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ERYTHROID APLASIA AND PLATELET ABNORMALITIES IN CATS
INDUCED BY THE KAWAKAMI-THEILEN STRAIN OF
FELINE LEUKEMIA VIRUS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
John Thomas Boyce, D.V.M.

The Ohio State University
1983

Reading Committee: Approved by

Dr. Gary J. Kociba
Dr. Richard G. Olsen
Dr. Robert Jacobs

Dr. Gary J. Kociba
Adviser
Department of Veterinary Pathobiology
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VITA

John Thomas Boyce

August 5, 1946

Born - Moscow, Idaho

1971

D.V.M., Washington State University
Pullman, Washington

1971-1976

Captain, Veterinary Corp., United States Army

1974-1976

Staff Member, Armed Forces Institute of Pathology, Washington, D.C.

1976-1978

Resident, Regional Primate Research Center, Seattle, Washington

1979-1983

Graduate Student, Department of Veterinary Pathobiology, The Ohio State University, Columbus, Ohio

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CHAPTER I
FELINE LEUKEMIA VIRUS-INDUCED ERYTHROID APLASIA:
IN VITRO HEMOPOIETIC CULTURE STUDIES

INTRODUCTION

The culture of colony forming hemopoietic cells has provided insights into normal and disordered hematopoiesis and has helped identify stem cell defects, microenvironmental disorders, and lymphoreticular-hemopoietic cell interactions in congenital murine anemias and in human aplastic anemias (1-4). Limitations inherent in the investigation of the pathogenesis of human aplastic anemia, however, include difficulty in conducting prospective studies and complicating effects of transfusion and other therapy. Erythroid aplasia is a common spontaneous disease of cats and has been induced experimentally with certain isolates of feline leukemia virus (FeLV) (5,6). The disease is characterized by progressive non-regenerative anemia and severe depletion of marrow erythroid cells (5). Although FeLV-infected bone marrow cells bear FeLV-associated cell membrane neoantigens (7), neither a regenerative marrow response nor positive direct antiglobulin reactions are present in FeLV-induced anemia (5,6). FeLV-related anemia, therefore, may represent a model of acquired erythroid aplasia which can be used to probe the mechanisms
of normal and disordered hemopoiesis. We describe here culture of marrow colony forming cells from normal cats and from cats of various ages inoculated with FeLV and explore the relationship between age and susceptibility of cats to FeLV-induced anemia (8).
METHODS AND MATERIALS

Animals. Specific-pathogen-free (SPF) cats were obtained from a closed breeding colony of gnotobiotic ancestry (9). Donors of normal cells were young adults. Neonatal (<1 week old), 4, and 8-week-old kittens were used to study age-related susceptibility to FeLV-KT. Anesthesia with ketamine hydrochloride (Vetalar, 10 mg/kg, Parke-Davis, Detroit, MI) was used for all procedures.

Virus-inocula. The inocula used were 0.4 μ filtrates of 10% (w/v) homogenates of splenic tissue from cats with anemia induced by in vivo passage of FeLV-KT as described (5). The FeLV infectivity titer of the inocula were assessed by the S+/L- focus induction assay described by Fischinger et al (10) and modified by Schaller and Olsen (11) (Table 1).

Experimental protocol. Five litters of neonates, 2 litters of 4-week-old kittens, and 24 8-week-old kittens were used to evaluate the age-related susceptibility to FeLV-KT. The susceptibility studies were run concurrently with the development of the feline hemopoietic assays.

Kittens were inoculated with FeLV-KT (1 ml intraperitoneally) prepared as a 10% (w/v) splenic homogenate from cats which developed erythroid aplasia in previous serial passages of FeLV-KT (Table 1). One uninoculated sibling from each inoculated litter as well as other uninoculated 8-week-old kittens served as controls for hemopoietic assay studies. Beginning at week 2, blood was collected weekly for detection of FeLV group-specific antigen (GSA) in circulating
leukocytes and determination of FOCMA antibody in serum using immuno-
fluorescence assays (12-14). Study of non-viremic, non-anemic
animals was terminated at 12 weeks after FeLV inoculation. Anemia
was defined as a packed cell volume (PCV) of 15 or less in kittens
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Bone marrow was aspirated from either the proximal humerus or
the proximal femur of cats anesthetized with ketamine HCl. No bone
was sampled more than once. CFU<sub>GM</sub> data only were obtained for the
anemic neonates receiving passage 4, 5, or 6 virus. CFU<sub>GM</sub> and CFU<sub>E</sub>
assay data were obtained at 6, 8, 9, and 10 weeks post-inoculation
from 2 neonatal litters receiving the 7th passage of FeLV-KT. Feline
hemopoietic assays were performed as described below.

