Chronic Feline Leukemia Virus Infection Alters Arachidonic Acid Proportions *In Vivo* and *In Vitro* (43533)

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Abstract. **The polyunsaturated ω-6 fatty acid, arachidonic acid ([AA] 20:4n-6), is both the key of the immunoregulatory substances, prostaglandins, and leukotrienes, and an essential component of immune cell membrane phospholipids, providing stability and flexibility to ensure cellular function. To explore possible effects of the physiological burden of viral replication in chronic viral infections on AA availability, plasma total esterified fatty acid (FA) proportions were measured in the feline leukemia (FeLV) model. Plasma FA profiles of 12 specific-pathogen-free cats with chronic infections with Rickard strain feline leukemia virus (FeLV-R) were compared with 12 age- and sex-matched uninfected specific-pathogen-free cats at 4 months after infection. A significant decrease from normal of average AA proportion was found in FeLV-R-infected cat plasma, while other major FA (palmitic, stearic, and oleic and ω-3 FA normally remained present until near death. Since plasma FA content rapidly affects circulating immune cell membrane composition and since FeLV infection also targets immune cells, we compared FA profiles of feline T4-thymic lymphoma 3201 cell membranes that were infected with virulent FeLV-R or less virulent FeLV-A, at 20 days after viral inoculation with sham-inoculated uninfected 3201 cells. Significantly altered FA proportions and ratios of saturated to unsaturated FA found in infected cell membranes were similar to plasma FA changes and paralleled the virulence of the FeLV inoculum. Altered postinfection FA proportions may impart serious functional defects to the immune cells of chronic FeLV-infected cats, contributing to the inability of their immune systems to eliminate FeLV by depleted plasma AA stores and modified cell membrane composition. Decreased AA availability may be an important factor in the cachexia and fatal outcome of FeLV infection.** [P.S.E.B.M. 1993, Vol 202]

Acute infections can dramatically alter host lipid metabolism (1–3). Fatty acids (FA), the essential building blocks of membrane and circulating lipoproteins and prostenoids, are utilized for muscle energy during fever and for tissue repair as well as the formation of additional immunoregulatory substances (1–4). Similar changes in FA metabolism probably accompany chronic viral infections as the need for immunoregulatory control and tissue replacement continues over time, albeit at a slower pace (5). Initially during a slow deprivation, the infected host's tissues would adjust to unusual demands of key FA metabolites by mobilization of lipid stores. Apparently normal cellular functioning that shields underlying deficiencies has been shown in chronic stress (6). However, with time, a persistent, low-grade viral infection could deplete host FA resources, exhausting the host's ability for metabolic compensation, leading finally to rapid irreversible decompensation and death (7–11).

This mechanism has not been thoroughly explored in chronic viral diseases (5, 10, 11). Of particular interest are FA proportions of plasma and immune cell membranes that may accompany chronic infection with immunosuppressive retroviruses (8–11). Long chain, polyunsaturated fatty acids, especially arachidonic acid (AA), have key roles in maintaining mem-

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brane integrity, fluidity, and lymphocyte function (4, 12). In people and cats chronically infected with their respective retroviruses, the human immunodeficiency virus (HIV) and the feline leukemia virus (FeLV), severe immunodeficiency is associated with loss of membrane-dependent functions of lymphocyte capping and blastogenesis, interleukin production, neutrophil chemiluminescence, and antibody responsiveness to immunogens (9, 10, 13–17). In addition, retroviruses HIV and FeLV demonstrate *in vitro* an ability to shut down membrane phospholipid synthesis and to divert oleic acid to neutral lipids leading to impaired lymphocyte function or overt lymphocyte death by apoptosis (10, 11).

