and 567 experimentally infected with EIAV were quantitated in a ConA ELISA as described in Materials and Methods using secondary polyclonal antibodies specific for IgM, IgG, IgGa, IgGb, IgGc, and IgG(T). The $\log_{10}$ of the reciprocal dilution that was 2 standard deviations above background was plotted for each time point.

crease in antibody directed to conformationally dependent envelope determinants. Interestingly, the conformational dependence value observed at 10 months postinfection was maintained in three (561, 564, and 567) of the four ponies up to the end of the 3-year observation period. In contrast, pony 562 antibody conformation continued to increase to a value of 2.0 at about 2 years postinfection, before declining to a steady-state level of 1.5, as observed for the other ponies.

Taken together, these longitudinal analyses of antibody avidity and conformational dependence demonstrate dynamic changes in envelope-specific antibody populations during the first year postinfection, long after the quantitative levels of antibody have reached a maximum after 2 months postinfection. Following the evolution of antibody during the first year postinfection, antibody responses appear to reach steady-state values that are maintained indefinitely in longterm asymptomatic infections. Interestingly, a similar maturation of antibody responses is observed with all four ponies, regardless of the

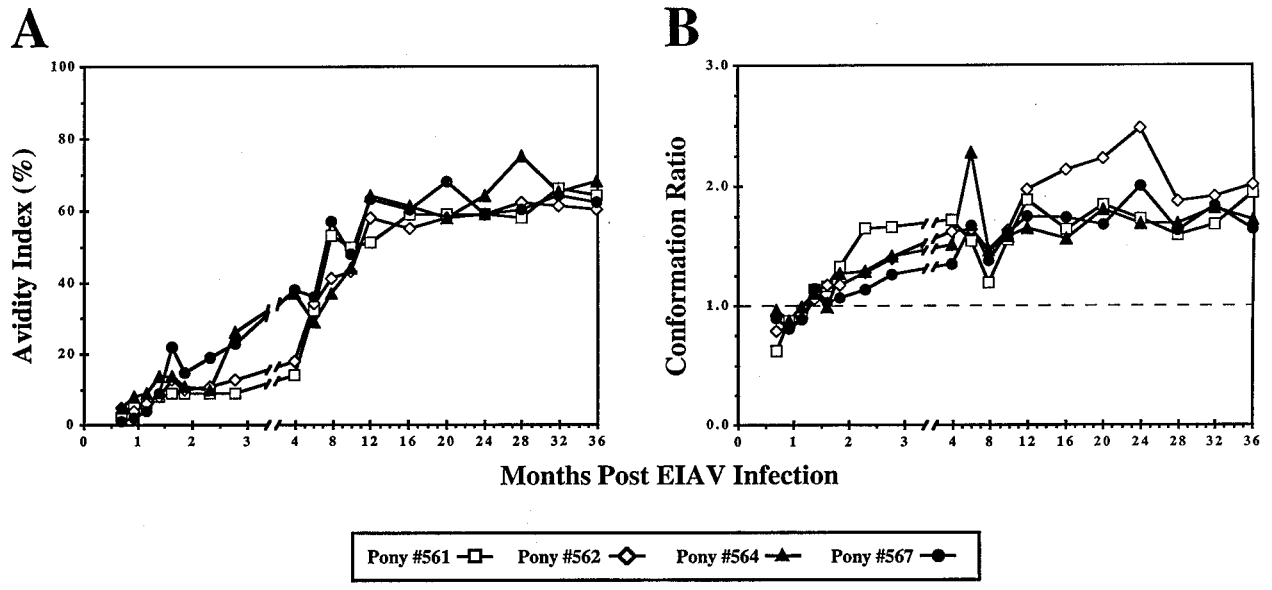

FIG. 6. Avidity and conformational dependence of Env-specific IgG in EIAV-infected ponies. By longitudinal analyses, the avidity index (A) and conformational dependence of Env-specific polyclonal IgG (B) were measured in a ConA ELISA as described in Materials and Methods for ponies 561, 562, 564, and 567. (A) Avidity index measurements are presented as percentages of the antibody-antigen complexes resistant to washes with 8 M urea. (B) Conformation ratio was calculated by measuring 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. Conformation ratios of >1.0 indicated antibody reactivities to epitopes which were predominantly conformational in nature, while ratios of <1.0 denoted antibody reactivities to epitopes which were predominantly linear in form.

very different clinical progressions or levels of virus replication observed in these persistent infections. Thus, the antibody avidity and conformational dependence assays do not appear to provide correlates of disease progression or virus replication during persistent infection, although they have proven useful in distinguishing experimental EIAV vaccine efficacy (7).