**Bone marrow culture technique.** The methylcellulose assay systems
developed by Iscove et al (15) and Worten et al (16) were modified
and applied to feline hemopoietic cells. Gradient-separated bone
marrow mononuclear cells were used for all assays. Marrow was
aspirated into a syringe containing preservative-free heparin in 8 ml
of RPMI 1640 culture medium (Gibco, Grand Island, NY) and 10 ml of
phosphate buffered saline (PBS), layered over 10 ml of lymphocyte
separation medium (Ficoll-diatrizoate, density 1.077 g/ml, Litton
Bionetics, Kensington, MD), and centrifuged at 400 X g for 20 min at
room temperature. The cell layer at the interface was aspirated,
washed once with PBS and suspended in 2 ml of RPMI 1640. Mononuclear
cell numbers rather than total nucleated cells were counted because a
greater proportion of granulocytes were present in the gradient-
separated cells from anemic cats with increased myeloid/erythroid
ratios. The mononuclear cell concentration was determined by differential counting with 0.05% new methylene blue in a hemocytometer. Yield from 1-2 ml of marrow aspirate was 1.0 to $10 \times 10^7$ mononuclear cells and cell viability (estimated by trypan blue exclusion) was greater than 90%. The cell concentration was adjusted with RPMI 1640 and the cells were held on ice until incorporation into the final culture medium. Time from bone marrow aspiration to initiation of cultures was 2 h or less.

Stock methylcellulose medium was prepared by the method of Worton et al (16) with minor modifications. Methylcellulose (0.16 g A4M premium grade, Dow Corning, Midland, MI) was dispersed in 3.3 ml of 90-100°C sterile water, boiled for 30 min, and allowed to cool to room temperature. Then 3.3 ml of 4°C sterile 2 X RPMI 1640 medium were added and mixed by vortexing. This 2.4% (w/v) methylcellulose stock was allowed to hydrate at least 48 h at 4°C before dilution with RPMI 1640 and other materials as described below to a final concentration of 0.8% in all assay procedures. Optimum concentrations of fetal calf serum (FCS), growth-stimulating conditioned media, erythropoietin (Ep) and marrow mononuclear cells are described in Results.

Granulocyte-macrophage progenitor assay. The optimum medium consisted of 0.8% methylcellulose in RPMI 1640 and contained 0.2% bovine serum albumin (BSA), glutamine ($2 \times 10^{-4}$ M), 1% antibiotics with mycostatin (AB/M, 100 U/ml penicillin, 100 µg/ml streptomycin and 20 U/ml mycostatin final concentration), 10% fetal calf serum (FCS) (Kansas City Biological Co., Kansas City, KS), and 20% medium
conditioned by peritoneal macrophages (see below). Unless stated otherwise, 5 X 10^5 mononuclear cells per ml were plated in 96 well tissue culture plates (0.1 ml/well) (Costar, Cambridge, MA). The cultures were incubated at 37°C in humidified air with 6% carbon dioxide. Colonies containing 20 or more cells on day 6 were counted in situ using an inverted microscope. The granulocyte/macrophage composition of the colonies was confirmed both by staining for myeloperoxidase and by cytological examination of colonies. Myeloperoxidase activity was demonstrated in situ by adding 0.1 ml of 3-amino-9-ethylcarbazole prepared as described by Kuplow (17) to the well and incubating for 10 min. Positive colonies were stained dark brown. Aspirated colonies were cytocentrifuged through FCS onto slides (Cytospin, Shandon Southern, Sewickley, PA), air dried, and Wright-stained for cytological examination.

Erythroid progenitor assays. Both early (day 2) and late (day 7) erythroid colonies (CFU_E) were cultured in RPMI 1640 medium containing 0.8% methylcellulose, 0.2% BSA, glutamine (2 X 10^-4 M), 1% AB/M, 30% PCS, 10% macrophage conditioned medium, and 10^-6 M alpha-thioglycerol. Unless stated otherwise, all assays were plated at 5 X 10^5 cells/ml. All assays except the Ep dose response study contained 1 unit Ep/ml (Step III sheep erythropoietin, specific activity = 4.9 U/mg protein, Connaught Laboratories, Swiftwater, PA). Unstained colonies were counted on day 2 (early CFU_E) and day 7 (late CFU_E) using an inverted microscope. Early and late erythroid colonies were discerned from CFU_GM by their closely apposed clusters of small refractile cells with well defined cell membranes and red tint
indicative of partial hemoglobinization, confirmed by in situ benzidine staining using the method of Urabe and Murphy (18) and cytological examination of stained cytocentrifuged preparations.

