Lymphocyte Subset Alterations and Viral Determinants of Immunodeficiency Disease Induction by the Feline Leukemia Virus FeLV-FAIDS

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The FeLV-FAIDS strain of feline leukemia virus consistently induces fatal immunodeficiency. To investigate the immunopathogenesis and viral genetic determinants responsible for the induction of immunodeficiency disease *in vivo*, we have generated chimeras between the two major viral genomes in the original virus isolate, designated common form clone 61E and major variant clone 61C, which were molecularly cloned directly from DNA of the same animal and tissue. Each of three 61E/C chimeras, containing at minimum a 34-amino-acid segment (including a 6-amino-acid insertion and one amino acid substitution) near the C terminus of the 61C surface glycoprotein (gp70), induced fatal immunodeficiency disease in all (12 of 12) infected animals over a course of 33 ± 10 weeks. By contrast, animals infected with virus 61E, although persistently antigenemic, remained asymptomatic throughout a 48-week observation period. Beginning 14 weeks after infection, a significant decrease (8 to 10%) in the percent of circulating CD4⁺ T lymphocytes developed in the 61E/C chimera-infected cats, compared with either 61E-infected or control animals. At this time, no significant changes were seen in CD8 cells, B cells, or mitogen-induced blastogenesis. Prior to this initial decline in CD4 cells, the ability of all antigenemic 61E/C-infected cats to generate a primary antibody response to the T-cell-dependent antigen keyhole limpet hemocyanin was markedly impaired, whereas all 61E-infected cats, one 61E/C-infected but nonviremic cat, and all uninfected control cats produced normal antibody responses. The results reported here demonstrate that a major determinant of *in vivo* immunodeficiency induction by FeLV-FAIDS is contained within a 34-amino-acid C-terminal segment of its surface glycoprotein and that this gp70 alteration determines the early and persistent deficits in CD4⁺ T lymphocytes and T-cell-dependent antibody responses. We hypothesize that these early immunologic alterations could result from early deletion of a CD4⁺ helper T-cell subset.

The feline leukemia virus FeLV-FAIDS strain consistently induces immunodeficiency disease in experimentally infected cats after incubation periods of 25 to >500 days, depending on age at time of infection and virus inoculum used (18). Two genomes associated with this natural isolate have been molecularly cloned from the same tissue of the same animal (1161): 61E, which is replication competent but minimally pathogenic, and 61C, which is replication defective but highly pathogenic (29). The generation of a replication competent, T-cell cytopathic viral chimera EECC demonstrated that the regions of the 61C genome responsible for pathogenicity were localized to the extracellular glycoprotein (gp70) or 3' long terminal repeat (LTR) or both (29). Nucleotide sequencing revealed that 61C differed from 61E by 11 scattered amino acid substitutions and the combination of an 18-base pair (6-amino-acid) deletion near the 5' end and a 6-amino-acid insertion near the 3' extremity of that gene (8, 28). In addition, three nucleotide substitutions were present in the 3' LTR (8, 29).

The above changes in the FeLV-FAIDS 61C variant genome impart differences in pathogenicity, antigenic recognition, and posttranslational modification of its envelope glycoprotein (29, 34). Moreover, the chimeric virus EECC

proved to be replication competent, extremely cytopathic for feline 3201 T cells, and capable of consistently inducing acutely fatal immunosuppression/enteropathy syndrome within 30 days, significantly shorter than the survival of animals infected with the original FeLV-FAIDS isolate or with mixtures of the molecularly cloned 61C and 61E viruses produced by cotransfection of cat cells (29). These and subsequent studies of 61E/C chimeric viruses (P. R. Donahue, S. L. Quackenbush, M. V. Gallo, C. M. C. de Noronha, J. Overbaugh, E. A. Hoover, and J. I. Mullins, submitted for publication) have focused particular interest on a C-terminal segment of the 61C gp70, which contains a 6-amino-acid insertion and one adjacent amino acid substitution.

FeLV is well established as a cause of immune dysfunction and opportunistic infections in naturally and experimentally infected cats (2, 13, 14, 17, 19, 20, 33). Humoral and cell-mediated immune response abnormalities have been reported by many investigators, implicating in particular T-cell alterations (4, 5, 19, 22, 25, 35, 42, 43, 46). The latter include paracortical lymphocyte depletion in lymph nodes, thymic atrophy (2, 33), delayed allograft rejection (33), decreased T-cell colony formation (35), decreased responses to T-cell mitogens and antigens in blastogenesis assays (4, 5, 6), impaired function in lymphocytes incubated *in vitro* with FeLV transmembrane protein p15E (25), and reduced anti-

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body responses to immunization with a T-cell-dependent antigen (TGAL) in naturally infected cats (43, 46). The pathogenesis of the immune deficit caused by FeLV, however, remains incompletely understood.

