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A Primary Production Deficit in the Thrombocytopenia of Equine Infectious Anemia

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The purpose of this study was to identify the mechanisms responsible for the thrombocytopenia that develops following infection of horses by the lentivirus equine infectious anemia virus (EIAV). Immunocompetent Arabian foals and Arabian foals with severe combined immunodeficiency (SCID), which lack functional B and T lymphocytes, were experimentally infected with EIAV. Levels of viremia and a number of clinical and hematologic parameters were examined prior to and following infection. Thrombocytopenia was not dependent on the immune response: SCID foals were affected as severely as immunocompetent foals. Production of platelets, measured by metabolic incorporation of radioactive label, was significantly reduced. The decrease ranged from 35 to 89% in three SCID and two immunocompetent foals examined. Platelet survival, measured by ^{51}Cr labeling, also declined following infection in both SCID and immunocompetent foals: 51 and 68%, respectively, relative to the preinfection life spans. The difference between immunocompetent and immunodeficient foals was not statistically significant. The number of megakaryocytes (MK) per square millimeter of bone marrow, determined by digitizing morphometry, was not significantly altered in either SCID or immunocompetent thrombocytopenic foals. Numbers of denuded MK nuclei per unit area increased, but the elevation was not statistically significant. No evidence for viral replication in MK was found. Three different parameters of intravascular coagulation (activated prothombin time, fibrin degradation products, and one-step prothombin time) remained normal until after platelet numbers had declined significantly, arguing against an important role for disseminated intravascular coagulation. The findings indicate that EIAV induces thrombocytopenia principally through an indirect, noncytocidal suppressive effect on platelet production, the mechanism of which is unknown. A shortening of platelet life span apparently contributes moderately to the platelet deficit as well. The shortening of platelet life span is multifactorial in origin, including both mechanisms that depend on an active immune response and those that do not.

Thrombocytopenia follows infection by a wide variety of viruses, including human immunodeficiency virus type 1 (HIV). Bleeding disorders associated with thrombocytopenia represent a significant clinical problem in patients with AIDS (23). The mechanisms of the platelet deficits following viral infections are diverse, incompletely understood, and frequently multifactorial in nature (31). The pathogenesis of AIDS-associated thrombocytopenia, the subject of considerable study and discussion, was initially considered primarily to involve the immune system. Evidence for this conclusion includes elevated levels of immunoglobulin and complement on platelet membranes (10) and the frequently beneficial effect of therapeutic splenectomy. However, platelet-associated immunoglobulins are elevated in both thrombocytopenic and nonthrombocytopenic HIV-positive patients (28). Other evidence for nonimmunologic mechanisms includes the facts that megakaryocyte (MK) precursors are reduced in number (30) and that platelet levels often respond to azidothymidine therapy (8). Moreover, kinetic analysis of platelet survival suggests a depression of platelet production in thrombocytopenic HIV-positive patients (1). Thus, AIDS-related thrombocytopenia is now generally felt to be multifactorial in nature, the dominant mechanism at the time determining the appropriate therapeutic modalities (31).

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Thrombocytopenia is a prominent component of the hematologic abnormalities associated with infection of horses with equine infectious anemia virus (EIAV), a lentivirus that shares with HIV certain capsid antigenic epitopes (13) and similarity in nucleotide sequence, particularly in the *pol* and *gag* genes (26). The hallmark of equine infectious anemia (EIA) in normal horses is irregularly recurring episodes of clinical disease, during which exuberant replication of neutralization escape variants in highly permissive tissue macrophages produces high viremia, leading to fever, anemia, and thrombocytopenia (4, 9). The mechanisms of anemia are multifactorial, including both immune-mediated destruction of erythrocytes (19, 24) and direct bone marrow suppression (12, 27). The mechanisms of thrombocytopenia have been less well studied. Elevated levels of platelet-associated immunoglobulin G (IgG) and IgM in thrombocytopenic EIAV-infected horses has prompted others to propose an immunologic mechanism for the platelet deficit (4).

This study was initiated to define the mechanism(s) responsible for the thrombocytopenia of EIA. The availability of severe combined immunodeficiency (SCID) foals provided an opportunity to determine whether the thrombocytopenia observed in normal horses also developed in horses devoid of an immune response and, if it did, to examine the events associated with the development of thrombocytopenia without the obscuring influence of an intact immune response. This model has been used previously to establish that the immune response is necessary to suppress viral replication and render horses periodically asymptomatic: SCID foals maintained con-

stant, high levels of viremia until death (21). Moreover, the immunodeficient foals developed anemia, suggesting a suppressive influence on erythrocyte production. This study was designed to determine whether these animals, absent an immune response, developed thrombocytopenia and, if they did, to define the causes of that platelet deficit.

MATERIALS AND METHODS

Animals, viruses, and infectivity titration. Arabian foals for experimental infection were obtained from a breeding herd of asymptomatic carriers of the homozygous recessive SCID gene. The 13 SCID and 20 normal (immunocompetent) foals used in this study were housed in isolation, weaned at 4 weeks of age, and infected at 8 weeks of age. SCID foals were maintained under intensive management, including antibiotic therapy and weekly infusions with normal equine plasma for incidental bacterial infections, as previously described (20, 21). To minimize artifact, schedules were adjusted such that no plasma transfusions were administered within 5 days before or after experimental platelet labelings.

Physical examinations, hemograms, and platelet counts were performed daily, and samples of serum, platelet-free plasma, and platelet-rich plasma (PRP) were collected, processed on ice as rapidly as possible, and stored at −80°C. Bone marrow samples were obtained at biopsy prior to infection and immediately after onset of thrombocytopenia. Foals were routinely necropsied at 30 ± 3 days postinfection (dpi), at which time samples of major organs, including bone marrow, were collected and appropriately preserved for histopathology, electron microscopy, MK purification, bone marrow morphometry, and in situ hybridization. Buccal bleeding times were monitored by standard methods.

