Colony Forming T Lymphocyte Deficit in the Development of Feline Retrovirus Induced Immunodeficiency Syndrome

By Sandra L. Quackenbush, James I. Mullins, and Edward A. Hoover

The identification and molecular cloning of a feline leukemia virus (FeLV) isolate (FeLV-FAIDS) that consistently produces immunodeficiency syndrome has allowed prospective investigation of events that occur in the prodromal phase of disease. Using a T-lymphocyte colony forming assay (T-CFU-Ic) we have demonstrated that a drastic depletion of circulating T-CFU-Ic prefigures the development of clinical immunodeficiency disease in inoculated cats and correlates with the appearance and replication of the FeLV-FAIDS variant genome in serially collected bone marrow samples. During the same presymptomatic time period, no significant alterations in conventional mitogen-stimulated lymphocyte blastogenic responses or in circulating lymphocyte numbers were evident. Thus T-CFU-Ic assay but not conventional mitogen-driven blastogenesis identified animals destined to develop immunodeficiency syndrome. The correlation among T-CFU-Ic depletion, the replication of the lymphocytopathic FeLV-FAIDS variant genome in hematopoietic and lymphoid tissues, and the onset of clinical disease, infers that ablation of a colony-forming T lymphocyte progenitor subset is important in the early pathogenesis of feline retrovirus-induced immunodeficiency syndrome.

MATERIALS AND METHODS

Animals

Specific pathogen free (SPF) cats were from a breeding colony in the Department of Pathology, Colorado State University and are free of feline viral diseases. Control cats were housed in separate rooms from FeLV-infected cats. Nine age-matched cats served as control animals for those animals that developed acute immunodeficiency disease. The data from 17 cats (≥1 year of age) were pooled and served as control data for those animals that developed chronic disease.

Viruses and Inoculation

Cats were anesthetized with ketamine hydrochloride (25 mg/kg) and inoculated intraperitoneally with 1 × 10^7 focus forming units of FeLV-FAIDS [titrated in clone 81 cells1]. Thirteen of the 15 inoculated cats included in this study were between 55 and 67 (mean, 61) days of age when inoculated. The remaining two animals were 98 (no. 1275) and 118 (no. 1081) days of age when inoculated.

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Three different FeLV-FAIDS inocula were used: (a) the original FeLV-FAIDS tissue-origin inoculum (designated DQ); (b) the first in vivo passaged, FeLV-FAIDS-DQ (designated as FeLV-FAIDS-1220 spleen or -1315 lymph node); and (c) supernatants from feline embryo fibroblast cells transfected with molecularly cloned FeLV-FAIDS from cat no. 1161 (designated as FeLV-FAIDS common form [61E] or variant [61C], or mixtures of the two [61E+61C]) (see Table 1). Because all clones of the full-length pathogenic variant genome were replication defective, and thus required the common form virus to produce infectious virus, the cloned 61E common form was used to rescue the transfected 61C variant genome.

**Assay for Persistent Viremia**

Viremia was detected by a modification of the indirect immunofluorescence assay described by Hardy et al. Previous work has shown that the presence of FeLV p27 structural antigen in circulating leukocytes and platelets correlates with presence of infectious virus in plasma, and thus is an indicator of viremia.2

**Hematology**

Complete hemograms were performed on each cat either biweekly or monthly after inoculation using a Coulter S-Plus II (Hialeah, FL) electronic counter calibrated for feline blood.

**Blood Mononuclear Cell Collection**

Mononuclear leukocytes were separated from 5 ml of heparinized blood by ficoll-hypaque (Histopaque-1077, Sigma, St Louis) density gradient centrifugation. The mononuclear cell layer was washed, resuspended in RPMI 1640 medium, and then resuspended in RPMI 1640 medium and centrifuged at 60 g for five minutes at 4°C. The pellet was resuspended gently and one drop examined under 200× magnification for rosette formation using 0.5% new methylene blue as a differential stain for leukocyte identification. Lymphocytes with three or more attached erythrocytes were considered to be positive rosetting cells. The presence of macrophages in CFU-IC was assessed by nonspecific esterase staining according to the method of Yam et al. Harvested colonies were cytokentrifuged onto glass slides, dried, fixed, and stained using alpha-naphthyl butyrate substrate.