With the recent development of monoclonal antibodies to feline CD4, CD8, and a pan-T-cell surface marker (1, 21), investigations of specific lymphocyte subset deficits and the tropism of immunocytopathic FeLVs for these cells can be addressed. In the present study, we employ molecular chimeras between the FeLV-FAIDS 61E and 61C genomes and sequential lymphocyte immunophenotype analysis to investigate the pathogenesis and viral genetic determinants of immunodeficiency syndrome induced by FeLV.

MATERIALS AND METHODS

Animals, inoculation, and sampling. Sixteen 8-week-old cats from a breeding colony in the Department of Pathology, Colorado State University, Fort Collins, were used for virus inoculation studies. Four age-matched cats from the same source served as controls and were housed in separate isolation rooms from the inoculated animals. Cats from this colony were free of exposure to feline retroviruses and other feline viruses. These cats did, however, carry in their background flora, in addition to common bacteria, silent infection with a feline coronavirus. This feline coronavirus, while clinically silent in animals not infected with FeLV, may produce feline infectious peritonitis in immunosuppressed animals and thus serves as a sentinel opportunistic infection secondary to the immunodeficiency in FeLV-infected cats.

For inoculation, all cats were anesthetized with ketamine hydrochloride (25 mg/kg of body weight) and injected by the intraperitoneal route with 10^5 infectious units (11) of either FeLV-FAIDS 61E or one of the 61E/C virus chimeras described below. Inoculated cats were observed for evidence of immunodeficiency disease for up to 48 weeks postinoculation (p.i.). Animals which developed symptoms of immunodeficiency syndrome were euthanized by intravenous barbiturate overdose. Necropsy and histopathologic examination were performed on all animals euthanized in the study. Blood samples were collected every 2 weeks under anesthesia. At each sampling interval, complete hemograms, differential leukocyte counts, and lymphocyte immunophenotypes (see below) were determined.

Viruses. Three chimeras were constructed between the following two significant molecularly cloned components of FeLV-FAIDS: (i) 61E, the low-pathogenicity common form genome, and segments of the extracellular glycoprotein (gp70) and (ii) the 3' LTR of the variant 61C genome. The 61E genome was used as a background into which segments of the *env* 3' LTR of 61C were substituted by using the conserved restriction sites depicted in Fig. 1. Because the transmembrane proteins of the two viruses are identical, this results in substitutions only in the gp70- and 3' LTR coding regions. In the nomenclature for 61E/C chimeras, the *gag*, *pol*, *SU*, and *TM*-3' LTR whole gene segments are each designated by a single letter indicating the viral genome of origin for that segment (E, 61E; C, 61C). Within the surface glycoprotein-coding domain of 61E, substitution of any of 4 blocks of homologous sequences from the 61C gp70 is indicated by a four-letter designation within parentheses (illustrated in Fig. 1).

Infectious chimeric viruses were produced by transfection of the feline T-cell line 3201 (a gift from W. D. Hardy, Jr., and E. E. Zuckerman [40], Memorial Sloan Kettering Insti-

tute, New York, N.Y.). To increase viral titers for in vivo inoculation, the progeny viruses were amplified by short-term passage into the feline embryo adherent cell line, AH927 (originated by W. Nelson Rees, Naval Biological Laboratory). Groups of four cats each were inoculated intraperitoneally with either 61E or chimeras EE(EECC)C, EE(EECE)C, or EE(EECE)E, and four served as uninoculated controls.

Assays for antigenemia and viremia. Antigenemia and viremia are usually coincident in the FeLV system. Viral p27 *gag* antigen in circulating leukocytes and platelets was monitored by a modification (16) of the immunofluorescence assay of Hardy et al. (15). Soluble p27 antigen in serum was determined by the enzyme-linked immunosorbent assay described by Lutz et al. (24).

Blood mononuclear cell collection. Mononuclear cells were separated from 5 ml of heparinized blood by Ficoll-Hypaque density gradient centrifugation. The mononuclear cell layers were harvested, washed four times with phosphate-buffered saline (PBS), and suspended in lymphocyte medium composed of RPMI 1640 with 20% fetal calf serum (HyClone), 2% glutamine, 1% penicillin-streptomycin, and 2×10^{-5} M 2-mercaptoethanol (35).