Two strains of EIAV were used: the in vitro WSU5 fibroblast-adapted strain (18) and the more virulent horse-passaged Wyoming (WYO) field strain. This proved necessary because the relatively low virulence of the WSU5 strain failed to provide significant and predictable clinical disease in non-SCID foals (see Results). SCID foals were infected with only $EIAV_{WSU5}$, using $10^{6.0}$ 50% tissue culture infective doses ($TCID_{50}$) injected intravenously (i.v.). Of 17 immunocompetent foals infected, 7 received $EIAV_{WSU5}$ ($10^{6.6}$ $TCID_{50}$) and 9 received approximately $10^{3.0}$ horse infectious doses of $EIAV_{WYO}$, in the form of diluted plasma from an acutely ill pony.

$EIAV_{WSU5}$ was titrated by limiting-dilution assays on equine kidney cells in 96-well plates. Plasma samples were diluted in maintenance medium (minimal essential medium, 2% fetal bovine serum) containing 5 U of heparin per ml. Six wells per $\log_{10}$ dilution were inoculated and incubated for 1 h, the inocula were removed, and maintenance medium was added. Plates were fixed with ethanol at 10 dpi and stained by direct immunofluorescence for EIAV p26 antigen, and the $TCID_{50}$ was estimated by standard methods (22). Titers presented represent the mean values of three separate titrations. Viremia levels were not titrated in the immunocompetent foals infected with $EIAV_{WYO}$. In vitro titration of this strain requires resource-intensive monocyte cultures, which were judged to be not justifiable, given the substantial amount of existing data on this virus in horses and the fact that the primary focus of the study was the WSU5 strain in the SCID foals.

Platelet counts. Nine milliliters of blood was drawn into syringes containing 1.0 ml of 0.11 M sodium citrate. Preliminary trials established that platelet counts were relatively stable for the first 120 min following bleeding and began to decline thereafter, presumably from platelet aggregation. Time constraints were therefore rigidly observed, platelets being counted within 90 min of sample collection. Platelets were counted on a particle counter (Coulter model ZBI-10) from PRP obtained by centrifugation of 3-ml blood samples at 152 × *g* for 8 min, and the values were corrected to that for whole blood, using the method of Bull et al. (3). To address the issue of error arising from heterogeneity of platelet density during differential centrifugation to obtain PRP, parallel counts were performed on whole blood, using an automated cell counter (System 9000; Serono-Baker Diagnostics, Allentown, Pa.), until the protocols were satisfactorily optimized.

Platelet production. Metabolic radioisotope incorporation was used as a measure of platelet production by the MK. Initial experiments were conducted with a single dose of 5 μCi of [^{75}Se]selenomethionine (CIS, Inc., Bedford, Mass.) per kg of body weight (7) injected i.v. on day 0 of the experiment. Because this isotope unexpectedly became unavailable during the course of the study, subsequent experiments utilized [^{35}S]sodium sulfate (New England Nuclear-Dupont) (11), injected i.v. at 100 μCi/kg. Following label injection, 21-ml samples of blood were collected daily into acid-citrate-dextrose anticoagulant containing 2.2 μM prostaglandin E_1 (Sigma Chemical, Inc.). PRP was prepared from 20 ml of whole blood by centrifugation at 152 × *g* for 8 min, and the platelets were sedimented therefrom at 1,400 × *g* for 15 min. Erythrocytes were lysed from the platelet pellet with 1% ammonium oxalate, and platelets were resedimented. Following a repeat of the ammonium oxalate lysis, the platelets were washed three times in saline, the preparation was appropriately sampled for counting of platelets and for determination of leukocyte and erythrocyte contaminants, and radioactivity was quantitated on a gamma counter (Gamma 8000; Beckman Instruments). The amount of activity per platelet was then extrapolated to total body platelet mass, using that day's platelet count.

Life span determination. To examine platelet survival, blood was collected from foals before infection and immediately after becoming thrombocytopenic into a citrate-based anticoagulant (CPDA-1; Miles Laboratories) in quadruple bag sets. Platelets were labeled with [^{51}Cr]sodium chromate, using standard procedures (25). Following platelet reinfusion, 9-ml blood samples for ^{51}Cr counting were obtained at 2, 7, 18, 24, 41, 48, 72, 96, and 120 h. Platelet life spans were estimated by analyzing the decay curves of ^{51}Cr-labeled platelets by the multiple-hit gamma-function mathematical model (5, 14), using iteration to arrive at the minimum residual sum of squares. The analyses were performed by a computer program written by D. Bolling and E. Foster, Johns Hopkins University.

MK quantitation. To obtain quantitative estimates of the MK in the bone marrow, core biopsy specimens were taken from the tuber coxae of foals before infection, immediately after they became thrombocytopenic, and at necropsy. Samples were fixed in formalin, decalcified, and embedded in paraffin, and morphometric analysis of hematoxylin-and-eosin-stained sections was performed by using a digitizing pad (Hipad Digitizer; Houston Instruments, Austin, Tex.) and Bioquant System IV software (R & M Biometrics, Inc., Nashville, Tenn.). MK and denuded nuclei were counted in a minimum of 7 mm^2 of evaluable marrow area, excluding hemorrhage, fat, bone, cartilage, muscle, and periosteal connective tissue. The areas occupied by the MK and their nuclei also were recorded, and the nuclear/cytoplasmic localization ratios were calculated (29).