(iii) EIAV-specific serum neutralizing activity. To extend our quantitative and qualitative characterization of the evolution of the humoral immune responses to persistent EIAV infection, we analyzed the functional capacity of immune serum to neutralize infectious $EIAV_{PV}$. Longitudinal serum samples collected over the 3-year observation period were analyzed in a quantitative infectious center assay to estimate the dilution of serum which neutralized 50% of the input virus; analyses during the acute and chronic periods of an EIAV infection showed that neutralizing antibody activity developed slowly, becoming detectable in vitro between 2 and 3 months postinfection, and, in general, continued to increase in activity up to 10 months postinfection (6). The current extended longitudinal analysis of serum neutralization activity in the four infected ponies revealed that the levels of neutralizing antibody continued to increase and reached a steady-state level approximately 2 years postinfection, having 50% titers at dilutions ranging between 1:200 and 1:400 (Fig. 7). The slow evolution of neutralizing antibodies was similar in all four ponies, regardless of the number of disease episodes or steady-state levels of virus replication. In addition, it is important to note that serum neutralization was first detected and increased in level after the quantitative levels of antibody had reached maximum titer by 2 months postinfection. This observation demonstrates further the dynamic evolution in virus-specific antibody populations while relatively constant levels of serum antibody to EIAV are maintained.

Previous studies have measured the levels of serum neutralization in EIAV-infected horses (17, 18, 31, 32, 36), but to date there has not been a thorough analysis of the contribution of various antibody subclasses to this neutralization activity. With the development of antibody reagents to distinguish equine IgG subclasses, we examined for the first time the role of different Ig populations in serum neutralization activity. Differences in the levels of neutralizing antibody activity present in the IgGa and IgGb subclasses of IgG were measured by a subtractive absorption method to remove each subclass of IgG from the immune serum before testing. Serial absorption of immune serum, using polyclonal subclass-specific antibody covalently coupled to Sepharose beads, effectively and quantitatively removed each subclass of IgG to background levels as determined by ELISA (Table 2). EIAV-neutralizing activity of immune serum was still present after absorption with either anti-IgGa or anti-IgGb immunosorbent (Table 2). However, removal of both IgGa and IgGb from immune serum consistently eliminated all detectable virus-neutralizing activity (Table 2). These results indicated that EIAV-neutralizing antibody activity resided predominantly with the IgGa and IgGb subclasses of IgG. Furthermore, the data implied that the remaining antibody components [IgGc, IgG(T), IgM, and IgA] did not appear to have significant neutralizing activity. Similar results were obtained in absorption experiments with serum samples collected at various time points over the 3-year observation period (data not shown), indicating a consistent association of IgGa and IgGb subclasses with the observed serum neutralization.

CTL responses of EIAV-infected ponies. To complement the assays of antibody responses to persistent EIAV infection, we also performed a longitudinal analysis of cell-mediated immune responses specific for EIAV antigens for up to 3 years postinfection. The two methods utilized to characterize the cell-mediated responses were assays of T-cell proliferation to autologous EIAV-infected macrophages and measurements of memory cytolytic T-lymphocyte (CTLm) activity specific for EIAV Gag and Env antigens. In our previous analyses of cellular immune responses during chronic EIA, it was shown