Monoclonal antibodies to feline lymphocytes. Monoclonal antibodies to feline T-lymphocyte subsets were produced by immunizing mice with feline thymocytes and blood lymphocytes (1). B-cell monoclonal antibodies were produced by immunization with feline immunoglobulin G (IgG) or immunoglobulin M (21, 22). After immunization, mouse spleen or lymph node cells were fused with Ag8.653 myeloma cells, and positive clones were selected and further characterized by specific reactivity against feline blood, marrow, spleen, and lymph node lymphocyte populations and by radioimmunoprecipitation (1, 21, 22). The antibodies used here were fCD4 (fel7) and fCD8 (FT2), the feline equivalents of human CD4 and CD8; Mo43 (pan T), which recognizes a heterodimer common to both subsets of feline T cells; and BA6, which recognizes the lambda light chain of feline B cells (22).

Membrane fluorescence labeling of lymphocyte subsets. Mononuclear cells (10^6) were added to each tube and washed with 1 ml of PBS containing 2% fetal calf serum and 0.1% NaN_3 . Monoclonal antibody (25 μl) was added to each tube and incubated for 20 min at 4°C. Cells were washed three times with PBS-2% fetal calf serum-0.1% NaN_3 . Goat anti-mouse IgG fluorescein isothiocyanate (25 μl) (Kirkegaard and Perry) was added to each tube, incubated for 20 min at 4°C, and washed as described above. Cells were suspended in 1 ml of 2% paraformaldehyde in PBS and analyzed by flow cytometry.

Flow cytometry. Lymphocyte surface labeling was analyzed by using a Coulter EPICS Profile II flow cytometer equipped with a 488-nm Argon laser. Ten thousand lymphocytes were analyzed by electronic bitmap gating which was determined by forward angle versus side light scatter. Analysis gates were set so that less than 2% of lymphocytes labeled with an isotypic control were included in the positive analysis regions. Coulter ImmunoCheck beads were used to calibrate the flow cytometer such that the half-peak coefficient of variance was less than 2 and the mean peak channel intensity was within one standard deviation of established levels for the fluorescence and forward scatter photomultiplier tubes.

Lymphocyte blastogenesis. Lymphocyte blastogenesis assays in response to the mitogens concanavalin A, pokeweed mitogen, and staphylococcus protein A were performed as previously described for FeLV-FAIDS-infected cats (36).

TABLE 1. Terminal hematologic and lymphocyte subset data of FeLV-FAIDS cats

Virus Designation	Mean (range) survival (weeks p.i.)	Mean no. of total WBC (SD) ^a	Mean no. of total lymphocytes (SD)	Mean % CD4 (SD)	Mean no. of total CD4 (SD)	Mean % CD8 (SD)	Mean no. of total CD8 (SD)	Mean hematocrit (SD)
EE(ECCC)C	26 (20–32)	16,304 (8,044)	3,209 (1,001)	23 (5)	698 (151)	18 (2)	564 (198)	24 (9)
EE(EECE)C	36 (31–48)	14,937 (9,249)	2,606 (1,174)	26 (14)	567 (139)	15 (6)	349 (191)	23 (5)
EE(EECE)E	36 (22–46)	11,330 (2,296)	2,424 (848)	27 (10)	632 (155)	22 (11)	448 (111)	24 (5)
All chimeras ^b	33 (20–48)	14,190 (6,871)	2,746 (984)	26 (9)	632 (146)	18 (7)	454 (180)	24 (6)
61E EE(EEEE)E	48	11,331 (48)	6,287 (694)	25 (2)	1,533 (37)	25 (4)	1,587 (441)	47 (4)
Uninoculated controls	>48	13,511 (3,272)	7,155 (2,816)	33 (4)	2,415 (902)	18 (5)	1,407 (831)	37 (5)

^a WBC, Leukocytes.^b Mean data.

T-cell-dependent antibody generation and assay. Cats were immunized with 1 ml of 100 µg/ml keyhole limpet hemocyanin (KLH) (Calbiochem, Behring diagnostics) in PBS at 11, 12, and 13 weeks p.i. A stock solution (50 µl) containing 20 µg of KLH per ml of PBS was added to each well of Immunolon-2 (Dynatech Laboratories, Inc.) 96-well microtiter plates and dried overnight at 37°C. Washing buffer (150 µl) (0.2% Tween-20 in PBS) was added to each well and incubated for 10 min at room temperature. The washing buffer was discarded, and 50 µl of serum (1:200) was added to each well and incubated for 1 h at room temperature. Plates were then washed three times with washing buffer, and 50 µl of anti-cat immunoglobulin G-peroxidase (1:1,000) (Kirkegaard and Perry) was added to each well and incubated for 30 min at room temperature. Plates were washed as described above, 100 µl of substrate solution (4 mg of *o*-phenylenediamine, 10 ml of 80 mM citrate-phosphate, and 4 µl of 30% H₂O₂) was added per well, and plates were placed in the dark for 20 min. A 25-µl portion of 2 N H₂SO₄ was added to each well, and plates were read on a Dynatech 700 enzyme-linked immunosorbent assay reader at 490 nm.