**DNA Hybridization Analysis**

Isolation of DNA from feline tissues and Southern blot analysis were performed as described previously. Briefly, total cellular DNA (7.5 μg) was purified, digested with restriction endonuclease KpnI, electrophoresed in a 1.2% agarose gel, and transferred to nitrocellulose filters. Filters were prehybridized and then hybridized with an exogenous FeLVLTR specific probe (exU3) in the presence of 10% dextran sulfate and 50% formamide, washed, then exposed to x-ray film.

**RESULTS**

**Parameters of T-CFU-IC Assay**

**Cell concentration.** Lymphocyte concentrations ranging from 1 x 10^6 to 1 x 10^6 cells per well were seeded using a ConA concentration previously determined to be optimum in the liquid culture lymphocyte blastogenesis assay (1 μg/2 x 10^5 cells). The number of T-CFU-IC obtained correlated directly with both numbers of cells seeded and incubation time (through nine days). Five x 10^5 cells per well and an incubation period of seven days produced an optimal number of individual colonies that could be enumerated under phase contrast microscopy.

**Colony cell composition.** Lymphocyte colonies from cultures of seven normal cats were harvested and stained with Wright stain for morphologic examination. All the constituent cells were large (12 to 15 μm in diameter), with large nuclei, prominent nucleoli, and basophilic cytoplasm containing small vacuoles—characteristics of antigen- or mitogen-stimulated lymphocytes (Fig 1). The colony forming cells were feline T lymphocytes as indicated by spontaneous guinea pig E-rosetting by 89% of the cells (83% to 94%) and all cells were negative for nonspecific esterase staining. Although the erythrocyte rosette formation assay is
Clinical Syndromes in FeLV-FAIDS Inoculated Cats

Results from cats inoculated with FeLV-FAIDS viruses were analyzed in three groups, reflecting three clinical courses (Table 1).

1. Acute immunodeficiency syndrome developed in seven of 15 cats and was characterized by a survival period of <200 days postinoculation (dpi), a short asymptomatic period (2 to 10 weeks) followed by a progressive decrease in total blood lymphocytes, weight loss, diarrhea, and opportunistic infections (previously detailed in reference 13).

2. Chronic immunodeficiency syndrome developed in two of 15 cats and was characterized by survival periods >200 dpi. These animals had a long (6 months to >1 year) minimally symptomatic interval characterized by subnormal weight gain and fluctuations in blood lymphocyte number and blastogenic response to mitogens and eventually terminated by an accelerated wasting syndrome marked by weight loss and chronic severe diarrhea.

3. Six cats remained asymptomatic and chronically viremic. After observation periods ranging from 420 to >900 dpi, these animals had no clinical evidence of illness, slightly subnormal lymphocyte numbers, and normal blastogenic responses.

T-CFU-lc in Relation to Conventional Lymphocyte Blastogenesis and Onset of Clinical Acute Immunodeficiency Syndrome