Ultrastructural examination for MK-associated virus. MKs were affinity purified from bone marrow aspirates collected from the tuber coxae of foals before inoculation, immediately after the platelet counts reached thrombocytopenic levels, and at necropsy. Aspirates were collected into a solution composed of 1.0 mmol each of adenosine and theophylline per liter, 1.0% bovine serum albumin, 13.6 mmol of sodium citrate per liter, and 25.0 mmol of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) per liter in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution. Mononuclear cells were banded in Percoll gradients (1.05 g/ml; 220 × *g*, 20 min), washed, and incubated for 1 h with an anti-gpIIb,IIIa monoclonal antibody (AP2; a gift of T. Kunicki, Blood Center of Southeastern Wisconsin, Milwaukee) at 250 mg/10^6 cells at 0°C, and the MK were separated by using goat anti-mouse IgG-coated magnetic beads (Dynal, Inc., Great Neck, N.Y.). Purified MK were then pelleted, fixed in 1% glutaraldehyde in cacodylate buffer for 18 h, postfixed in osmium tetroxide, sectioned, stained with uranyl acetate, and examined on an electron microscope.

Platelet-associated viral antigen assay. Platelets were obtained from PRP by sedimentation as described above, fixed in 2% paraformaldehyde for 10 min, and reacted with a preoptimized quantity of high-titered anti-EIAV serum from a horse chronically infected with $EIAV_{WSU5}$. Following washing, platelets were reacted with fluoresceinated goat anti-equine IgG, washed, and analyzed by flow cytometry. Negative controls consisted of staining with preinoculation serum from the antiserum donor horse. Positive controls consisted of equine platelets reacted with monoclonal anti-human gpIIb,IIIa which had previously been shown to react with equine platelets, followed by fluoresceinated anti-mouse IgG.

Coagulation factor assays. To evaluate the contribution of the coagulation system to thrombocytopenia, three coagulation parameters were measured in appropriately collected and handled plasma samples, using commercial quantitation kits: fibrin degradation products (FDP; Murex Diagnostics, Norcross, Ga.), one-step prothrombin time (OSPT), and activated partial thrombin time (APTT; Baxter Diagnostics, Redmond, Wash.).

RESULTS

Clinical responses, platelets, and viremia. The mean normal body temperature, obtained from 558 separate daily readings on 32 uninfected foals, was 101.2°F (±0.51) [°C = (°F − 32) × 519], with only a slight, nonstatistically significant difference between immunocompetent and SCID foals. Fever and severe clinical disease were consistent in all foals infected with the highly virulent WYO strain of virus. Among foals infected with the relatively low virulence WSU5 strain, body temperature fluctuations in response to viral replication were somewhat greater in immunocompetent foals than in SCID foals (compare Fig. 2a with Fig. 4). The differences, however, were not statistically analyzed.

Normal platelet values were derived from 250 counts performed on 13 SCID and 18 immunocompetent uninfected foals 4 to 10 weeks old. An examination of histograms and sample moments indicated that the data were normally distributed. There were no consistent changes in platelet counts relative to age over this time period. There also was no statistically significant difference between the mean values from immunocompetent and SCID Arabian foals. Consequently, the data from the two groups were pooled to establish normal ranges. The

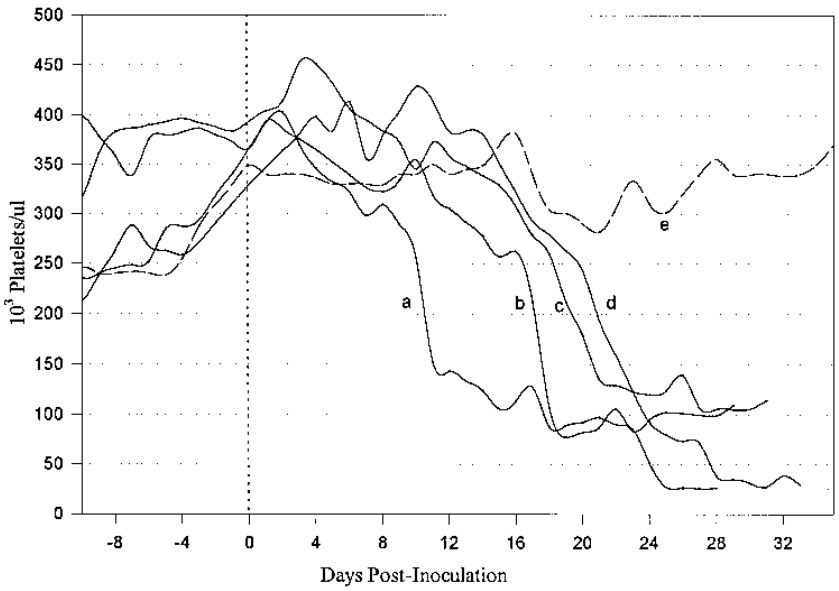

FIG. 1. Platelet counts in four EIAV-infected foals (a to d) and an uninfected control SCID foal (e). A total of $10^{6.0}$ $TCID_{50}$ of $EIAV_{WSU5}$ was inoculated i.v. on day 0, and platelet counts were monitored for the next 30 ± 3 days.

normal mean was 294,000 platelets per μl, and the upper and lower limits of the 95% confidence interval were 151,000 and 437,000/μl, respectively. Therefore, counts below 151,000/μl were considered to represent clinical thrombocytopenia.

Thrombocytopenia, accompanied by fever and a gradual decline in erythrocytes, was a prominent feature of onset of acute EIA in both SCID and immunocompetent foals. In SCID foals, which were infected with $EIAV_{WSU5}$, onset of thrombocytopenia was consistent and progressive, usually beginning between 12 and 16 dpi, with some as early as 8 dpi. The declines were generally dramatic, usually reaching thrombocytopenic levels (≤151,000/μl) within 4 to 9 days and levels of ≤50,000/μl within 10 to 14 days of onset of the decline (Fig. 1, foal a). Of 11 infected SCID foals, 8 reached extreme minima of less than 30,000 platelets μl (Fig. 1, foals b and d), and 3 plateaued at about 100,000 platelets per μl (Fig. 1, foals a and c) until termination at 30 ± 3 dpi. Uninfected SCID foals maintained normal platelet numbers throughout a similar experimental observation period (Fig. 1, foal e).