Statistics. Student's *t* test was used for all statistical analyses.

RESULTS

Disease induction. (i) Course of disease. Consistent with previous studies, FeLV-FAIDS 61E induced persistent antigenemia in all animals inoculated but did not induce disease throughout the 350-day observation period of the study. In total, 26 cats infected with 61E have been followed for a total of 12,611 animal days (mean, 485 days per cat; range, 350 to 812 days), with no cases of immunodeficiency disease and two cases of lymphoma developing (mean, 635 days p.i.), nor did control animals develop disease. By contrast, all 12 cats inoculated with the FeLV-FAIDS 61E/C chimeric viruses containing at least the third (102-base-pair) segment of the extracellular glycoprotein (gp70) with or without the 3' LTR from 61C developed persistent viremia and fatal immunodeficiency disease (Table 1). The mean survival period for 61E/C chimera-infected cats was 32.75 ± 10.02 weeks (229 ± 70 days). One of these animals (2203) developed initial antigenemia between weeks 2 and 4 p.i., then became negative for circulating p27 in serum and cells between weeks 4 and 20, finally reverted to persistent viremic and antigenemic status at week 22, and died of immunodeficiency

syndrome at week 46. The mean survival times of the animals in each of the three 61E/C chimeric groups (EE[ECCC]C, EE[EECE]C, and EE[EECE]E) (Fig. 1) were not significantly different. These results thus indicated that the third (102-base-pair 34-amino-acid-coding) segment of the 61C gp70, when transplanted into a 61E background, was sufficient to confer fatal immunodeficiency induction in vivo.

(ii) Symptoms. Symptoms of clinical immunodeficiency common to all 12 chimera-infected animals were progressive weight loss, persistent diarrhea, pallor (due to anemia), inactivity, and progressive debilitation. In addition, 6 of the 12 61E/C-infected cats developed the opportunistic infection

FIG. 1. FeLV-FAIDS 61E/C chimeric viruses used. The 61E genome was used as background into which segments of the *env* 3' LTR of 61C were substituted. The three 61E/C chimeras have 61E *gag* and *pol* regions in common. The *env* gene was segmented by using the designated restriction enzymes into four regions as illustrated. The viral genome contributing each segment is designated E for 61E or C for 61C. The vertical lines indicate single amino acid substitutions and the triangles represent 6-amino-acid deletion and insertion points.

feline infectious peritonitis (30–32), a polysystemic infection caused by an enteric coronavirus (30–32) which is carried asymptomatically in our specific-pathogen-free cats but causes disease in immunosuppressed FeLV-infected animals (see “Animals, inoculation, and sampling,” Materials and Methods). Terminal feline infectious peritonitis was characterized by severe fibrinopurulent ascites and icterus, characteristic of the classic wet form of the disease (47).

Hematologic alterations. (i) **Total leukocytes and lymphocytes.** While terminal total leukocyte counts of 61E/C-infected cats (mean, $14,190 \pm 6,871$ cells per μl) were not significantly different from those of controls (mean, $13,511 \pm 3,272$ cells per μl) or 61E-infected cats (mean, $11,331 \pm 48$ cells per μl at 48 weeks p.i.), terminal total lymphocyte numbers were significantly reduced in 61E/C animals (mean, $2,746 \pm 984$ cells per μl versus mean of controls, $7,155 \pm 2,816$ cells per μl ; $P < 0.0001$) (Table 1). By contrast, the mean number of total circulating lymphocytes of 61E-infected animals was $6,287 \pm 694$ cells per μl (at 48 weeks p.i.), slightly lower than uninfected controls but significantly higher ($P \leq 0.0002$) than the 61E/C animals. As early as 4 weeks p.i., the numbers of circulating lymphocytes of the 61E/C-infected cats were significantly less than those of controls, whereas the mean lymphocyte numbers of 61E-infected cats, while less than control mean, were still within the control range (defined by mean ± 2 standard errors of the mean) (Fig. 2B). In particular, lymphocyte counts of 61E/C cats decreased consistently and progressively from week 16 to termination (Fig. 2A). Animal 2203 was significant in that regression of its antigenemia between weeks 4 and 20 correlated with a rebound in lymphocyte numbers to above the control range (Fig. 2A). Reappearance of p27 antigenemia in animal 2203 at week 22, however, was followed by decline in lymphocyte count and then by symptomatic immunodeficiency.