Seven of the 15 FeLV-FAIDS inoculated cats developed the acute course of immunodeficiency syndrome and survived an average of 118 dpi (range, 67-199 dpi) (Table 1). In six of the seven cats a drastic reduction in the number of T-CFU-lc was seen either before (five animals) or concurrent with (one animal) initial clinical indications of feline immunodeficiency syndrome (weight loss, diarrhea, symptoms of bacterial rhinitis or pneumonia, ie, paroxysmal sneezing, dyspnea, mucoid nasal discharge) (Fig 2). The number of T-CFU-lc decreased from a mean of 692 (range, 206 to 1,648 [146% to 627% of control values]) to 14 (range, 4 to 34 [3% to 56% of control values]) (Fig 2, Table 2). At the same time, total blood lymphocytes decreased only slightly (10%) from a mean of 5,516 cells/μL (range, 3,040 to 8,170 [64% to 114% of control values]) to a mean of 4,807 cells/μL (range, 2,718 to 8,128 [36% to 120% of control values]) (Fig 2, Table 2). An elevation in T-CFU-lc, spanning an average duration of 31 days (range, 13 to 82 days),
preceded the precipitous decline in this cell population. At the time of initial T-CFU-lc decline, liquid culture ConA-induced lymphocyte blastogenic responses increased in four of six cats; blastogenic responses driven by pokeweed mitogen were elevated in three animals and subnormal in three, whereas the staphylococcus protein A responses were normal in two animals and subnormal in four (Table 2). In the one remaining cat which developed acute immunodeficiency disease, a more gradual and concurrent response to mitogens, and lymphocyte number occurred. Thus, in 86% (six of seven) of FeLV-FAIDS infected cats T-CFU-lc enumeration was more sensitive than liquid culture lymphocyte blastogenesis in predicting onset of acute immunodeficiency syndrome.

Fig 2. Comparison of total lymphocytes, ConA-driven lymphocyte blastogenesis and T-lymphocyte colony-forming assay before (14 days before) and at the time of initial depletion in T-CFU-lc in cats developing acute immunodeficiency syndrome (n = 6). Control = age matched SPF cats (n = 9).

T-CFU-lc in Relation to Detection of FeLV-FAIDS Variant Replication

Replication of FeLV-FAIDS common form viral genome was detected (by its characteristic 3.4 kb 3' internal 3' viral DNA fragment) in Southern blots of serially biopsied bone marrow cells from the onset of viremia (4 weeks postinoculation) and thereafter in all inoculated cats regardless of disease status. Replication of the FeLV-FAIDS disease-specific variant genome was detected by its signature 2.1 kb 3' internal viral DNA fragment in bone marrow biopsies either before or coincident with the initial decline in circulating T-lymphocyte colony-forming progenitor cells in three of the six cats (Figs 3 and 4). At necropsy the FeLV-FAIDS variant genome was also identified in lymphoid tissues (spleen, mesenteric, pharyngeal, colonic, mandibular lymph nodes), bone marrow, and intestine of all (six of six cats with acute FAIDS. Thus the appearance of FeLV-FAIDS variant genome replication in marrow correlated with onset of both blood T-CFU-lc depletion and clinical disease (summarized in Fig 5).

To determine whether the addition of IL-2 might stimulate growth of colony forming T lymphocytes, exogenous recombinant IL-2 was added to terminal T-CFU-lc assays of five cats. In four, no significant restoration of T-CFU-lc was observed. In one animal (no. 1607), however, IL-2 supplementation increased T-CFU-lc from 176 to 914, a restoration from 25% to 127% of control values. The animal with an increase in T-CFU-lc after addition of IL-2 survived for 26

Table 2. Lymphocyte Responses of FeLV-FAIDS-Inoculated Cats in Developing Acute Immunodeficiency Syndrome Before and at the Time of Depletion in T-Lymphocyte Colony-Forming Cells