**Conditioned media.** Conditioned media (CM) were prepared by culturing various feline cells for 72 h and harvesting the cell-free supernatants. Included were: (a) macrophage CM produced by incubating $8 \times 10^5$ thioglycollate-elicited peritoneal macrophages per ml in McCoy's 5A containing 40% equine serum, glutamine, and other amino acids as described (19); and (b) feline lymphocyte CM from spleen or lymph node ($5 \times 10^6$ cells), concanavalin A (Con A) ($50 \mu g/10^6$ cells), or phytohemagglutinin (PHA) ($7.5 \mu g/10^6$ cells). Post-endotoxin serum was collected from an SPF cat 3 and 24 h after it received 1.0 mg endotoxin (*E. coli* serotype 0127:B8, Difco, Detroit, MI) intravenously. The CM and the post-endotoxin serum were tested for CFU enhancing activity.
RESULTS

Normal CFU<sub>GM</sub> assay. Feline CFU<sub>GM</sub> (each containing 20-100 cells) developed readily when marrow mononuclear cells were plated at concentrations between 10<sup>5</sup> and 5 X 10<sup>5</sup> cells/ml. Within this range of cell densities, CFU<sub>GM</sub> production was linear (y = 2.3 + 8.1 X 10<sup>-3</sup>x) (Fig 1.1). Non-linear, sparse CFU<sub>GM</sub> growth occurred below 10<sup>5</sup> cells/ml. Above 5 X 10<sup>5</sup> cells/ml, the high colony density precluded accurate counting. In a system with 10% FCS, only macrophage conditioned medium had significant stimulatory activity compared to the control medium (Fig 1.2). The macrophage conditioned medium concentration was optimal in the range of 20% to 30% (Fig 1.3). With 20% macrophage conditioned medium, the optimum range of FCS was 5-10% (Fig 1.4, P<0.05).

CFU<sub>GM</sub> colonies contained cells with distinctly granular cytoplasm in phase contrast microscopy of viable cultures. Many of the cells contained abundant myeloperoxidase as demonstrated by in situ staining with 3-amino-9-ethylcarbazole. In the Wright-stained cytocentrifuged preparations, the early (day 2-5) CFU<sub>GM</sub> colonies were composed of predominantly maturing neutrophils or eosinophils with smaller numbers of macrophages and myelomonocytic blasts. Substantial degeneration and loss of granulocytes was evident by day 7 when colonies were composed principally of macrophages.

Early and late erythroid colonies. Two distinct populations of erythroid colonies developed. On day 2 small red clusters of 8-16 tightly arranged, small, refractile cells were evident. Benzidine
staining confirmed that the cells contained hemoglobin and Wright-stained specimens of isolated colonies contained normoblasts and erythrocytes (Plate I). The early CFU_E were short-lived and by day 4 few hemoglobinized colonies could be identified. On days 6 to 8 a second population of erythroid colonies could be discerned. These were larger (often 32-64 cells) than the day 2 CFU_E and were composed of tightly clustered, refractile cells, often located on the periphery of CFU_GM. Due to the high colony numbers and the clustering adjacent to CFU_GM, each discrete erythroid cell cluster or colony was counted as a separate unit rather than as subunits of a larger erythroid burst or mixed cell colony. Because the morphology of the day 7 feline erythroid colonies overlapped with that of the day 2 CFU_E and was not equivalent to that described for the murine BFU_E by Iscove (20), we have used the designation late, or day 7, CFU_E rather than BFU_E. Examination of Wright-stained cytocentrifuged preparations of late CFU_E revealed both colonies containing erythroid cells with occasional macrophages (Plate II) and colonies containing mixtures of erythroid cells, granulocytes, and macrophages.