(ii) **Red blood cells.** Hematocrits of 61E/C-infected cats were significantly lower (mean, 23.5 ± 6.2) than those of either the control (mean, 36.6 ± 4.5 ; $P < 0.0001$) or the 61E-infected group (mean, 47 ± 4.2 ; $P < 0.0002$ at 48 weeks p.i.) Of the 12 61E/C cats, 75% (9 of 12) were anemic (hematocrit < 30) when frank disease occurred (Table 1).

Thus, cats infected with the 61E/C chimeras, in contrast to those infected with the 61E parent virus, had consistent presymptomatic decreases in both total circulating lymphocytes and red blood cell numbers.

Lymphocyte subsets. (i) **T cells.** Beginning 14 weeks p.i., there was a progressive decrease in total number of T lymphocytes of 61E/C cats which paralleled the decrease seen in the number of total lymphocytes (Fig. 2C). The mean percent of T lymphocytes in the 61E/C cats at week 14 was $41.55 \pm 7.24\%$ versus the control mean of $56.25 \pm 4.99\%$ ($P < 0.001$) (Fig. 3A). Transient increase in T-cell numbers occurred in one cat (2203) during the period of viremia regression and was followed by T-cell depletion when viremia recurred and terminal disease developed (Fig. 2C). The total number of T cells in the 61E animals, while lower than the mean value of controls, were within the control range through most of the course of infection (Fig. 2D). The percent of T cells in 61E animals remained within control values through week 37, then decreased through week 48 (Fig. 3B).

(ii) **CD4 cells.** Beginning 14 weeks p.i., the percent of CD4 cells in 61E/C infected cats became significantly decreased (mean, $25.08 \pm 8.19\%$ versus $33 \pm 3.65\%$ for controls; $P < 0.04$) and remained so through 33 weeks p.i. (Fig. 3C). A transient increase occurred in three cats surviving to 41

weeks p.i., although the total number of CD4 cells (mean, 708 ± 254 cells per μl) remained lower than controls ($2,758 \pm 1,412$ cells per μl ; $P < 0.03$). The terminal mean percent of CD4 cells of 61E/C cats was $25.5 \pm 9.32\%$, significantly lower than that of controls ($33.47 \pm 3.56\%$; $P < 0.0001$) (Table 1). Overall, decrements in total CD4 cell numbers in 61E/C cats paralleled those in total lymphocytes and T cells in 61E/C cats (Fig. 2E). The mean terminal CD4 count of 61E/C cats was 632 ± 146 cells per μl , significantly lower than that of the control (mean, $2,415 \pm 902$ cells per μl ; $P < 0.0001$) or 61E-infected groups (mean, $1,533 \pm 37$ cells per μl at 48 weeks p.i.; $P < 0.0001$); the latter were generally below the control mean but remained within the control range throughout the study (Table 1) (Fig. 2F). No significant differences in time of onset or magnitude of CD4 cell depletion were evident among the 3 chimeric viruses.

(iii) **CD8 cells.** Changes in percent of CD8 cells of 61E/C cats were less significant than those in either pan-T or CD4 cells (Fig. 3E and H). Terminal percentages of CD8 cells in the 61E/C cats were similar to those of controls (mean, $17.92 \pm 7.35\%$ versus $18.30 \pm 5.19\%$ for controls) (Table 1). In contrast to the percent of CD8 cells, the total numbers of CD8 cells in 61E/C cats were significantly lower than either controls ($1,407 \pm 831$ cells per μl) or 61E cats ($1,587 \pm 441$ cells per μl) (Table 1) (Fig. 2G and H). Total numbers of CD8 cells of 61E cats were similar to control values (Fig. 2H).

(iv) **B cells.** We were able to evaluate B-cell numbers and percentages only through week 26 p.i. for the 61E/C cats. During this period, both the mean total and percent of B cells were slightly greater than control values, although not significantly so (data not shown). Likewise, B-cell numbers in the 61E group were within the range of control cats when evaluated between 10 and 16 weeks p.i.

In summary, early depletion in relative and absolute numbers of CD4 cells and total T lymphocytes (but not CD8 lymphocytes) characterized the immunodeficiency induced by all three FeLV-FAIDS 61E/C chimeric viruses.