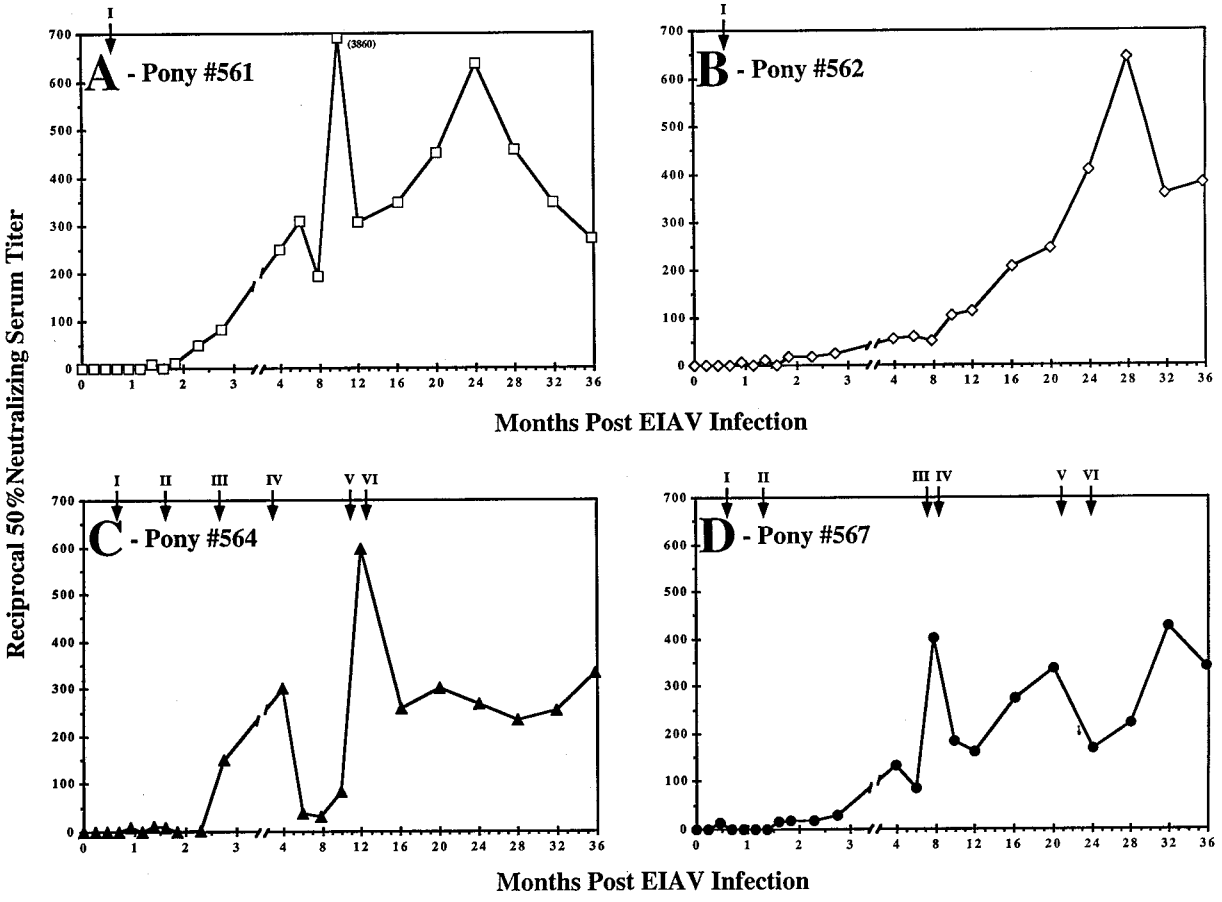

FIG. 7. EIAV-Specific serum neutralizing activity in EIAV-infected ponies. The mean reciprocal dilutions of serum which neutralized 50% of input $EIAV_{PV}$ as measured in an infectious center assay are presented for ponies 561, 562, 564, and 567. The number in parentheses in panel A indicates the reciprocal dilution at which 50% neutralizing serum activity was measured for data points that would not fit on the graphs using the presented scale. Data presented are representative of three separate experiments. Febrile episodes for each animal are indicated at the top of each panel in consecutive roman numerals.

that T-lymphocyte proliferation to EIAV-infected macrophages was first detected (stimulation index, >10) by 3 weeks postinfection, concurrent with the acute febrile episode (6). During the initial 10 months postinfection, T-cell proliferative responses increased gradually and reached a steady-state level (stimulation index between 20 and 60) by about 9 months postinfection (6). Extending these longitudinal lymphoproliferation assays over the entire 3-year observation period demonstrated relatively constant stimulation indices ranging from 20 to 60 (data not shown). There was no significant difference in the levels of proliferation detected in the four ponies, indicating a lack of apparent correlation with clinical progression or levels of virus replication.