The relationship between the decline in platelet numbers and the onset of viremia, as detected by fibroblast infectivity assay, was relatively consistent. The onset of detectable viremia in SCID foals ranged from 9 to 11 dpi, with a mean of 10.2 ± 1.1 dpi (Table 1). The maximum virus titer observed in SCID foals was 5.1 $\log_{10}$ $TCID_{50}$/ml of plasma, with a mean maximum among six foals examined of 4.6 ± 0.4. In five immunocompetent foals examined, the earliest detectable viremia was at 5 dpi, with a mean of 8.0 ± 1.6 dpi. The highest virus titer observed in immunocompetent foals was 4.7 $\log_{10}$ $TCID_{50}$/ml, with a mean of 3.6 ± 0.6. The differences in viremia onset and maxima were significant ($P \leq 0.05$) by both the Wilcoxon rank sum and Student's *t* tests. SCID foals thus developed viremia more slowly but eventually experienced higher levels than normal foals. As observed previously in these animals (21), viremia was not episodic. Elevated plasma virus titers remained relatively constant until termination of the experiment at 30 ± 3 dpi.

In immunocompetent foals infected with the relatively low virulence $EIAV_{WSU5}$, the periods of marked platelet decline were, as with the SCID foals, consistently correlated with appearance of detectable viremia. Platelet counts rebounded promptly upon declines in viremia in the immunocompetent foals, often reaching normal levels within 3 to 5 days (Fig. 2b). A reciprocal relationship was often evident between body temperature and platelet counts in immunocompetent foals infected with $EIAV_{WSU5}$, one declining as the other rose (Fig. 2a), a phenomenon previously observed by others (4). However, immunocompetent foals infected with $EIAV_{WSU5}$ often failed to develop sufficiently reliable and severe clinical episodes to support effective labeling experiments (Fig. 2). Consequently, labeling experiments for platelet production and survival in immunocompetent foals utilized $EIAV_{WYO}$, which produced disease episodes of greater severity and predictability (Fig. 3b).

Occasional unexpected rises in platelet counts were observed both before (Fig. 3a) and after (Fig. 4a) infection. These were transient, usually lasting only a few days, and apparently

TABLE 1. First day of detectable viremia and maximum titers in SCID and immunocompetent foals infected with $EIAV_{WSU5}$

Group	Foal	Detection of viremia (day)[a]	Highest titer ($\log_{10}$ $TCID_{50}$/ml of plasma)
SCID	2053	9	4.9
	2057	12	4.9
	2064	10	4.7
	2073	9	4.3
	2086	10	3.9
	2090	11	5.1
Mean ± SD		10.2 ± 1.8	4.6 ± 0.4
Immunocompetent	2052	9	3.9
	2051	9	2.7
	2054	7	3.7
	2072	10	4.7
	2079	5	3.1
Mean ± SD		8.0 ± 1.6	3.6 ± 0.6

[a] First day on which viremia became detectable.

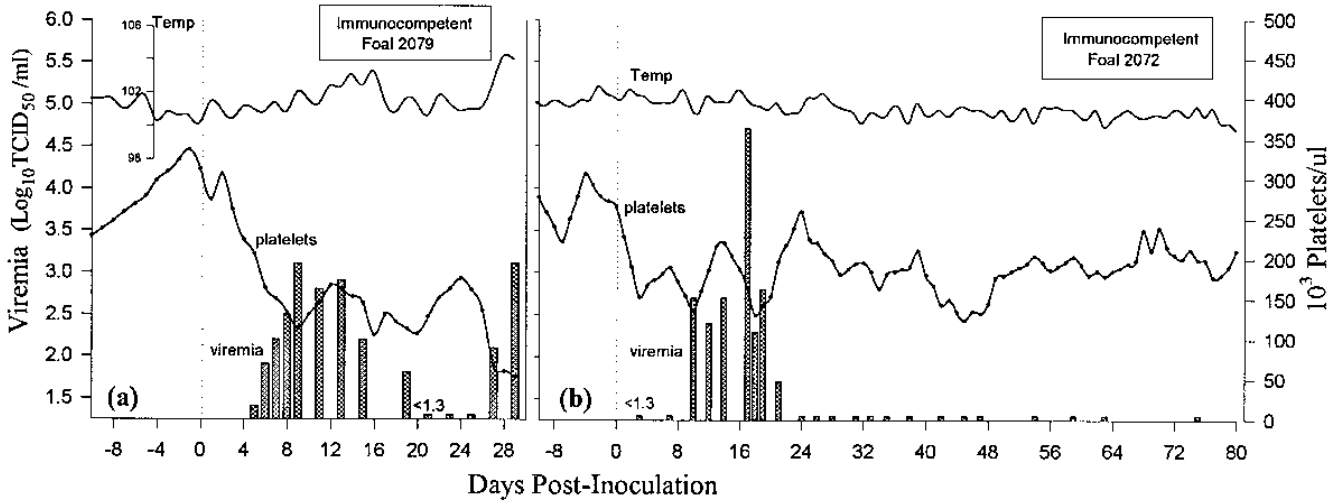

FIG. 2. Platelet counts, body temperatures, and viremia levels in two immunocompetent (non-SCID) foals following infection with $EIAV_{WSU5}$.

unrelated to EIAV. Also, in several of the immunocompetent foals, a phenomenon of moderate but dramatic declines in the platelet counts was observed in the first 1 to 3 days following infection (Fig. 2) that appeared not to be due to EIAV, as it was also seen in one sham-inoculated inoculated control. These declines, however, were short-lived. Counts returned to normal within 3 or 4 days in the sham-inoculated control (data not shown). This phenomenon was typically not seen in SCID foals and, when present, was much milder (Fig. 1 and 4).

The mean bleeding time in normal foals was 3.94 min, with a range of 3.0 to 8.0 min and standard deviation of 1.57 min. Above 50,000 platelets per μl, there was little correlation between bleeding times and platelet counts. Predictably, bleeding times increased markedly as platelet counts dropped below 50,000/μl.