We propose that the profound cachexia and immunosuppression that accompany leukemogenic retrovirus infection may be in part due to chronic depletion of key polyunsaturated FA, particularly AA (20:4n-6). This unique FA has a dual role in being both the precursor of immunoregulatory cytokines, prostaglandins, and leukotrienes (18, 19), and the largest unsaturated FA component necessary to maintain fluidity and function in membrane phospholipids (12, 20). To test this hypothesis, we compared plasma total esterified FA profiles of specific-pathogen-free (SPF) cats chronically infected with Rickard strain FeLV (FeLV-R) FA profiles of age- and sex-matched, uninfected SPF control cats (21, 22). Finding altered total plasma FA proportions in viremic cats 4 months after primary inoculation with FeLV-R, we then evaluated FA directly in FeLV-infected T4 target lymphocytes. FA profiles of cultured feline T4-thymic lymphoblastic 3201 cell membranes inoculated with virulent FeLV-R (23) or less virulent Glasgow-1 FeLV, strains (24, 25) were compared with FA profiles of sham-inoculated 3201 cells (for controls).

Materials and Methods

Cats, Viral Agents, and Plasma Sample Collection. Twelve weanling (6–8 weeks old) SPF cats were inoculated intraperitoneally with 10^5 focus-forming-units of Rickard strain FeLV (23). The 10% w/v suspension of thymic lymphoma homogenate of FeLV-R used induces persistent viremia by 2 weeks to 2 months after inoculation (postinoculation [PI]) (26). Transient lymphopenia, neutropenia, and thrombocytopenia occur 1–2 months PI, with chronic immunosuppression and thymic atrophy by 3–4 months PI, and T cell lymphoma appearance by 8–14 months PI in 85% of cats inoculated as weanlings (9, 10, 22, 26). Both FeLV-inoculated and sham-inoculated, age- and sex-matched control SPF cats were maintained under identical environmental conditions of light and temperature. The cats were fed free choice commercial kibble (Carnation classic cat chow; Manno Pro Corp., Los Angeles, CA), which meets the National Research Council recommendations for cats and has the following specifications on a dry matter basis: 30% protein, 8.6% total fat, and free fatty acid proportions of caprylic (8:0), (0.4%); capric (10:0), (0.6%); lauric (12:0), (0.3%); myristic (14:0), (1.5%); myristoleic (14:1), (0.3%); pentadecanoic (15.0), (0.4%); palmitic (16:0), (22.5%); palmitoleic (16:1), (6.3%); margaroleic (17:1), (0.1%); stearic (18:0), (7.0%); oleic (18:1), (36.1%); linoleic (18:2), (23.8%); and linoleic (18:3) acid, (1.5%). The arachidonic acid (20:4n-6) content had not been evaluated directly by the manufacturer, but was determined to be adequate to support feline growth and maintenance in controlled studies (A. Aydan and G. Douglass, personal communication). Care and usage of all cats conformed to standards established by the Institutional Laboratory Animal Care Committee of The Ohio State University and by Department of Health, Education, and Welfare (Department of Health and Human Services) publication no. NIH 74-23, *Guide for the Care and Use of Laboratory Animals*. Viremia status was determined by indirect immunofluorescence assay for the major core protein of FeLV (FeLV p27) in circulating neutrophils and platelets as described (27).

Ten milliliters of heparinized blood were collected by jugular venipuncture from 12 viremic and 12 uninfected cats at 4 months PI for determination of plasma total esterified FA. All blood samples were obtained at the same time each morning. In the sequential study, 10 ml of heparinized blood were obtained by jugular venipuncture from three viremic cats at three biweekly intervals beginning at 4 months PI.

Total Lipid Extraction for Gas Liquid Chromatography. A modification of the method of Love (5) was used for extraction of total esterified and nonesterified fatty acids. Overnight stirring of 0.5 ml of plasma or cell pellet in 19 ml of chloroform:methanol (2:1) containing butyl hydroxytoluene (1 μg/50 ml) was followed by 4 ml of 0.1 *M* KCl and a Folch wash containing chloroform:methanol:0.1 *M* KCl, 3:48:47, v/v/v. After drying under N_2 hydrolysis was performed with 2 ml of 1 *M* KOH in 90% ethanol for 30 min, followed by acidification with 2 *M* HCl, extraction with diethyl ether and drying under N_2. Methylation was accomplished by heating to 60°C in a dry bath for 10 min with 0.5 ml of boron trifluoride-methanol (Alltech Assoc., Deerfield, II). The methylated FA were transferred to a separatory funnel with hexane, washed with saturated NaCl, dried with Na_2SO_4, dried under N_2, and kept at −20°C until use.