TABLE 2. EIAV-neutralizing activity of the IgGa and IgGb subclasses in immune serum[a]

Treatment of serum[c]	EIAV-specific reciprocal endpoint titer[b]			Reciprocal 50% neutralizing serum titer
	IgG	IgGa	IgGb	
Beads	2.5×10^5	2×10^5	5×10^3	273
IgGa-beads	1×10^4	$<10^2$	3×10^3	64
IgGb-beads	2×10^5	1×10^5	$<10^2$	76
IgGa and IgGb-beads	1×10^3	$<10^2$	$<10^2$	<10

[a] Data presented in this table are representative of multiple immune serum samples tested in a similar manner.

[b] Serum collected from pony 561 10 months post-EIAV infection and treated with immunosorbents as listed was analyzed in a ConA ELISA for the levels of EIAV-specific antibody as described in Materials and Methods, except that the polyclonal secondary antibody used was specific for either total equine IgG or equine isotype IgGa or IgGb.

[c] Serum collected from pony 561 10 months post-EIAV infection was treated with agarose beads alone (Beads) or polyclonal antibody specific for equine IgGa and/or IgGb covalently coupled to agarose beads before quantitation of EIAV-specific titer and neutralizing activity.

EIAV-specific CTL were analyzed in a ^{51}Cr release assay against target cells that expressed both major histocompatibility complex class I and major histocompatibility complex class II to measure contributions of both $CD4^+$ and $CD8^+$ CTL. Antigens were introduced into the target cells by infection with recombinant vaccinia virus vectors encoding the genes for β-galactosidase, EIAV Gag, or EIAV Env. Attempts to detect CTL activity in circulating peripheral blood mononuclear cells isolated at various time points during the course of the experimental infections were uniformly negative, indicating a low level of circulating CTL. Therefore, effector T cells were stimulated in vitro with autologous macrophages infected with EIAV to activate and expand the CTLm populations specific for EIAV. The results of these studies (Fig. 8) demonstrated detectable CTLm activity in all ponies within 3 to 4 weeks postinfection, concurrent with the initial acute disease episode,