Platelet production. Production was measured by metabolic incorporation of isotope by MK into platelets. Data presented are from paired labelings only, wherein the same foal was labeled both before infection and immediately after onset of thrombocytopenia, so that efficiency of label incorporation could be examined independently of interanimal variation. To examine early events, labeling experiments for both production and life span (see below) were initiated promptly after thrombocytopenic thresholds (≤151,000/μl) were reached. Platelet counts at the time of label injection (day 0) ranged from 115,000 to 141,000/μl (Table 2). Maximal incorporation peaks consistently occurred about day 5 (± ca. 0.5 day) after injection of label (Fig. 5). Maximal incorporation, as a percentage of the injected dose, was reduced by 35 to 89% in the postinfection period compared with preinoculation incorporation levels (Table 2). There was no apparent difference in abilities of the two labels, ^{75}Se and $^{35}SO_4$, to serve as effective measures of platelet production: levels of suppression as revealed by the two labels were generally similar.

Platelet life spans. Thirty-two platelet labelings by ^{51}Cr, 13 in SCID foals and 19 in immunocompetent foals, were performed. Platelet counts of infected foals at the time of labeling ranged from 68,000 to 193,000/μl. Experiments on infected immunocompetent foals were considered valid only if platelet counts were either stable or falling at the time of labeling and during the succeeding 5 days. Any experiments in which platelet counts suddenly started rising after the platelets were labeled were excluded from the data set. After excluding experiments in which data did not meet acceptable standards (25) for other reasons (e.g., excessive erythrocyte contamination or insufficient recovery following reinjection), we analyzed a total of 21 sets of data (Table 3).

Survival times of platelets, as estimated by the multiple-hit model, decreased in both SCID and immunocompetent foals following infection (Fig. 6). SCID foal platelet life spans decreased about 51%, from a mean of 3.5 ± 0.4 days ($n = 6$) before infection to 1.7 ± 0.3 days ($n = 3$) after infection. In immunocompetent foals, life spans decreased about 68%, from 4.1 ± 0.9 days ($n = 8$) to 1.33 ± 0.7 days ($n = 4$) in uninfected and infected groups, respectively (Table 3). Data were examined for significance by the two-sample t and Wilcoxon rank-sum tests. The difference in life spans between infected and uninfected groups was highly significant in both SCID and immunocompetent foal groups ($P = 0.004$ and 0.0008, respectively; two-sample t test). The differences between SCID and immunocompetent groups, however, were not statistically significant in either uninfected or infected groups.

Bone marrow MK. MK and denuded nuclei per square millimeter of evaluable bone marrow were counted in preinoculation, newly thrombocytopenic (≤151,000 platelets per μl), and necropsy samples. For data analysis, only foals in which complete sets of all three time point samples were available were included. Analysis of variance revealed no statistically significant differences between infected and uninfected foals except in nuclear areas (29). No differences were found between SCID and non-SCID foals; the data from the two groups were therefore pooled. The pooled mean values were 22.2 ± 8.1 and 18.2 ± 6.3 MK per mm^2 of evaluable marrow before infection and at necropsy, respectively. The mean numbers of denuded nuclei were 0.5 ± 1.5 and 1.5 ± 1.1 at these same time points.

Electron microscopy. Examination of 30 sections of fixed pellets of purified platelets from four different SCID foals during high viremia, as well as sections of MK concentrated by affinity purification, failed to reveal any budding structures suggestive of viral replication.

Membrane-associated viral antigen. Examination by flow cytometry of SCID foal platelets taken during periods of high viremia for membrane-bound viral antigen were uniformly negative. In contrast, positive controls consisting of anti-gpIIb,-IIIa antibody labeled the surface of equine platelets intensely.