<table>
<thead>
<tr>
<th>Cat No.</th>
<th>Weeks Post Inoculation</th>
<th>T-CFU-lc</th>
<th>Total Lymphocytes</th>
<th>ConA Blastogenesis†</th>
<th>SPA Blastogenesis†</th>
<th>PWM Blastogenesis†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1448</td>
<td>Before At</td>
<td>Before At</td>
<td>Before At</td>
<td>Before At</td>
<td>Before At</td>
<td>Before At</td>
</tr>
<tr>
<td></td>
<td>10 14</td>
<td>206 6</td>
<td>4,374 3,526</td>
<td>39,940 46,441</td>
<td>5,584 10,506</td>
<td>4,636 10,386</td>
</tr>
<tr>
<td></td>
<td>% control†</td>
<td>146 16</td>
<td>65 56</td>
<td>143 174</td>
<td>23 49</td>
<td>66 157</td>
</tr>
<tr>
<td>1449</td>
<td>Before At</td>
<td>452 4</td>
<td>3,040 2,718</td>
<td>90,585 38,901</td>
<td>15,005 4,146</td>
<td>22,608 3,841</td>
</tr>
<tr>
<td></td>
<td>% control</td>
<td>415 3</td>
<td>64 40</td>
<td>339 139</td>
<td>57 17</td>
<td>383 52</td>
</tr>
<tr>
<td>1539</td>
<td>Before At</td>
<td>1,648 34</td>
<td>6,351 5,729</td>
<td>42,441 20,831</td>
<td>98,912 6,852</td>
<td>75,024 2,865</td>
</tr>
<tr>
<td></td>
<td>% control</td>
<td>627 56</td>
<td>64 85</td>
<td>162 53</td>
<td>195 8</td>
<td>753 30</td>
</tr>
<tr>
<td>1540</td>
<td>Before At</td>
<td>366 22</td>
<td>6,123 8,128</td>
<td>23,049 74,379</td>
<td>120,470 85,070</td>
<td>7,499 12,458</td>
</tr>
<tr>
<td></td>
<td>% control</td>
<td>574 36</td>
<td>104 120</td>
<td>88 188</td>
<td>237 101</td>
<td>75 131</td>
</tr>
<tr>
<td>1541</td>
<td>Before At</td>
<td>348 4</td>
<td>5,035 5,595</td>
<td>60,641 62,528</td>
<td>40,144 20,437</td>
<td>8,600 6,093</td>
</tr>
<tr>
<td></td>
<td>% control</td>
<td>570 4</td>
<td>74 118</td>
<td>153 234</td>
<td>48 79</td>
<td>91 103</td>
</tr>
<tr>
<td>1607</td>
<td>Before At</td>
<td>1,128 10</td>
<td>8,170 3,146</td>
<td>11,071 34,218</td>
<td>7,440 16,008</td>
<td>2,309 5,912</td>
</tr>
<tr>
<td></td>
<td>% control</td>
<td>246 8</td>
<td>114 36</td>
<td>32 50</td>
<td>63 22</td>
<td>25 35</td>
</tr>
<tr>
<td>Mean</td>
<td>Before At</td>
<td>692 14</td>
<td>5,516 4,807</td>
<td>44,621 46,216</td>
<td>47,926 23,837</td>
<td>20,113 6,893</td>
</tr>
<tr>
<td>SEM</td>
<td>Before At</td>
<td>232 4</td>
<td>725 842</td>
<td>11,528 7,960</td>
<td>20,362 12,485</td>
<td>11,357 1,544</td>
</tr>
<tr>
<td>Mean % of control</td>
<td>Before At</td>
<td>430 21</td>
<td>81 76</td>
<td>153 140</td>
<td>104 46</td>
<td>232 85</td>
</tr>
<tr>
<td>SEM</td>
<td>% control</td>
<td>80 9</td>
<td>9 16</td>
<td>42 31</td>
<td>36 15</td>
<td>117 22</td>
</tr>
</tbody>
</table>

*2.5 μg ConA was added to 5 x 10⁴ cells, values are expressed as no. colonies/5 x 10⁴ cells seeded.
†Values are expressed as mean cpm of incorporated ³H-thymidine.
‡Percent control, inoculated cat data divided by the mean of age-matched control SPF cat data.
weeks post inoculation, whereas the other four cats survived a mean of 12.5 weeks (range, 10 to 14 weeks).

*T-CFU-Ic in Animals With Chronic Immunodeficiency Syndrome*

The chronic course of feline immunodeficiency syndrome in two of the 15 cats (no. 1081 and 1239) developed and was characterized by a prolonged prodromal period of minimally symptomatic viremia followed by an accelerated wasting syndrome and ultimately terminating in extranodal lymphoma (at 741 and 899 dpi, respectively). In cat no. 1239, a sequential comparison of conventional lymphocyte blastogenesis and T-CFU-Ic assays was performed over a 14-month period beginning at the time of initial detection of the feline AIDS variant genome in the bone marrow at 3.8 months (466 dpi). During this period T-CFU-Ic numbers averaged 18% of control values, whereas ConA blastogenic responses averaged 90% of control values (Fig 6). As in animals with the acute course of disease, the appearance of the FeLV-FAIDS variant in bone marrow biopsies and the decline in circulating T-CFU-Ic numbers heralded the development of clinical immunodeficiency syndrome, even when the prodromal period was extended to as long as 14 months.