Gas Liquid Chromatography of Total FA Methyl Esters. Detection of the methylated FA fractions and the internal 15:0 standard was performed on a Hewlett-Packard 5840-A gas liquid chromatograph equipped with a dual hydrogen flame-ionization detector and an automatic integrator as described (5). A 30 M SP 2330 fused silica capillary column with a 0.20-μm film thick-

ness and 0.25 mm i.d. (Supelco, Bellfonte, PA) was temperature-programmed from 180°C to 240°C at 2°C/min. The injection port temperature was 250°C, the detection temperature was 250°C, and the linear flow velocity was 10 ml/min, with the carrier gas being He, at a split ratio of 1:100. Peaks on the chromatogram were identified by comparing retention times with reference standards (Supelco, and Nuchek Prep, Elysian, MN). The percentage of each FA was calculated by the ratio of its area to the total area of the sample. Normal controls were included in daily runs. Variation in FA percentages among duplicate samples was less than 2%. Proportions of FA, known as the FA profile, were compared between FeLV viremic and uninfected, age-matched control SPF cats and between infected and sham-inoculated 3201 cells by an analysis of variance utilizing the CRISP IBM statistical program and the Student's *t* test. The ratio of saturated to unsaturated FA (but not including the 10–14:1 fraction) was calculated by dividing the total percentage of saturated FA by the total percentage of unsaturated FA.

In vitro Infection of Feline T4-Thymic Lymphoma 3201 Cells with FeLV. Feline T4-thymic lymphoma 3201 cells are an established cell line that is negative for exogenous FeLV sequences and negative for replicating FeLV (26, 28) and that expresses feline CD4 epitopes but few other feline lymphocyte antigens (26, 29). The 3201 cells, a generous gift of Dr. W. D. Hardy, Jr. (New York, NY), were maintained in exponential growth by daily adjustment of cell number to 10^6 cells/ml of complete medium consisting of a 1:1 volume mixture of RPMI 1640 and Leibowitz's L-15 medium supplemented with 5% fetal calf serum (Gibco, Grand Island, NY), 2 m*M* Na pyruvate, 2 m*M* L-glutamine, and 50 μg/ml of gentamycin as described (26). Exponentially growing 3201 cells were infected with the Rickard strain of FeLV (23) or FeLV-A/Glasgow-1 (24, 25) at a multiplicity of infection of 1.0 using sucrose-density-gradient-purified virus. Controls were exponentially growing 3201 cells exposed to sucrose-banded medium as described (10, 30).

After exposure to FeLV or sham inoculum, cells were maintained in logarithmic phase by adjusting the cell number to 10^6 cells/ml in fresh complete medium daily. Infection, monitored by examining the supernatant for infectious FeLV at 20 days PI, was quantified by its ability to rescue the defective sarcoma virus genome from, and initiate transformed foci in, target S + L- clone 81 cells, as described (30, 31). At 20 days PI, seven to eight replicate samples of 5×10^7 infected and uninfected 3201 cells were harvested, washed three times in Ca^{2+}-free, Mg^{2+}-phosphate-buffered saline (Gibco), and frozen as dry pellets at −20°C until extracted, as described above.

Results

Plasma Total Esterified FA Profiles of FeLV-R Viremic Cats. A marked reduction in the average proportion of the plasma ω-6 FA, arachidonic acid (20:4n-6) (mean percentage ± SD of total esterified FA plasma extract), was found in 12 FeLV-R viremic cats tested at 4 months PI compared with values of uninfected, age- and sex-matched control cats ($P < 0.001$) (Table I). Other altered FA proportions in viremic cats included significant increases in medium chain (10–14:1) and palmitoleic acid (16:1) FA ($P < 0.005$). However, the majority of FA were within the values of uninfected cats. A reduction in ω-6 linoleic acid (18:2n-6), the essential FA precursor of AA, was variable among infected cats (as seen by the large SD), but the average value was not significantly lower than in uninfected cats (Table I). The ratio of saturated to unsaturated FA in uninfected cat plasma was 0.43, while in FeLV-R chronically infected cat plasma, the saturated to unsaturated FA ratio was 0.47. A higher ratio indicates that a higher proportion of saturated FA was present in FeLV-infected cat plasma.