Maximal growth of erythroid colonies required 20-30% FCS (Fig 4) and 1 U/ml of Ep (Fig 1.5), although some colonies formed without added fetal calf serum or erythropoietin. With 30% FCS and 1 U/ml Ep colony formation for both day 2 and day 7 CFU_E was linear at 1-5 X 10^5 plated marrow mononuclear cells (Fig 1). For early CFU_E, y = -27.67 + 6.7 X 10^{-3}X and for late CFU_E, y = -6.3 + 6.2 X 10^{-3}X.
Induction of anemia and age-related susceptibility in kittens.

Ninety-three percent of FeLV-KT-inoculated neonatal kittens (13/14) became viremic (FeLV GSA-positive) and 85% (12/14) developed anemia (Table 1.1). The mean period from inoculation until anemia was first evident was 5.4 weeks (range - 3 x 8 weeks). Only 4.5% (1 of 22) of inoculated 4 to 8-week-old kittens became anemic within 12 weeks after inoculation, although 23% (5/22) of these kittens developed persistent viremia and 26% (6/22) were transiently viremic (Table 1.1). FCMMA antibody titers of ≥1:8 developed in all (16/16) nonviremic 4-8-week-old kittens tested; none of these kittens developed anemia (Table 1.1). Four in vivo passages of the virus did not appear to alter the pattern of age-related resistance to induction of viremia or anemia in kittens.

Sequential in vitro hemopoietic precursor cell assays were done on marrow of neonates receiving 7th passage FeLV-KT. The mean PCV of this group of infected kittens was significantly decreased compared to controls (P<0.005, paired t statistic) at week 8 post-inoculation (Fig 1.6). No circulating reticulocytes were detected in the FeLV-infected anemic kittens. The mean marrow myeloid-erythroid cell ratio of the infected kittens was 10.4 (range = 5.2-17.8) versus 1.6 (range = 0.96-2.2) in littermate controls (P<0.01, paired t statistic). All anemic kittens were lymphopenic at week 9 (P<0.025) (Fig 1.7), although lymphopenia occurred before this interval in individual cats.
Hemopoietic colony data from FeLV-KT-infected kittens. Marrow mononuclear cells from 12 anemic (PCV 15%, weeks 4-9) kittens produced 674 ± 135 (mean ± SE) CFU<sub>GM</sub> colonies per 5 X 10<sup>4</sup> mononuclear cells, a significant increase compared to the 309 ± 45.7 of controls (P<0.005, paired t statistic). CFU<sub>GM</sub> from inoculated kittens were significantly increased compared with controls (P<0.005, Student's t statistic) at weeks 9 and 10 (Fig 1.8). CFU<sub>GM</sub> dose-response curves for macrophage-conditioned media had similar profiles in FeLV-inoculated versus control cats (Fig 3), however, more CFU<sub>GM</sub> were produced per 5 X 10<sup>4</sup> mononuclear cells of FeLV-inoculated cats (0.05>P>0.01, paired t statistic).

The mean number of late CFU<sub>E</sub> in the marrow FeLV-infected neonatal kittens was significantly reduced (P<0.05, paired t statistic) at all post-inoculation intervals assayed (Fig 1.9). This late CFU<sub>E</sub> depletion occurred several weeks before a significant decrease in mean PCV was demonstrable (Fig 1.6). The early CFU<sub>E</sub> numbers in FeLV-infected kittens were significantly reduced at week 8 (P<0.005, paired t statistic) and remained so thereafter (Fig 1.9). Increasing the Ep concentration to up to 10 U/ml failed to increase the numbers of CFU<sub>E</sub> obtained from the marrow of FeLV-infected kittens.
DISCUSSION

The standard for comparison of colony-forming assays has been the mouse wherein CFU
GM, CFU
E and BFU
E numbers are dependent on exogenous growth factors and are linear over a wide range of cells plated (20,21). We found that some formation of feline erythroid and myeloid colonies occurred in the absence of GM or Ep and that the ratio of colonies obtained to marrow mononuclear cells plated was linear only within a range of comparatively high cell densities. Similar observations have been reported in other species (22) and probably reflect both endogenous growth factor production by marrow mononuclear cells and low levels of Ep and growth factors present in FCS (22,23). It has been shown that human CFU
GM are multi-clonal when plated at high cell density (24). Consequently, although our feline hemopoietic CFU assays gave reproducible data, the monoclonality of assays remains unproven. The relatively high ratio of CFU
GM produced per marrow cell plated probably reflects both our lower limit of 20 cells in defining a CFU
GM and the expression of our data as CFU per marrow mononucleated vs nucleated cell. Considering these factors, the 380 CFU
GM per 5 X 10^4 feline marrow mononuclear cell probably is comparable to the 100 CFU
GM/5 X 10^4 murine bone marrow nucleated cells reported by others (25,26).