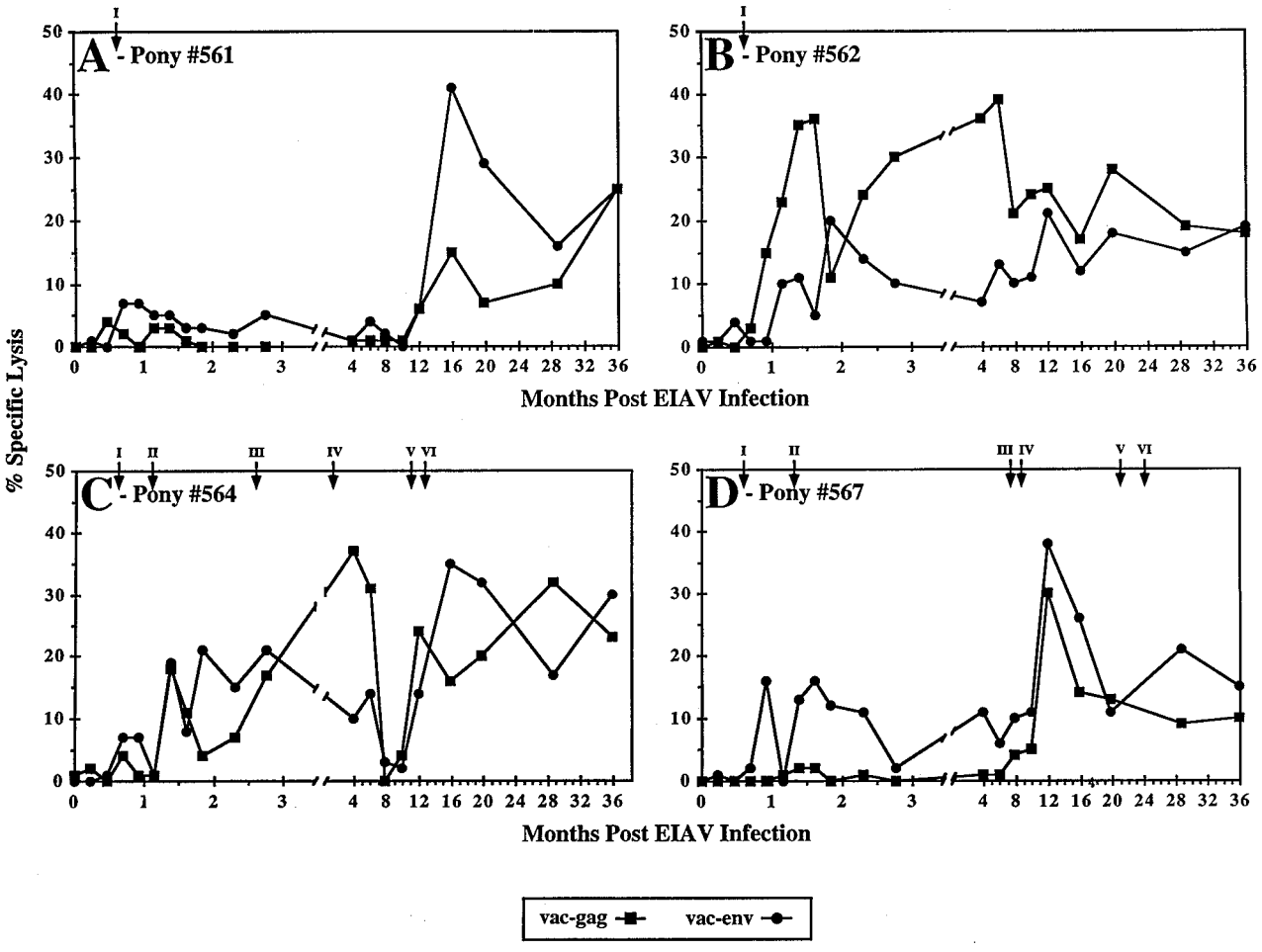

FIG. 8. EIAV-specific cytolytic T-cell activity in EIAV-infected ponies. EIAV-specific CTLm activity was measured using fresh peripheral blood mononuclear cells that had been activated and expanded by in vitro coculture with recombinant human interleukin-2 and autologous EIAV-infected macrophages from ponies 561, 562, 564, and 567. The net specific lysis was determined by subtracting the level of lytic activity of the T cells against autologous cells expressing a control antigen, β-galactosidase, from autologous cells expressing either EIAV Gag (vac-gag) or Env (vac-env). The standard error of the mean percent specific lysis was always less than 3%. Febrile episodes for each animal are indicated at the top of each panel in consecutive roman numerals.

as noted previously (6). After the initial observation of CTLm activity, however, the levels and specificity of the observed CTL activity differed greatly among the four infected ponies. Pony 561 displayed minimal CTLm activity until approximately 1 year postinfection (Fig. 8A). At about 12 months postinfection, both Gag- and Env-specific CTLm activities were first detected, and these CTLm activities remained at relatively high levels at all subsequent time points tested during the following 2 years of infection. Ponies 562 and 564 in general consistently displayed significant levels of both Gag- and envelope-specific CTLm over the entire observation period (Fig. 8B and C). In contrast, pony 567 had only envelope-specific CTLm activity (Fig. 8D) during the first year postinfection. Interestingly, both Gag- and envelope-specific CTL activities were then consistently detected up to 3 years postinfection.