Sequential Changes in Plasma FA Profiles in Three FeLV-R Viremic Cats. To examine FA patterns sequentially over time, plasma FA profiles of three FeLV-R viremic cats were measured 4 times at 2-week intervals after 4 months of FeLV-R infection. As before, the ω-6 AA proportion in all three cats was lower than the normal range for age- and sex-matched uninfected control cats (Fig. 1 and Table I). Although all three viremic cats had elevated C10–14:1 medium chain FA proportions compared with controls (Fig. 2), greater fluctuations were noted between cats (Fig. 2). Linoleic acid values were not significantly different from controls, except in the final sample obtained from cat 3344 (Fig. 3). Deterioration from the normal range is seen in

Table I. Plasma Total Esterified Fatty Acids in FeLV-Infected and Uninfected Control Cats

Fatty acids[a]	Uninfected cats ($n = 12$)	FeLV-infected cats[b] ($n = 12$)	P[c]
C10:14:1	0.4 ± 0.4[+]	3.6 ± 2.1	0.005
C16:0	12.6 ± 1.5	11.6 ± 1.7	NS
C16:1	3.2 ± 1.0	5.7 ± 2.3	0.005
C18:0	16.3 ± 0.9	17.6 ± 1.9	NS
C18:1	23.9 ± 2.9	22.7 ± 2.9	NS
C18:2n-6	33.8 ± 4.0	29.7 ± 5.2	NS
C20:4n-6	4.7 ± 0.6	2.4 ± 0.9	0.001
C20:5n-3	0.5 ± 0.1	0.6 ± 0.2	NS
C22:6n-3	1.1 ± 0.4	1.2 ± 0.6	NS

[a] Percentage of the total plasma FA; unlisted FA percentages were normal.
[b] Cats were inoculated with 10^5 focus-forming units of FeLV at 8 weeks of age. Plasma was collected 4 months later during chronic phase.
[c] *P*-values were determined by Student's *t* test (NS, not significant).

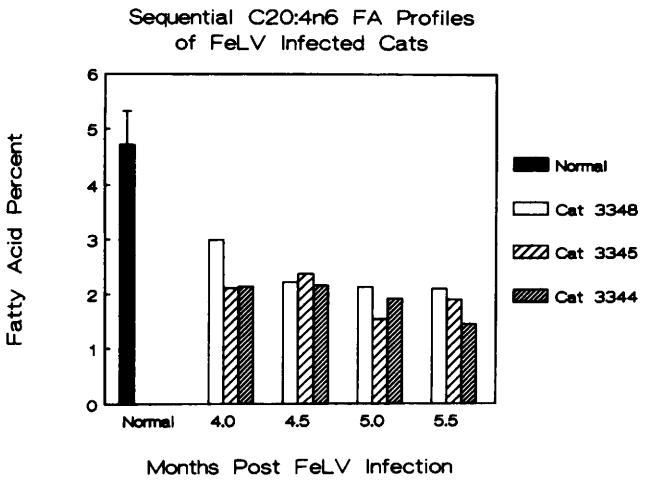

Figure 1. Arachidonic acid (C20:4n-6) proportions of three FeLV-infected cats measured at 2-week intervals beginning at 4 months after inculation with FeLV-R (see Materials and Methods). Cat 3344 died 2 weeks after the final sample.