The abundant early and late CFU
E numbers in kittens reflect their physiologically expanding erythron which is approximately 50% adult level at birth. It has been shown that expansion of the erythron by phlebotomy or administration of Ep increases murine CFU
E by 5 to 10-fold (27). The feline late CFU
E may represent either a
less mature population of CFUE or subunits of larger burst equivalent units. If, as reported in humans (28), feline bursts contained up to 10 subunits, the ratio of early CFUE to late CFUE would approach the 10:1 early CFUE:BFUE ratio reported in other species (29). The marked individual variation in CFUE dose response to Ep in cats is similar to that noted in man in Ogawa et al (30).

Metcalf and Johnson (31) have stressed that the in vitro phenotypic expression of hemopoietic precursor cells is dependent on environmental conditions. In this respect, the mixed nature of the feline day 7 CFUE and the intimate association between many late CFUE and CFUM was notable. Recent reports concerning human and murine CFUE and BFUE stimulation by conditioned media from macrophage-containing cultures (32-34) also implicate regulation of erythropoiesis by factors other than Ep. That cell-to-cell interactions are critical to hemopoiesis in vitro has been inferred by ultrastructural studies of Daniels (35), and other studies implicating macrophages as the source of burst-promoting factors (33,36). The relationship of lymphocytes to erythroid CFU also has yet to be determined (23,37, 38). Further studies of the feline late CFUE/macrophage interactions are indicated to determine the extent to which the mixed nature of the late CFUE/macrophage interactions reflect inherent participation of bone marrow macrophages versus the presence of a mixed colony-forming precursor cell (39) in the bone marrow of cats.

Neonatal kittens were considerably more susceptible to FeLV-induced anemia than were kittens 4 or 8 weeks of age. In this respect, age-related susceptibility of cats to FeLV-KT is even more
dramatic than that demonstrated for the Rickard strain of FeLV, which transforms feline T cells and produces thymic lymphosarcoma (8,40).
In both anemia-inducing and lymphosarcoma-inducing FeLV infections, susceptibility to disease induction is correlated with induction of persistent viremia and failure of antibody response to F0CM and FeLV (8,41). The physiologic erythroid hyperplasia of the neonatal kitten may be important in the host susceptibility to infection with anemia-inducing strains of FeLV, although our recent ability to induce anemia in adult cats by administration of low doses of corticosteroids at the time of FeLV inoculation (42) does not support this tenet. In the anemia associated with Rauscher and Friend murine leukemia virus infections, a block of erythroidic maturation has been demonstrated. The maturation arrest occurs after generation of the erythropoietin-responsive cell and therefore is associated with increased in CFU_E, BFU_E growth in vitro (43,44). Suppression of erythropoiesis before inoculation of Friend virus prolongs survival time of mice, whereas pre-inoculation stimulation of erythropoiesis accelerates the onset of anemia by increasing the number of viral target cells available (45,46). In contrast to the murine leukemia viruses, FeLV-KT may affect an earlier erythroid precursor cell since both early and late erythroid colony-forming units are reduced in vitro.

Marrow erythroid colonies from pre-anemic and anemic FeLV-KT-infected kittens were drastically reduced, whereas CFU_GM were normal or increased. In this respect, FeLV-related erythroid aplasia shares features of human red cell aplasia (47). The mechanism of impairment
of feline erythroid cell growth may involve either direct viral-induced inhibition of cell proliferation (48) or host immunopathologic reactions against FeLV-infected erythroid progenitors (49). The sustained myeloid precursor cell production in FeLV-KT-infected cats implies both a viable pluripotent stem cell compartment and selective viral pathogenicity for erythroid precursors. In human cases of immune-mediated erythroid aplasia, inhibition of erythroid cell proliferation was evident only when patients' sera were added to marrow cultures (50-52). Erythroid colony formation in FeLV-infected anemic kittens was decreased in the absence of autologous serum and in other experiments sera from anemic cats have failed to suppress normal CFUE growth (42). FeLV-KT, therefore, may cause erythroid progenitor cell inhibition by a relatively direct mechanism such as inhibition of cell functions by viral gene products as has been demonstrated with the FeLV pl5E protein and feline lymphocytes (48,53). Alternatively, FeLV-KT infection of, or interaction with, accessory lymphoreticular cells could mediate the suppression of erythropoiesis. FeLV-induced thymic and lymphoid atrophy and immunosuppression have been documented (5,40) and accessory cell growth factor production appears to be important in feline CFUE growth. Lipton et al found that the early but not the late phases of human BFUE formation was T-cell dependent (54), therefore it is plausible that FeLV-KT interactions with bone marrow lymphocytes or macrophages may alter the early events in erythropoiesis.
SUMMARY