T-dependent antibody and lymphocyte blastogenesis responses. At 10 weeks p.i., when total lymphocytes of the 61E/C and 61E cats were near the lower extremity of the control range, integrated immune system function was evaluated by immunizing animals with 100 μg of the T-cell-dependent antigen KLH at 11, 12, and 13 weeks p.i. and measuring specific antibody generation. Whereas the four control and the four viremic 61E animals generated an immune response to KLH, none of the 11 61E/C viremic cats were able to do so. Significantly, the only 61E/C cat able to produce a primary antibody response to KLH (2203) was virus-negative during the period of immunization (Fig. 4).

Lymphocyte blastogenesis in response to the mitogens concanavalin A, staphylococcus protein A, and pokeweed mitogen was assessed in the 61E/C cats at this same interval (16 weeks p.i.). All 61E/C-infected, viremic cats produced responses comparable to control cats and to the one 61E/C regressor (nonviremic) cat 2203 (Fig. 5).

Thus, as early as 11 weeks p.i., before significant deficits in circulating T-cell numbers or blastogenic response were detectable in 61E/C chimera-infected animals, a substantial deficit in ability to generate a primary T-cell-dependent immune response was present.

Histologic lesions. In all (12 of 12) of the 61E/C cats, severe lymphocyte depletion was present in the cortical, paracortical, and medullary regions of the lymph nodes (mesenteric, pharyngeal, colonic, and mandibular) and in the spleen (Table 2). In nodes, cortical lymphoid follicles were small, no secondary follicles were present, paracortical areas were

FIG. 2. Serial analysis of total lymphocytes and lymphocyte subsets of FeLV-FAIDS 61E/C chimeras, 61E, and control cats. Control values are depicted in the shaded area and represent the mean $\pm$ standard deviation ($n = 4$). Symbols: (A, C, E, and G) ----, control; —■—, 61E/C chimeras; ----◆----, 2203; (B, D, F, and H) ----, control; —■—, 61E.

FIG. 3. Serial analysis of the percent of positive circulating lymphocyte subsets of FeLV-FAIDS 61E/C chimeras, 61E, and control cats. Control values are depicted in the shaded area and represent the mean $\pm$ standard deviation ($n = 4$). Symbols: (A, C, and E) ----, control; —■—, 61E/C chimeras; ----◆----, 2203; (B, D, and F) ----, control; —■—, 61E.

depleted, and vessels were more prominent because of background cell depletion. Of the 12 61E/C animals, 25% (3 of 12) had evidence of chronic enteritis, characterized by atrophy and fusion of absorptive villi, distortion and regen-

eration in the germinal (crypt) zone, residual necrotic cellular debris in the crypts, and focal infiltrations of mixed leukocytes in the jejunum or ileum. In the bone marrow of 58% (7 of 12) of the 61E/C cats, depletion of both myeloid

FIG. 4. Primary antibody generation in response to immunization with KLH in cats infected with FeLV-FAIDS chimeras, 61E, and control cats. Data represent the mean optical density (OD) $\pm$ standard deviation for each group.

and erythroid progenitor cells was accompanied by multifocal follicular infiltration of lymphocytes. In each of the 6 animals (6 of 12 total animals) in which the secondary disease, feline infectious peritonitis, was recognized clinically and at necropsy, granulomatous lymphadenitis affecting the mesenteric nodes and fibrinopurulent serositis were present (Table 2). No significant difference in frequency or severity of lymphoid depletion or opportunistic infections could be discerned among the three 61E/C chimera groups. None of the above lesions were present in either 61E-infected or control animals.

DISCUSSION

The results from these in vivo studies of FeLV-FAIDS chimeric viruses reinforce those focusing on the determinants of T-cell cytopathicity in vitro (P. R. Donahue et al., submitted for publication). Viral chimeras which contain at least a 3' 102-base-pair segment of the 61C variant gp70 gene inserted within the framework of common form genome 61E are both T-cell cytopathic and induce immunodeficiency disease. Thus, it appears that the C-terminal 34 amino acids

FIG. 5. Blastogenic response to mitogens in cats infected with FeLV-FAIDS chimeras and control cats. Data represent the mean cpm $\pm$ standard deviation. Abbreviations: ConA, Concanavalin A; SpA, staphylococcus protein A; PWM, pokeweed mitogen.