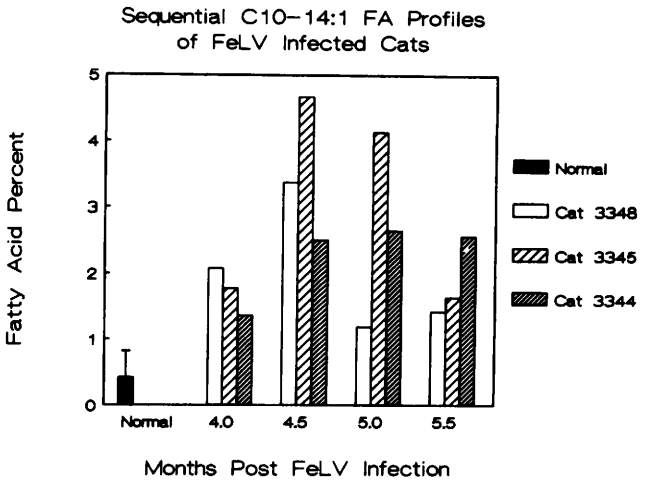

Figure 2. Medium chain C10–14:1 FA proportions of three FeLV-infected cats measured at 2-week intervals beginning at 4 months after inculation with FeLV (see Materials and Methods). Cat 3344 died 2 weeks after the final sample.

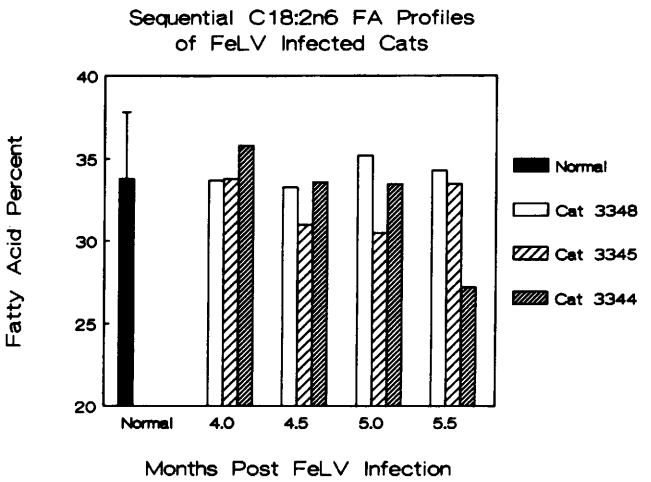

Figure 3. Linoleic acid (C18:2n-6) proportions of three FeLV-infected cats at 2-week intervals after inculation with FeLV (see Materials and Methods). Cat 3344 died 2 weeks after the final sample.

Table II. Total Esterified Fatty Acids of FeLV-Infected and Uninfected Feline T4 Thymic Lymphoma 3201 Cells

Fatty acids[a]	Uninfected 3201 cells ($n = 8$)	FeLV-R Infected 3201[b] ($n = 8$)	FeLV-A Infected 3201[b] ($n = 7$)
C16:0	21.1 ± 1.5	20.0 ± 1.2	21.4 ± 0.4
C16:1	8.2 ± 1.1	9.8 ± 1.0	9.8 ± 0.4[c]
C18:0	11.5 ± 1.0	10.2 ± 0.6	11.2 ± 1.5
C18:1	44.6 ± 1.8	47.2 ± 2.5[c]	43.8 ± 2.4
C18:2n-6	2.0 ± 0.1	1.8 ± 0.1[c]	1.8 ± 0.1[c]
C20:4n-6	2.6 ± 0.1	2.2 ± 0.2[d]	2.3 ± 0.2[c]
C22:6n-3	1.8 ± 0.1	1.5 ± 0.2[c]	1.6 ± 0.1[c]

[a] Mean percentage ± SD of total esterified fraction compared with uninfected 3201 cells; unlisted FA were not different.
[b] FeLV-R- and FeLV-A-infected thymic lymphoma 3201 cells were as described in Methods.
[c] Determined by Student's t test, $P < 0.01$.
[d] Determined by Student's t test, $P < 0.001$.

each of the FA proportions in this final sample (Figs. 1–3). Interestingly, cat 3344 died 1 week after this sample was obtained. At necropsy, severe cachexia, serous atrophy of fat, and lymphoid depletion were present.