Colony forming unit (CFU) assays were developed for feline granulocyte-macrophage (CFU<sub>GM</sub>), early erythroid (day 2 CFU<sub>E</sub>), and late erythroid (day 7 CFU<sub>E</sub>) colonies in methylcellulose medium. Feline CFU<sub>GM</sub> and both day 2 and day 7 CFU<sub>E</sub> were enhanced by feline macrophage conditioned medium and late CFU<sub>E</sub> often were intimately associated with macrophages. Kittens were inoculated with the Kawakami-Theilen (KT) strain of feline leukemia virus (FeLV) and sequential changes in marrow CFU determined. Erythroid aplasia, characterized by progressive non-regenerative anemia, lymphopenia, and a profound decrease in early and late CFU<sub>E</sub> but not CFU<sub>GM</sub> was induced by 3 to 5 weeks after FeLV-KT inoculation. The susceptibility of kittens to FeLV-induced erythroid aplasia was strongly age-related; neonatal kittens were most sensitive and substantial natural resistance developed by 4 weeks of age. The results demonstrate that FeLV-Kt infection induced a rapid and selective suppression of erythroid progenitor cells and represents a suitable model of experimentally-induced acquired erythroid aplasia.
Table 1.1 Age-related susceptibility of cats to FeLV-KT-induced anemia

<table>
<thead>
<tr>
<th>Age of cat</th>
<th>Virus passage</th>
<th>Virus titer(^a)</th>
<th>FeLV viremia(^b)</th>
<th>Anemia</th>
<th>FOCMA(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neonate</td>
<td>4</td>
<td>ND(^d)</td>
<td>3/3</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Neonate</td>
<td>5</td>
<td>2 X 10(^3)</td>
<td>3/3</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Neonate</td>
<td>6</td>
<td>1.5 X 10(^1)</td>
<td>3/3</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Neonate</td>
<td>7</td>
<td>1.2 X 10(^4)</td>
<td>2/2</td>
<td>2/2</td>
<td>0/2</td>
</tr>
<tr>
<td>Neonate</td>
<td>7</td>
<td>1.2 X 10(^4)</td>
<td>2/2</td>
<td>2/2</td>
<td>0/2</td>
</tr>
<tr>
<td>4 weeks</td>
<td>5</td>
<td>2 X 10(^3)</td>
<td>0(2)(^e)/6</td>
<td>0/6</td>
<td>6/6</td>
</tr>
<tr>
<td>4 weeks</td>
<td>6</td>
<td>1.5 X 10(^1)</td>
<td>2/3</td>
<td>0/3</td>
<td>ND</td>
</tr>
<tr>
<td>8 weeks</td>
<td>5</td>
<td>2 X 10(^3)</td>
<td>1(4)/6</td>
<td>1/12</td>
<td>11/12</td>
</tr>
<tr>
<td>8 weeks</td>
<td>7</td>
<td>1.2 X 10(^4)</td>
<td>2/7</td>
<td>0/7</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^a\) Focus-forming units/ml in the clone 81 FeLV infectivity assay (10,11).

\(^b\) FeLV group specific antigen in blood leukocytes (12,13).

\(^c\) FOCMA = feline oncornavirus-associated cell membrane antigen (14), titer of \(\geq 1:8\) considered as a positive response.

\(^d\) ND = no data available.