of the surface glycoprotein, which includes a 6-amino-acid insertion and one other amino acid substitution, are sufficient to mediate the immunodeficiency characteristic of FeLV-FAIDS. It would appear from the work of Donahue et al. (submitted) that the cytopathicity of the 61C genome is enhanced by other regions of its gp70 gene. Thus, the chimera EE(CCCC)C, which contains the entire *env* 3' LTR of 61C fused to the 5' *gag-pol* LTR of 61E, is so acutely pathogenic that fatal disease results in approximately 1 month, which is less than the mean survival time observed in transmission studies with the original tissue-origin FeLV-FAIDS isolate (18) and less than that found in similar studies with mixtures of the 61C and 61E molecular clones (29). In these previous studies, we defined two courses of infection which were distinguished by the latent period from infection to clinical immunodeficiency induction: an acute course characterized by a mean survival of 86 ± 42 days and a chronic course with a mean survival of 396 ± 113 days, the latter occurring in older animals and better resembling the slower progression of immunodeficiency caused by FeLV in naturally infected cats and by human immunodeficiency virus (HIV) in people (9, 18). By reducing the component of the 61C *env* 3' LTR in 61E/C chimeras to as little as 34 amino acids, it would appear we have more closely and uniformly simulated the more gradually progressive panoramic im-

TABLE 2. Lesions in cats infected with FeLV-FAIDS virus chimeras^a

Cat. No.	Chimera	Lymph nodes	Spleen	Bone marrow	Small Intestine
2194	EE(EECC)C	LD, GL	LD, FP	NE	E
2197	EE(EECC)C	LD	LD	LI, HD	NSL
2199	EE(EECC)C	LD, GL	LD, FP	NE	NSL
2200	EE(EECC)C	LD, GL	NE	LI, HD	NSL
2201	EE(EECE)E	LD, GL	LD, FP	LI, HD	NE
2202	EE(EECE)E	LD, GL	LD, FP	HD, LI	FP
2203	EE(EECE)E	LD	LD	LI	NSL
2204	EE(EECE)E	LD	LD	LI, HD	NSL
2205	EE(EECE)C	LD, GL	LD	LI, HD	NSL
2206	EE(EECE)C	LD	LD	MH	E
2207	EE(EECE)C	LD	LD	NSL	E
2208	EE(EECE)C	LD	NE	LD	NSL

^a LD, Lymphoid depletion; E, enteropathy; GL, granulomatous lymphadenitis; LI, follicular lymphoid infiltrate; HD, hemopoietic cell depletion; NSL, no significant lesion; FP, fibrinous peritonitis; NE, not examined; MH, myeloid hyperplasia.

mune deficiency which develops in adult cats infected with FeLV-FAIDS or other virus strains under natural conditions.

That the 61E/C chimera-infected animals developed a disease characteristic of retroviral immunodeficiency syndrome was established by quantitation of the two major feline T-lymphocyte immunophenotypes and by documentation of the features of hematologic suppression, immunologic deficit, and opportunistic infection established in previous studies (18) with FeLV-FAIDS. Both the preclinical and clinical phases of disease were manifested by progressive decreases in T lymphocytes, total and relative CD4 lymphocytes, and, to a lesser extent, CD8 lymphocytes. The CD4 cell subset was most severely affected in the 61E/C-infected cats. A significant decrease in the percent of CD4 cells was detected 14 weeks after infection, when no change in CD8 cells, B cells, or lymphocyte blastogenesis were discernible. CD8 cells became significantly depleted only in the later stage of disease, when decreases in total lymphocytes were present. The total numbers of lymphocytes and CD4 cells of 61E-infected animals, while less than those of controls, were significantly higher than those of the 61E/C group and did not decline below 900 cells per μ l throughout the 48-week course of the study. The mean CD4 cell count of 61E/C chimera-infected cats at the time symptoms of immunodeficiency were first documented was 915 cells per μ l. Thus, CD4 cell depletion to levels below 900 cells per μ l appeared to be a significant correlate of clinical immunodeficiency induced by FeLV-FAIDS 61E/C chimeras and may serve as a guideline for assessing immunodeficiency states associated with FeLV infections in nature.

Another prominent early indicator of infection by FeLV-FAIDS chimeric viruses was early loss of ability to generate a primary antibody response to T-cell-dependent antigens. This deficit was first documented in FeLV-FAIDS-infected cats by Pardi et al. (D. Pardi, E. A. Hoover, S. L. Quackenbush, J. I. Mullins, and G. N. Callahan, *Vet. Immunol. Immunopath.*, in press) in an earlier study employing mixtures of the cloned 61E and 61C viruses. However, Trainin, Wernicke, and colleagues (43, 46) first reported this abnormality in 1983 in cats naturally infected with FeLV. Recent evidence suggests that a similar deficit exists in HIV-infected people (23, 26, 41). We are currently investigating the possibility that the deficit in T-cell-dependent antibody formation in FeLV-FAIDS-infected animals may reflect early deletion of a CD4 subset responsible for help in antibody production. Precedent for a selective loss of CD4+ helper T-cell function in retrovirus-induced immunodeficiency is found in the work of Shearer and colleagues (3, 39) in patients with presymptomatic HIV infection in which loss of major histocompatibility complex-restricted proliferative responses to influenza virus and tetanus toxoid is among the earliest immunologic abnormalities detected.