T4-Lymphoid FA Profiles after Infection with FeLV-R or FeLV-A. To determine whether FeLV infection directly alters the FA membrane composition in target lymphocytes, feline T4-thymic lymphoma 3201 cells were infected with either virulent FeLV-R (23) or less virulent FeLV-A (Glasgow-strain; 24, 25) or sham-inoculated *in vitro* and cultured for 20 days. At this time, the cells infected with virus were producing 10^5-10^6 focus-forming units of helper FeLV per ml as assayed against S + L- clone 81 cells. Compared with uninfected cells, 3201 cells chronically infected with either strain of FeLV showed significantly altered FA proportions that were strikingly similar to the altered *in vivo* plasma FA profiles of FeLV-R-infected cats (Table II). These changes included significantly decreased proportions of AA and its metabolic pathway precursor, linoleic acid (18:2n-6). In addition, T4 cells infected with both strains of FeLV had significantly lower docosahexaenoic acid (22:6n-3) proportions than controls. As in plasma, increased palmitoleic acid (16:1) was found in FeLV-A-infected 3201 cells. These results indicate that FeLV could induce similar altered FA proportions *in vitro* and *in vivo*.

Further analysis of infected 3201 cell FA profiles suggests that FA changes of cells infected with virulent FeLV-R or less virulent FeLV-A could be correlated with the clinical virulence of these viral strains (23–25) (Table II). A significantly lower AA fraction in FeLV-R-infected 3201 cells than in FeLV-A-infected cells

suggests a more severe drain on AA stores by the virulent FeLV-R infection. In the less virulent FeLV-A-infected cells, the saturated to unsaturated FA ratio is 0.55, which is identical to the control 3201 cell membrane ratio (0.55). In contrast, the saturated to unsaturated ratio of FeLV-R-infected cell membranes is 0.48, showing greater FA unsaturation than control cells. This result is surprisingly unlike the higher than control FA saturation index in FeLV-R-infected cat plasma. However, it is important to note that the high unsaturated portion of FeLV-R-infected cell membranes is supplied by an elevated fraction of oleic acid (18:1), which has 1 double bond, rather than by AA (20:4n-6), which has four double bonds (Table II). Since the number of double bonds in the polyunsaturated FA of membrane triglycerides and phospholipids controls the flexibility and confirmation of the membrane (4), this change may be important in membrane function (12).

Discussion

Immunosuppression is a well-documented component of infection with the retrovirus FeLV in cats (10, 11, 13–17, 22, 32–34). Our data support the possibility that one mechanism underlying poor cellular immunoregulation and physiologic decompensation in FeLV chronically infected cats may be decreased proportions of key esterified polyunsaturated ω-6 fatty acids in FeLV-infected immune cell membranes directly and in their plasma environment. Chronic infection with the immunosuppressive and oncogenic Rickard strain of FeLV (23) in viremic cats led to significant altered plasma FA profiles when compared with age- and sex-matched SPF control cats. In addition, virulent FeLV-R or less virulent FeLV-A (24, 25) infections directly altered FA profiles in membranes of target lymphocytes *in vitro* in parallel with their clinical virulence *in vivo*.

We considered that the lowered plasma stores of arachidonic acid in FeLV-R viremic cats might arise from host variables, such as decreased intake or poor absorption. However, adequate dietary FA composition and availability of FA are implied by the normal growth and immune responses in uninfected, age- and sex-matched SPF control cats, and by food offered *ad libitum*. Although cats with chronic FeLV viremia may be anorexic and have decreased intake usually coinciding with febrile episodes (W. D. Hartke, L. E. Mathes, and J. L. Rojko, manuscript in preparation), it is unlikely that intermittent anorexia in viremic cats alone caused these disproportions. Both viremic and control SPF cats are able to compensate for periods of decreased fat consumption by increasing fat absorption (W. D. Hartke, L. E. Mathes, and J. L. Rojko, manuscript in preparation). Moreover, normal proportions of other plasma FA in FeLV-infected cats, including palmitic, stearic, oleic, and the ω-3 FA, suggested adequate FA intake and absorption processes in experimental cats.