\(^e\) ( ) = number with transient viremia.
Figure 1.1 CFU numbers per marrow mononuclear cells. The relationship of feline CFU<sub>GM</sub>, day 2 CFU<sub>EL</sub>, and day 7 CFU<sub>EL</sub> to marrow mononuclear cells was linear between 1 and 5 x 10<sup>5</sup> cells/ml when cultured in microtiter plates (0.1 ml/well; • = CFU<sub>GM</sub>; ▲ = day 2 CFU<sub>EL</sub>; ■ = day 3 CFU<sub>EL</sub>, n = 6 cats, brackets = SE). CFU<sub>GM</sub> medium contained 20% macrophage CM/ml and CFU<sub>EL</sub> medium contained 1 U Ep/ml.
Fig. 1.1
Figure 1.2 CFU<sub>GM</sub> stimulating activity of conditioned media. Feline macrophage conditioned medium had significant CFU<sub>GM</sub> colony stimulating activity (0.05 > P > 0.01) compared to unconditioned medium, but post-endotoxin feline serum and media conditioned by lectin-stimulated feline lymphocytes did not (n = 5, brackets = SE) (CM, conditioned medium; PHA, phytohemagglutinin; Con A, concanavalin A; PW, pokeweed mitogen). Cultures contained 20% conditioned or control media, 10% PCS, and 5 X 10<sup>5</sup> cells/ml.
Fig. 1.2

CONTROL MEDIUM
ENDOTOXIN SERUM
PHA/LYMPHOCYTE CM
CON A/LYMPHOCYTE CM
PW/LYMPHOCYTE CM
MACROPHAGE CM

100 200 300 400
CFU-GM/5x10^4 CELLS
Figure 1.3 CFU<sub>GM</sub> dose response to macrophage conditioned medium; FeLV inoculated cats (■, P.I. week 6) had significantly more CFU<sub>GM</sub>/5 X 10<sup>4</sup> cells than did control cats (○ = 0.05 > P > 0.01, paired t statistic). Cultures contained 10% FCS and 5 X 10<sup>5</sup> cells/ml.
Fig. 1.3
Figure 1.4 Effect of PCS concentration on feline CFU assays. The optimum PCS concentration was 5-10% for $\text{CFU}_{\text{GM}}$ (○) and 20-30% for day 2 (▲) and day 7 (■) $\text{CFU}_{E}$ (P<0.05 for 5-10% PCS in $\text{CFU}_{\text{GM}}$ assay and 20-30% PCS in day 2 $\text{CFU}_{E}$ assay, with no significant differences between test concentrations for day 7 $\text{CFU}_{E}$ by one-way analysis of variance and Duncan's multiple range test, brackets = SE).
Fig. 1.4
Figure 1.5 Feline CFUE response to erythropoietin (Epo) concentration. 1.0 unit/ml was significantly (P<0.05) stimulatory for day 2 CFUE (○). Marked individual variation in response to Epo was noted (n = 6 for day 2 CFUE; n = 5 for day 7 CFUE (□), brackets = SE). Cultures contained 5 X 10^5 cells/ml.
Fig. 1.5
Figure 1.6  Packed cell volume (PCV) changes in FeLV-KT-infected kittens. The mean PCV of infected kittens (A) (n = 4) was significantly less than that of controls (C) (n = 2) at weeks 8 thru 10 after virus inoculation (P<0.005). Brackets = SE.
Fig. 1.6

The graph shows the percentage of packed cell volume (PCV) over weeks post-inoculation. Two lines are plotted, labeled 'C' and 'A', with error bars indicating variability. The x-axis represents weeks post-inoculation (6 to 10), while the y-axis represents PCV (%).

Fig. 1.6
Figure 1.7 Peripheral blood lymphocyte counts in FeLV-infected kittens. Lymphocyte numbers were significantly decreased ($P < 0.025$) in FeLV-KT-infected kittens (A) compared with controls (C) at weeks 9 and 10. Brackets = SE.
LYMPHOCYTE NUMBER (X 10^{-3})

WEEKS POST-INOCULATION

Fig. 1.7
Figure 1.8 Sequential CFU<sub>GM</sub> assays following FeLV-KT inoculation. CFU<sub>GM</sub> were significantly increased (P<0.005) in FeLV-KT-infected cats (A, n = 4) compared with controls (C, n = 2) at weeks 9 and 10. Cultures contained 5 X 10<sup>5</sup> cells/ml.
Fig. 1.8
Figure 1.9 Sequential CUFE assays following FeLV-KT inoculation: Day 2 (○) and Day 7 (■) CUFE from FeLV-KT-infected kittens (n = 4) were significantly reduced compared with Day 2 (○) and Day 7 (□) CUFE from control kittens.
Fig. 19
Plate I. Day 2 CFU\textsubscript{E} morphology in Wright-stained cytocentrifuged preparation. (200X)
Plate II. Day 7 CFU<sub>P</sub> morphology in Wright-stained cytocentrifuged preparation. (200X)
CHAPTER II