Other signs of immunodeficiency caused by the FeLV-FAIDS chimeric viruses were progressive weight loss, intermittent diarrhea, nonregenerative anemia, and the opportunistic infection feline infectious peritonitis, which occurred as a preterminal disease in 50% (6 of 12) of the 61E/C-infected cats but in none of the 61E or control animals. The cats in our colony carry the causative coronavirus as an asymptomatic infection; however, in 20 years of experience with animals from this closed colony, we have never observed feline infectious peritonitis in an animal not infected with an immunosuppressive retrovirus. The concordance of FeLV and feline infectious peritonitis infections in nature is well established (30–32). The results of the present

study suggest that FeLV immunodeficiency predisposes the cat to development of the clinical syndrome of feline infectious peritonitis. Histopathologic lesions common to all animals infected with the pathogenic 61E/C chimeras were generalized lymphoid depletion, hemopoietic cell depletion and nodular lymphoid infiltration in bone marrow, and chronic enteritis characterized by residual crypt cell damage and villous atrophy, a panel of lesions observed previously in FeLV-FAIDS, HIV, and other retroviral immunodeficiency syndromes (10, 12, 18).

One animal in the present study illustrated especially well the relationship between 61E/C chimera replication, antigenemia, and impaired immune function. Cat 2203, which was able to generate an effective antiviral immune response and suppress circulating antigenemia early (weeks 4 to 20) after infection, was also capable of generating a T-cell-dependent antibody response similar to that of control and 61E-infected cats during this period. However, subsequent to the KLH immunization regimen, this animal evidently lost its ability to curtail viral replication, again became antigenemic, developed a progressive drop in circulating CD4 cells, and progressed to frank immunodeficiency disease by 33 weeks. Perhaps the immune stimulation produced by KLH immunization also stimulated a burst of cytopathic viral replication in latently infected T cells (38) and a consequent loss of immunologic containment of virus infection, as is hypothesized to occur in HIV infection (9).

Previous investigations of FeLV-FAIDS pathogenesis in tissues suggest that both B and T cells are infected *in vivo* (18, 37), although T-cell deficits have been most associated with induction of disease (4–6, 19, 33, 35, 42, 43, 46). In current studies of the feline T-cell subsets targeted for infection and killing by FeLV-FAIDS and other FeLV isolates, we have found surprisingly little expression of FeLV-FAIDS gp70 on natural T lymphocyte populations *in vivo*. However, studies with purified CD4 cells reveal that surface expression of viral glycoprotein is rapidly inducible *in vitro*, again suggesting that activation of lymphocytes may also activate viral gp70 expression and cytopathicity in feline CD4 lymphocytes.

The mechanism of immune dysfunction in cats infected with FeLV-FAIDS is unknown at present. High levels of unintegrated viral DNA are found in cells infected *in vivo* and *in vitro* with both the original FeLV-FAIDS isolate and the pathogenic 61C clone (27), a phenomenon which may result from failure to establish superinfection interference (44, 45; Donahue et al., submitted). It has been demonstrated for some time that exposure of feline lymphocytes to the purified transmembrane protein of FeLV (p15E) (7, 25, 28) impairs the function of these cells *in vitro*. The amino acid sequences of the 61E and 61C p15E proteins are identical; however, this does not preclude involvement of the transmembrane protein in cytopathicity if, for example, the configuration of gp70-p15E complex alters the presentation and interaction of p15E with cell membranes. Decreased production of interleukin-2 by lymphocytes from cats naturally infected with FeLV also has been demonstrated (42), further suggesting that as with HIV infection, a cascade of immunologic deficits evolve in FeLV immunodeficiency. In that the major *in vitro* T-cell-killing and immune-deficiency-inducing properties of FeLV-FAIDS reside in the gp70 gene, we are focusing our work toward the interactions of the extracellular glycoprotein with its thus far uncharacterized cell receptor.

In conclusion, we demonstrate that a major immunodeficiency-inducing determinant of the variant FeLV-FAIDS

pathogenic clone 61C is contained within a 3' 102-base-pair segment of the *env* gene which codes for the C-terminal 34 amino acids of the surface glycoprotein, gp70. Virus chimeras containing these determinants are distinguished by their ability to mediate early and persistent deficiencies in CD4+ T lymphocytes and T-cell-dependent antibody responses which presage development of fatal immunodeficiency syndrome in vivo.

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