The highly divergent patterns of CTL activity observed during the 3-year observation period fail to establish any correlation between the CTL levels and specificity and the severity of disease (number of disease cycles) resulting from the experimental infection. For example, ponies 561 and 562 both experienced only a single acute disease episode. However, only minor levels of CTLm activity were detectable in pony 561 during the first year postinfection, while pony 562 consistently displayed high levels of CTLm starting at 1 month postinfection and continuing through the 3-year observation period (Fig. 8A and B). Similarly, the two ponies (564 and 567) experiencing six disease episodes displayed markedly different levels of CTLm during the first year postinfection (Fig. 8C and D).

In contrast to the early stages of EIAV infection that were characterized by diverse CTLm levels, there were uniformly high levels of Env- and Gag-specific CTLm detected in all of the infected ponies after 1 year postinfection. While it is tempting to correlate high levels of CTLm with the maintenance of long-term inapparent infections, it must be noted that pony 564 and pony 567 continued to experience disease episodes in the presence of high levels of CTLm activity (Fig. 8C and D).

DISCUSSION

The current study describes for the first time a comprehensive longitudinal analysis of the dynamics of EIAV replication and host immune responses in experimentally infected ponies as they progress from chronic disease to long-term inapparent infections. These studies complement and extend our previous analyses of the early stages of EIAV infection in the same four

experimentally infected ponies, including the evolution of genomic quasispecies during sequential febrile episodes (20) and the development of host immunity during chronic EIA (6). The results of these analyses reveal a number of new insights into virus-host interactions in this lentivirus system but also raise a number of important questions that require further investigation.

The first unexpected finding from the current study was the wide range of virus replication levels observed in the four ponies during long-term inapparent infections. Numerous studies from our lab (6) and others (16, 18, 29, 38) have previously reported a lack of detectable infectious virus in the plasma of long-term inapparent carriers of EIAV. Based on these studies, it has been assumed that host immune responses effectively suppress EIAV replication to minimal levels during asymptomatic periods of chronic EIA and in long-term inapparent infections. We recently used sensitive PCR assays in a cross-sectional analysis to monitor the levels of virus infection and replication in various tissues of experimentally infected equids during the initial acute disease and during long-term inapparent infections (8). The results of these studies indicated plasma RNA levels of 10^5 to 10^8 copies per ml during acute disease and less than 100 copies per ml in the two inapparent carriers examined in the study. These latter observations appear to support the concept that the lack of detectable infectious virus in the plasma of inapparent carriers is in fact due to effective suppression of virus replication and not only to the presence of high levels of serum neutralizing antibodies. In contrast to these earlier observations, however, the current study clearly demonstrates dramatic differences in the steady-state levels of EIAV replication associated with long-term inapparent infection, suggesting the development of very different levels of immune control during persistent infection. While all four of the ponies were consistently negative for infectious virus during asymptomatic infections, the two ponies that experienced only a single disease episode typically contained an undetectable number to $<10^3$ copies of RNA per ml, apparently reflecting effective suppression of virus replication. In contrast, the other two ponies that experienced multiple disease cycles usually displayed plasma RNA levels ranging from 10^4 to 10^6 copies per ml during the long-term asymptomatic infection, evidently indicating a rather tenuous control of the viral infection to subclinical levels. Although the number of animals used in the study is relatively small, the range of steady-state virus replication levels revealed in this study is similar to the range of steady-state virus replication levels observed with SIV-infected monkeys (4, 9, 13) and HIV-1-infected patients (35). As in these latter lentivirus infections, the basis for the difference in steady-state EIAV replication levels in inapparent carriers remains to be determined.