Decreased AA proportions are of particular importance in the cat because the cat lacks appreciable Δ-6-desaturase, an important FA enzyme necessary to convert linoleic acid to AA (35–37). Cats quickly show signs of AA deficiency, which include decreased platelet aggregation, thrombocytopenia, infertility, and, possibly, poor wound healing and increased susceptibility to infections (35–37). These changes, frequently seen in chronic FeLV viremia, have been of unknown pathogenesis (9). A virally induced block of the FA enzyme, Δ-6-desaturase, may also limit FA incorporation (38), but this effect has not been directly attributed to FeLV (9, 10). As with HIV, FeLV infection directly decreases membrane phospholipid synthesis and increases neutral lipid synthesis detected by [^{14}C]oleic acid in infected T4 lymphocytes (10, 11).

The lower plasma stores of AA could result from their increased consumption of AA by a continual demand for AA-derived immunoregulatory substances, prostaglandins, and leukotrienes (18, 19) during chronic FeLV infection (10). Activation of the arachidonic acid cascade follows the direct binding of FeLV to the first component of complement (C1q) and the generation of C3 and C5 inflammatory mediators after virus-directed complement consumption (39–41). FeLV antigen-antibody complex activation of the classical complement pathway (42) also leads to a continued demand for immunoregulatory prostenoids with progressive depletion of their AA precursor molecule (18, 19). In addition, the increased medium chain FA fractions in chronically FeLV-infected plasma suggests an accumulation of prostenoid metabolic breakdown products, since these are extracted with authentic C10–14:1 and C16:1 FA (5).

Decreased immune function of lymphocytes found in cats with viremic FeLV-R infections (10, 13–17, 34) may result specifically from these altered FA proportions. During AA depletion, substitution of oleic acid (18:1, containing a single double bond) for AA (20:4n-6, containing four double bonds) in membranes of FeLV-R-infected cells (43) could result in a less flexible configuration (4, 12) and thereby contribute to their previously observed impaired capping, secretory abilities, and blastogenesis (13–17, 34). The high oleic and low AA acid composition of FeLV-R infected cell membranes appears to correlate with its greater clinical viral virulence (45, 46). Lesser effects on 3201 cell membrane FA profiles and FA saturation ratios were found during infection with the less virulent FeLV-A strain (44, 45).

A need for adequate AA for prostenoid production in lymphocyte immunoregulation during FeLV infection is supported by two sets of data. The AA drain during FeLV infection can be replaced with prostaglandin supplements (15). Moreover, AA-sparing treat-

ments, such as nonsteroidal anti-inflammatory drugs that inhibit cyclo-oxygenase, are effective additions to restore function in viremic cat lymphocytes (16). These results implicate AA as a key component in immune cell action. Increased production of immune prostenoid metabolites via both the lipoxygenase and cyclo-oxygenase pathways is also found in another immunosuppressive retrovirus, HIV (46).

Similar FA profiles of decreased AA, its precursor, linoleic acid, and increased medium chain FA in plasma and target T4 lymphocytes infected with FeLV suggest that FeLV activates similar FA metabolic processes *in vitro* and *in vivo*.

Immunosuppression with decreased ability to control viral spread seen in FeLV viremia (reviewed in Ref. 9) appears to be influenced by these dual effects on both target lymphocytes and plasma. Seven viremic cats of this study, examined for immune function, had decreased neutrophil chemiluminescence and lowered titers of antibody to FeLV, while three developed lymphoma and three had opportunistic bacterial or viral infections (L. J. Lafrado, unpublished data). Our sequential study of three cats with chronic FeLV viremia shows the progressive, slowly increasing effects of chronic viral infection on plasma AA stores. Recently, we have shown that below normal AA proportions and an inability of the host to normalize a reversed ratio of serum linoleic to oleic FA paralleled delayed recovery from Epstein-Barr virus infection and may offer insight into its pathogenesis (5). We suggest that continually altered proportions of plasma total esterified FA may be an early indicator of the effects of deleterious chronic viral infection.

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