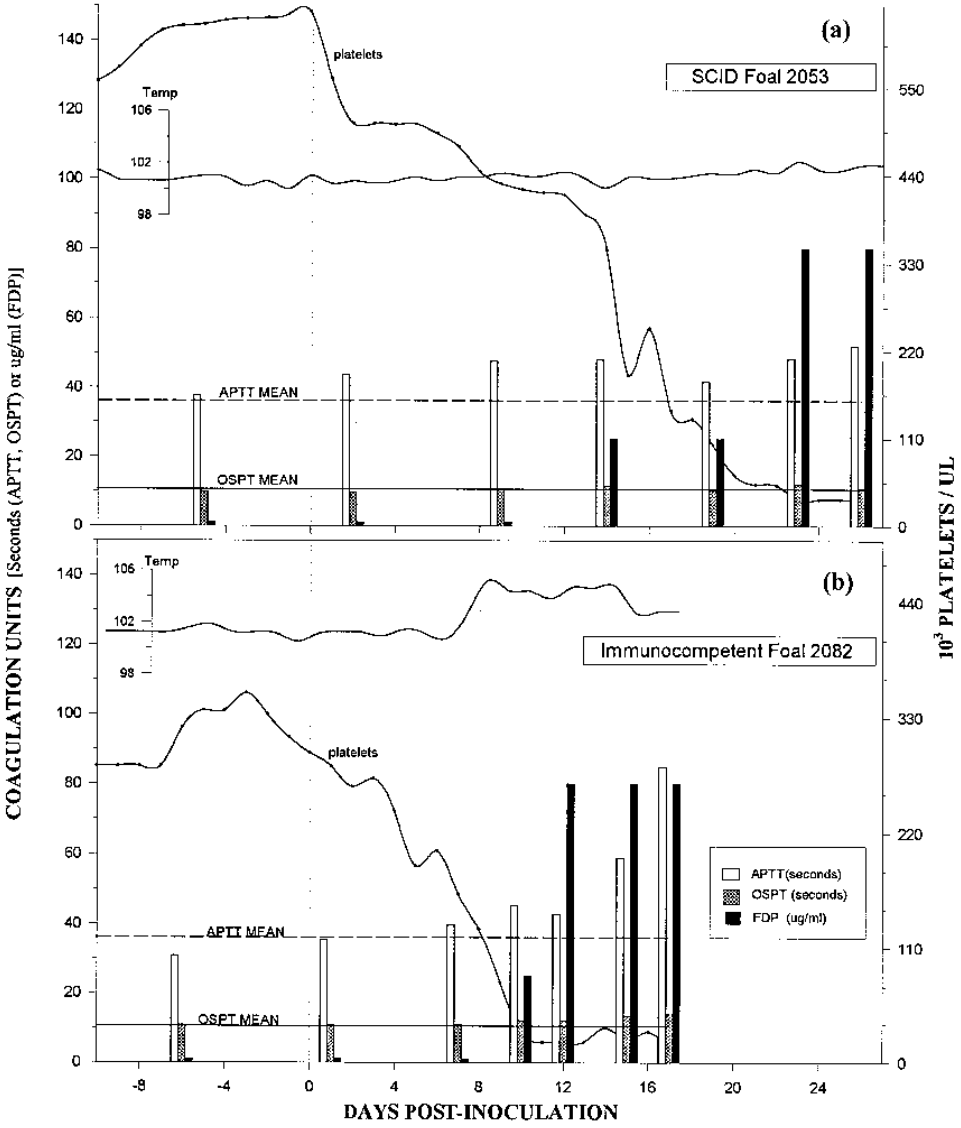

FIG. 3. Coagulation factor levels in plasma following EIAV infection. SCID foal 2053 (a) was infected with $EIAV_{WSU5}$ and immunocompetent foal 2082 (b) was infected with $EIAV_{WYO}$ on day 0, and levels of APTT, OSPT, and FDP were assayed before and during the decline in platelet counts. APTT and OSPT mean levels in noninfected foals are shown for reference.

Coagulation factors. FDP, APTT, and OSPT levels showed no appreciable increase until after platelet decline was well under way. APTT and FDP levels became elevated at about the time of clinical thrombocytopenia and increased progressively thereafter (Fig. 3). No significant alterations in OSPT levels occurred.

DISCUSSION

The focus of investigation into mechanisms of thrombocytopenia is increasingly turning to encompass mechanisms other than the traditional immune system-mediated consumption and direct infection of MK. The notion that positive or negative regulation of MK production maturation and function is important and perhaps predominant in acute viral disease is receiving heightened and much-deserved attention (16). This study establishes that the predominant mechanism of thrombocytopenia in EIA is related to production, not excessive consumption, and that it is not immune system dependent.

Metabolic radiolabel incorporation showed a marked suppression of platelet production during the early phases of the platelet decline. Incorporation of label into platelets was reduced between 35 and 89% during the early stages of thrombocytopenia. MK numbers per unit area of bone marrow, however, did not appreciably change, and no significant alterations in their morphology were found (29). By electron microscopy, no evidence of viral replication in MK was found. Even among retroviruses that replicate in MK such as the murine leukemia viruses, budding appears to occur preferentially across internal, rather than plasma, membranes (6). However, in the present study, only rarely were virion-like structures seen in MK, and no budding was found, either at the cell surface or in interior membranes. Neither our results nor those of Clabough et al. (4) yielded any evidence of viral replication in MK, either

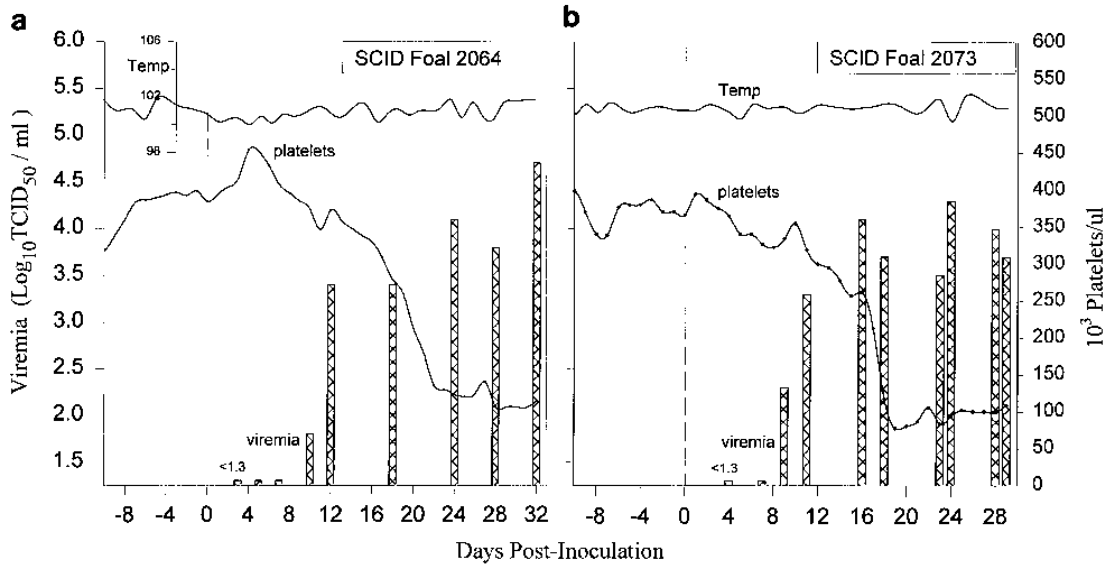

FIG. 4. Platelet counts, body temperatures, and viremia levels in two SCID foals following infection with $EIAV_{WSU5}$.

by electron microscopy or by in situ hybridization. This has been further confirmed recently in this laboratory by in situ PCR experiments, which also failed to find proviral DNA in MK (unpublished observations).