The capacity of exogenous IL-2 to restore T-CFU-Ic was examined in one animal with chronic immunodeficiency syndrome (no. 1239) beginning 619 dpi. IL-2 supplementation (2.5 U/12 x 10⁶ cells) increased T-CFU-Ic numbers from 3% (26 colonies) to 86% (660 colonies) of non-IL-2 supplemented control values. Over the following 9 months, the average number of T-CFU-Ic detectable without IL-2 addition was 26 (3% of control) x 397 (52% of control) with IL-2 (Fig 7). Thus, in one animal with the chronic course of immunodeficiency syndrome, proliferation of colony forming T-lymphocyte progenitors was greatly augmented by exogenous recombinant IL-2.

*T-CFU-Ic in Cats With Chronic Asymptomatic Viremia*

Six of the 15 FeLV-FAIDS inoculated cats remained persistently viremic and asymptomatic after 420 to >900 dpi (Table 1). Total blood lymphocytes, lymphocyte blastogenic responses, and T-CFU-Ic were assessed throughout the course of infection. T-CFU-Ic depletion has been detected in only one of six cats (no. 1240, at 556 dpi)—the only cat in

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**Fig 3.** Sequential analysis of viral DNA present in the bone marrow of a cat inoculated with FeLV-FAIDS. SPF cat (no. 1448) was inoculated with 1 x 10⁶ ifu of FeLV-FAIDS, which had previously been passed in vivo in animal no. 1315. Bone marrow aspirates were obtained at 2-week intervals; total cellular DNA was extracted and analyzed by Southern transfer and hybridization using the FeLV exU3 probe (see Materials and Methods section). Days PI refers to the number of days post viral inoculation. MLN and CLN correspond to mesenteric and colonic lymph nodes, respectively; Spl. and SG correspond to spleen and salivary gland, respectively. The relative molecular mass of DNA is shown in kilobase pairs adjacent to the markers in lane M. RDF corresponds to a human cell line RD(FeLV)-2 that was infected with FeLV and cloned in vitro. In the RD(FeLV)-2 lane the 3.6 kb band corresponds to approximately 22-copy intensity, whereas the remaining bands correspond to single-copy per-cell intensity. The common form genome is recognized by the 3.4 kb band visible at 13 days PI and thereafter, whereas the variant genome is recognized by the 2.1 kb fragment observed at 76 days PI and thereafter. The 5 kb band visible in the 76 and 98 day lanes corresponds to a partial enzyme digestion product not observed in repeated analyses of these samples, whereas the 1.2 kb band in the 174 day sample may correspond to a recombinant virus.

**Fig 4.** Serial comparison of total blood lymphocytes, ConA lymphocyte blastogenesis, and T-CFU-Ic assay during the course of immunodeficiency disease development in cat no. 1448 as compared with data from age-matched normal SPF cats. Arrows represent the detection of the immunodeficiency-specific FeLV-FAIDS genome in bone marrow (nd, not done).
which the FeLV-FAIDS variant has been detected. FeLV-FAIDS common form viral genome has been detected throughout the course of infection in all the cats. Liquid culture blastogenic responses have varied from subnormal to elevated through the course of infection in all the cats. The absence of immunodeficiency syndrome in this group of animals is consistent with observations in separate studies demonstrating that the FeLV-FAIDS common form viral genome, although highly replication competent and infectious, fails to induce immunodeficiency syndrome when segregated from the defective FeLV-FAIDS variant genome by molecular cloning.19,77