A primary focus of the current study was to characterize the development of host immune responses during the progression of persistent EIAV infection from chronic EIA to long-term inapparent carriage, with the goal of identifying immune responses that establish sustained control of virus replication and disease. Our previous study of humoral and cellular immune responses during the first 10 months postinfection in these four experimental infections revealed a complex and lengthy evolution of antibody and cellular immune responses, regardless of the clinical course of the infection (6). The current studies demonstrate a continued progression in EIAV-specific antibody and CTL responses to about 12 months postinfection. At this time, there appears to be an establishment of relatively stable humoral and cellular immune responses that is maintained for the remainder of the 3-year observation period. The virus-specific immunity is associated with consistently high levels of serum antibodies that are characterized by high avidity (>60%), predominant specificity for conformational envelope determinants (conformational ratios, >1.5), and effective virus neutralization. In addition, inapparent infections are associated with consistently high levels of CTL activity to EIAV Gag and envelope proteins. Thus, the immune responses associated with the maintenance of long-term inapparent EIAV infections are in distinct contrast to immune responses observed early during persistent infections that are characterized by high-titer antibody that is of low avidity and poorly neutralizing and relatively inconsistent CTL activity (6). Taken together, these studies demonstrate a maturation of immune responses to persistent EIAV infection that is similar to that reported previously for SIV and simian-human immunodeficiency virus infection of monkeys and HIV-1 infection of humans (2). However, the 12-month time required for immune maturation in the EIAV system appears to be substantially longer than the average of 8 months required for immune maturation in the other lentivirus systems. The basis for this difference is uncertain at this time.

While the current studies define the dynamics of immune maturation to persistent EIAV infection, they do not appear to define specific immune properties that correlate with the development of immune control of virus replication and disease during a persistent infection. For example, a similar evolution of humoral and cellular responses was observed for all four experimentally infected ponies, regardless of the number of disease cycles or the steady-state levels of virus replication observed during the observation period. In certain cases, cycles of disease were controlled in the early stages of infection in the absence of detectable neutralizing antibodies or virus-specific CTL, raising a number of questions about the mechanisms by which the host pony establishes control of the aggressive virus replication associated with disease cycles. In addition, pony 567 experienced disease episodes at about 21 and 24 months postinfection, long after the establishment of steady-state immunity (and maximum antibody and cellular immunity) evident at 12 months postinfection. We have previously demonstrated the utility of antibody avidity and conformational dependence assays to differentiate immune responses to experimental SIV and EIAV vaccines and to establish an association between vaccine efficacy and the capacity of the immunization protocol to induce mature antibody responses (3, 7). However, the current studies indicate that, while these parameters may relate to protection from virus exposure, they do not reliably correlate with control of established EIAV infections. This discrepancy suggests fundamental differences between immune mechanisms necessary to protect against viral exposure and those required for controlling a persistent infection.

Despite extensive efforts to identify reliable immune predictors of virus replication and clinical progression in HIV-1-infected patients (2, 10) and SIV-infected monkeys (1–3, 15), there has been to date no consensus definition of reliable immune correlates. However, there have been recently a number of studies that indicate the importance of virus-specific $CD8^+$ CTL and $CD4^+$ lymphoproliferative responses in controlling established lentivirus infections (14, 24, 30, 34, 35, 39). The current longitudinal analysis of persistent EIAV infections does reveal the maintenance of a high level of virus-specific lymphoproliferation and CTL in long-term inapparent carriers, consistent with the essential role of these cellular responses in enduring suppression of virus replication and disease. However, it should also be noted that long-term inapparent infections are equally characterized by sustained high levels of EIAV-specific neutralizing antibodies that may also contribute to the control of the persistent infection. Thus, these studies

emphasize the need to develop new assays that can measure novel aspects of antibody and cellular immune responses to persistent lentivirus infections that may then define reliable immune correlates of control of lentivirus infections.

ACKNOWLEDGMENTS

This work was supported by NIH grant 5RO1 AI25810 and by funds from the Lucille P. Markey Charitable Trust and the Kentucky Agricultural Experiment Station. S.A.H. was supported by NIH AIDS Training Grant 5T32 AI07487.

We thank Walter Storkus from the Department of Medicine at the University of Pittsburgh School of Medicine for providing the recombinant human interleukin-2; Sekhar Chakrabarti and Bernard Moss of the NIAID and the NIH AIDS Research and Reference Reagent Program for the recombinant vaccinia virus vector vSC8; and Gary Thomas, Brian Meade, and William Arnold at the University of Kentucky for excellent care and handling of the animals.

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