Perhaps not unexpectedly, platelet counts occasionally exhibited bizarre and unexplainable behaviors. Some foals experienced abnormal rises in counts that had no apparent cause. We attempted to guard against the known tendency for platelet values to fluctuate with stress and ill-defined changes in the environment by avoiding management-associated disturbances. All foals were housed in small quiet, air-conditioned isolation buildings, and personnel were consistent and gentle in their care of the foals. The causes for these spurious platelet fluctuations were not identified. The explanation for marked declines in platelets immediately following inoculation in the immunocompetent foals also was not found. It is possible, although speculative, that the nascent active immune response of the immunocompetent foals was involved, producing an antibody specificity that reacted with some nonviral antigen in the cell culture-derived inoculum. The exquisite sensitivity of platelets to activation by immune complexes could accelerate their sequestration, giving rise to the rapid decline.

Significant alterations in coagulation parameters did not appear until relatively late in the disease process, suggesting that disseminated intravascular coagulation did not play a major role in the development of thrombocytopenia.

The lack of platelet membrane-bound viral antigens is consistent with the conclusions of Clabough et al. (4). In their study, using immunocompetent adult horses, thrombocytopenia and reciprocal variations between platelets and viremia were first reported. They were unable to demonstrate virus on platelets by immunofluorescence, Western blotting (immunoblotting), or in situ hybridization. The elevation of platelet-associated immunoglobulins was attributed to adherence of virus-antibody complexes to platelets membranes, with the inability to demonstrate viral antigens resulting from masking by antibody or assay insensitivity (4). The fact that platelets from thrombocytopenic SCID foals in the present study were negative for membrane-bound viral antigen by flow cytometry argues against significant binding of EIAV components directly to platelet membranes, in that no masking by antiviral antibody would be present in the SCID foals. However, the membrane-bound immunoglobulin in immunocompetent foals could easily represent, as observed by Clabough et al. (4), antiviral

TABLE 2. Suppression of platelet production during acute EIA

Foal	Immune status	Virus	Infection status	Platelet count[a] (10^3)		Label[b]	Maximum label incorporation (%, 10^3)	% Suppression[c]
				Day 0	Day 5			
2053	SCID	WSU5	Pre	490	462	^{75}Se	252.3	
			Post	123	33	^{75}Se	83.2	68
2057	SCID	WSU5	Pre	288	271	$^{35}SO_4$	2.3	
			Post	124	38	$^{35}SO_4$	1.3	44
2064	SCID	WSU5	Pre	318	387	$^{35}SO_4$	2.6	
			Post	128	106	$^{35}SO_4$	1.7	35
2087	Immunocompetent	WYO	Pre	299	347	$^{35}SO_4$	5.7	
			Post	115	22	$^{35}SO_4$	0.6	89
2089	Immunocompetent	WYO	Pre	305	290	$^{35}SO_4$	5.2	
			Post	141	32	$^{35}SO_4$	1.0	82

[a] Platelet count per microliter of blood on days 0 and 5 following isotope injection.
[b] ^{75}Se, [^{75}Se]Selenomethionine; $^{35}SO_4$, $Na^{35}SO_4$.
[c] Calculated as (1 − postinfection percent incorporation/preinfection percent incorporation) × 100.

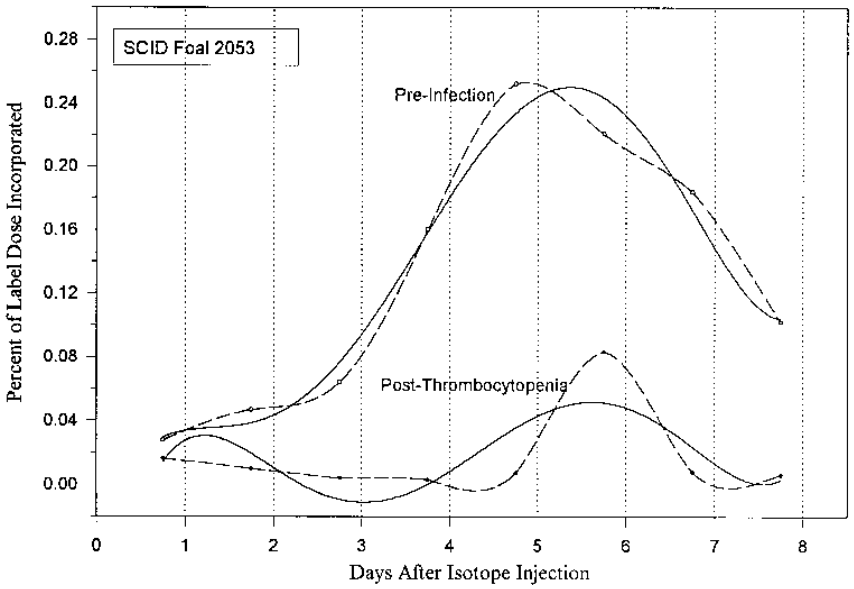

FIG. 5. Isotope incorporation into platelets of a SCID foal before and after infection with $EIAV_{WSU5}$. [^{75}Se]selenomethionine was injected i.v. at a dose of 5 μCi/kg before (○), and after (●) infection, during early thrombocytopenia. The percentage of the initial injected dose of isotope incorporated into washed platelets was determined daily. Dashed lines represent actual datum points; solid lines represent sixth-order regression plots.

antibody-viral antigen complexes bound to the platelet through immune adherence. In this regard, HIV antigen-antibody complexes appear to bind readily to human platelet membranes via Fc receptors (10). This mechanism could be at least partly responsible for the decreases in platelet half-lives observed herein. Life spans in immunocompetent foals were shortened somewhat more than in SCID foals, consistent with the notion that the effects of the immune clearance component and the nonimmune components evident in the SCID foals were additive.