**DISCUSSION**

This study demonstrates a correlation between the depletion of T lymphocyte colony forming progenitors, the onset of clinical immunodeficiency syndrome, and the replication of an immunodeficiency disease-associated feline leukemia variant virus genome in hematopoietic and lymphoid tissues. Drastic reduction in circulating T-CFU-lc numbers (to 21% of control values) occurred in six of seven cats that developed acute clinical immunodeficiency disease. During the same period mitogen-stimulated liquid lymphocyte blastogenesis remained normal or elevated and total blood lymphocyte numbers decreased only slightly (from 81% to 76% of control values). Depletion of circulating T-CFU-lc has been observed previously in AIDS patients and in patients with systemic lupus erythematosus.13,26 Possible reasons for the greater sensitivity of T-CFU-lc assay versus conventional lymphocyte blastogenesis in signaling the development of immunologic deficit include: (a) colony growth is restricted to T cells, whereas both T and B cells may proliferate in the suspension cultures: (b) T-CFU-lc represent approximately 1% of blood lymphocytes, whereas 30% of lymphocytes proliferate in the suspension cultures; and (c) the requirement for cytokine production is more crucial in the semisolid matrix where cell to cell contact is minimized.13,16,29,30 Those lymphocytes responding in the T-CFU-lc assay have greater proliferative ability than cells in suspension cultures and as suggested by others18,39 may represent lymphoid progenitor cells.

![Fig 5. Sequential correlation of T-lymphocyte colony-forming units, detection of FeLV-FAIDS variant virus replication in bone marrow cells, and onset of clinical symptoms of acute immunodeficiency syndrome in cats inoculated with FeLV-FAIDS.](image)

![Fig 6. Comparison of ConA-induced lymphocyte blastogenesis (ConA) and T-lymphocyte colony-forming assays (CFU-Ic) examined in the period after the appearance of FeLV-FAIDS variant (at 323 days PI) in bone marrow during the development of chronic immunodeficiency syndrome in cat no. 1239.](image)
forming assays in a cat (no. 1239) during the course of chronic disease. 

...the mechanism of lymphocyte ablation by FeLV-FAIDS remains undetermined, the correlation between high levels of unintegrated FeLV-FAIDS replication in lymphoid tissues of cats and the onset of lymphoid depletion merits further study. In cytopathic avian leukosis virus infections, progeny virus rapidly superinfects surrounding cells leading to production of unintegrated viral DNA and subsequent cell death. Our current work indicates high levels of unintegrated FeLV-FAIDS variant viral replication also occurs in feline T-lymphoblasts cytopathically infected in vitro and that membrane display of the viral extracellular glycoprotein (gp70) is increased (Quackenbush et al, unpublished data) while cleavage of its precursor (gp85) is retarded (Poss et al, unpublished data).

Addition of human recombinant IL-2 significantly increased the number of T-lymphocyte colonies formed in two of six cats with immunodeficiency syndrome. These results are similar to those obtained in human AIDS patients and suggest that progressive deficiency of IL-2-producing cells and eventually IL-2-responsive cells as well occurs during the progression of feline AIDS.

That assay of circulating T-CFU-lc appears to be a useful indicator of subclinical immune system deficit and lymphoid system reserve is suggested by data from human bone marrow transplant recipients. Whereas blood lymphocyte counts of transplant-reconstituted patients were normal or slightly decreased, mean T-CFU-lc numbers were only 5% of controls—a deficit that persisted for up to 2 years after reconstitution and could not be corrected by addition of IL-1, IL-2, or normal lymphocytes to T-CFU-lc assays, thus suggesting that an intrinsic deficiency in lymphocyte progenitors was present. The developing immunologic deficit in the pathogenesis of retroviral immunodeficiency disease also is revealed by a deficit in circulating colony-forming T-lymphocyte progenitors. Investigation of the effects of FeLV-FAIDS on T-CFU-lc, and other lymphocyte subpopulations using monoclonal reagents currently being developed should provide further insight into the mechanisms of lymphoid depletion in retrovirus-induced immunodeficiency syndrome.

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REFERENCES


Colony forming T lymphocyte deficit in the development of feline retrovirus induced immunodeficiency syndrome

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