TABLE 3. Platelet life spans in EIAV-infected and uninfected foals

Foal	Immune status	Virus	Platelet count[a] (10^3)		Platelet life span[b] (days)
			Day 0	Day 4	
2036	SCID	None	369	370	2.9
2044a	SCID	None	378	347	3.4
2044b	SCID	None	374	315	3.4
2056	SCID	None	247	199	3.6
2060	SCID	None	247	286	4.2
2073	SCID	None	338	387	3.6
Mean ± SD					3.5 ± 0.4
2046	Comp[c]	None	305	364	5.1
2052	Comp	None	242	259	3.3
2054	Comp	None	323	402	3.2
2055	Comp	None	318	260	5.6
2084	Comp	None	399	235	5.0
2085	Comp	None	497	465	4.2
2091	Comp	None	397	432	3.2
2092	Comp	None	204	352	3.3
					4.1 ± 0.9
2053	SCID	WSU5	145	63	1.5
2056	SCID	WSU5	193	58	1.4
2060	SCID	WSU5	82	35	2.2
					1.7 ± 0.3
2084	Comp	WYO	70	25	2.4
2085	Comp	WYO	68	44	0.6
2091	Comp	WYO	176	75	1.3
2092	Comp	WYO	126	40	1.0
					1.3 ± 0.7

[a] Platelet count per microliter of blood on days 0 and 4 following platelet labeling.
[b] Average platelet life span as estimated by the multiple-hit gamma-function model.
[c] Comp, immunocompetent.

Mechanisms of the shortened platelet life span in the SCID foals are more problematic. Possibilities include direct interaction between the virus and the platelet, reminiscent of platelet activation by feline coronavirus (2), or enhancement of platelet destruction by excessive elaboration of a cytokine (15). Moreover, activation of endothelial cells by viral infection or by increased levels of inflammatory mediators such as platelet-activating factor, interleukin-1, and tumor necrosis factor alpha could increase platelet adhesion, activation, and degranulation, thus shortening platelet life span. In this regard, EIAV proviral DNA sequences have recently been demonstrated in vascular endothelium by in situ hybridization in this laboratory (17).

Replication of EIAV in SCID foals is not subject to immunologic controls and thus does not display the episodic pattern seen in normal foals. In this study and in a previous study (21), viremia levels rose to the $10^{3.0}$ to $10^{4.5}$ range and persisted there until termination. In contrast, foals with functional immune systems experienced viremia in bursts or episodes, usually lasting only a few days before subsiding. In these animals, platelet counts generally followed a pattern reciprocal to that of virus titers, rebounding rapidly as viremia declined, as observed by Clabough et al. (4). The dramatic recovery of the platelets in the non-SCID foals does not seem compatible with a significant amount of damage to the MK population. The rate of the platelet rebound is most consistent with the existence of nonlethal, short-lived factors suppressive to the MK produced during the periods of active viral replication rather than with a deficiency of positive regulators. Attractive candidates include cytokines such as alpha interferon and tumor necrosis factor alpha.

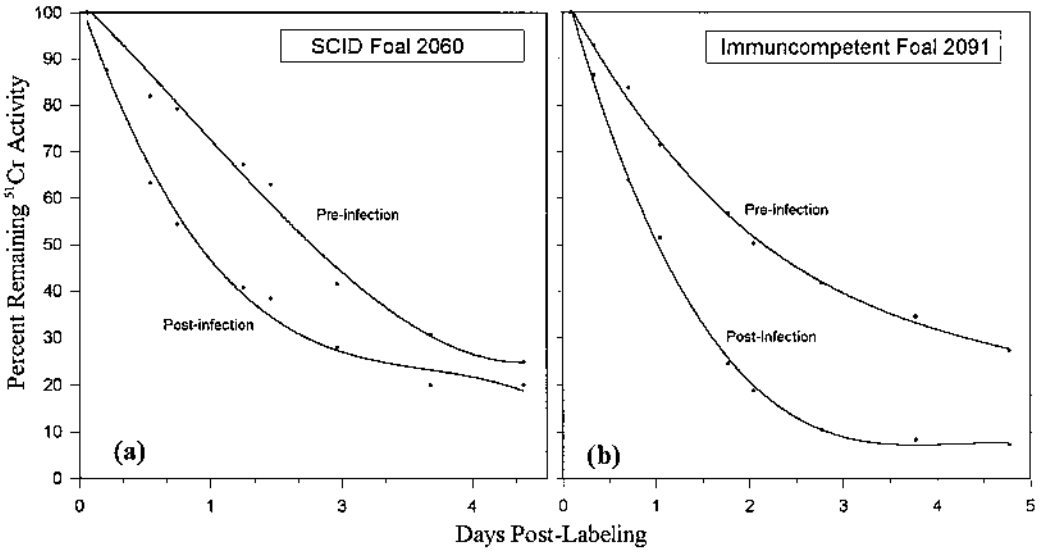

FIG. 6. Platelet survival curves. Platelets from a SCID (a) and an immunocompetent (b) foal before and after infection with EIAV, taken during the early phases of thrombocytopenia, were labeled with ^{51}Cr and reinjected, and isotope activity in 3-ml blood samples was assessed daily thereafter. The SCID foal (a) was infected with $EIAV_{WSU5}$, and the immunocompetent foal (b) was injected with $EIAV_{WYO}$.

Ballem et al., using indirect analysis of platelet kinetics in thrombocytopenic AIDS patients, found evidence of a reduction in both production and survival of platelets (1), reminiscent of observations made in the current study. It appears that thrombocytopenias of at least these two lentiviral infections share pathogenetic mechanisms to a significant degree. The concept that production deficits due to MK dysfunction are common and clinically important yet poorly understood phenomena recently has been emphasized (16).

The existence of the SCID foal model has allowed unequivocal demonstration of nonimmunologic suppression of the MK axis as a major mechanism of thrombocytopenia in this lentiviral disease. The earlier in vivo ferrokinetic studies of McGuire et al. (12) and in vitro marrow culture studies of Swardson et al. (27) both have implicated hematopoietic bone marrow suppression in the anemia of EIA. These studies used ponies or horses, neither of which were juveniles. The constraints of the SCID model prevent studying these same processes in more mature immunodeficient animals, but there is no apparent reason to believe that the mechanisms shown herein would not be operative in adult animals. Any age-related discrepancies would likely be quantitative, related to maturation, and not represent fundamental qualitative differences.

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