FELINE LEUKEMIA VIRUS-INDUCED MACROTROMBOCYTOSIS
IN CATS

INTRODUCTION

The mechanisms of platelet volume regulation are complex and poorly understood (1). Increased thrombopoiesis frequently results in macrothrombocytosis (2) and most acquired macrothrombocytic disorders of man involve increased platelet turnover and reduced lifespan (1,3,4). However, in some acute leukemias, acquired intrinsic megakaryocytic abnormalities rather than platelet age are critical determinants of platelet volume (1,5) and in human hereditary thrombocytopenias accompanied by macrothrombocytosis the platelet lifespans may be normal, decreased or increased (1). Animal models of macrothrombocytosis are needed to further investigate the regulatory systems of the megakaryocyte-platelet axis.

Naturally occurring FeLV infections in cats induce a wide spectrum of hematological and immunological dysfunctions (6), some of which mimic human diseases, eg. preleukemia and acute leukemia, in which macrothrombocytosis and platelet dysfunction are frequently noted (1,7,8). Macrothrombocytosis in FeLV-diseased cats is not
generally recognized but bizarre macrothrombocytes have been described (6) and a transient thrombocytopenia has been reported in viremic cats after infection by contact exposure to FeLV (9). In viremic cats the megakaryocytes and platelets are productively infected (10) and acquire FeLV group-specific antigens (11,12). This paper describes a prospective study of platelet volume changes in cats experimentally infected with the Kawakami-Theilen strain of feline leukemia virus (FeLV-KT). We found that FeLV-KT induced a marked macrothrombocytosis which has potential for studies of platelet volume control mechanisms.
MATERIALS AND METHODS

All cats studied were 2 to 4 kg young adult females from a FeLV-free closed breeding colony (17). Ketamine HCl (Vetalar, 10 mg/kg IM, Parke Davis, Morris Plains, NJ) anesthesia was used for all procedures. The FeLV-KT-inoculated cats were given 1.5 ml of a splenic homogenate containing $7.5 \times 10^4$ FFU of FeLV-KT virus intraperitoneally in conjunction with corticosteroid to abrogate the natural adult resistance to infection. The corticosteroid (methyl prednisolone acetate, Depomedrol, 5 mg/kg IM, Kalamazoo, MI) was given 7, 4, and 0 days prior to and 4 days post-inoculation (13). Eight inoculated and 2 steroid-treated age-matched controls utilized in sequential studies of mean platelet volumes and concentrations. An additional 10 cats with progressive FeLV-KT infection (post-infection weeks 8-10) induced as above and 11 control cats from the same colony were used in the glutaraldehyde fixed and/or hypotonically swollen platelet volume studies.

Cats were bled weekly under anesthesia (Ketamine HCl, 10 mg/kg IM, Parke Davis, Detroit, MI) via the jugular vein beginning 3 or 4 weeks prior to inoculation. Blood anticoagulated with ethylenediamine tetracetic acid ($K_3$ EDTA, $1.5 \times 10^{-3}$ g/ml) was routinely collected. For studies of shape change blood was drawn into acid citrate dextrose NIH formula A (ACD-A, 15% v/v) and mixed immediately after collection with 4 volumes of 1.3% glutaraldehyde in 0.5M NaCl with 0.0M phosphate buffer at room temperature. Platelet rich plasmas (PRP) were prepared from anticoagulated blood by centrifugation in vertical tubes for 5 minutes of progressively decreasing centrifugal
force (Coulter Thrombofuge, Coulter Electronics, Hialeah, FL). Platelet counts and size distribution histograms were determined with a single channel electronic counter and 100 channel particle size analyzer (Coulter Counter Model ZBI and Channelyzer C-1000, Coulter Electronics, Hialeah, FL) interfaced with a microcomputer for analysis of size distributions (Apple II-plus, Apple Computers Inc., Cupertino, CA). The electronic counter was equipped with a high resolution 70 μm aperture (#7040, Coulter Electronics, Hialeah, FL) and instrument settings included amplification 0.5, aperture current 0.5, lower threshold 5, upper threshold 100, and gain trim 5. Channelyzer settings included base channel threshold 5, window width 100, peak channel 1000, and edit function on. The size distribution scale was calibrated routinely with 16 fl polystyrene spheres. Log-probit plots of platelet size distributions were linear and therefore were compatible with log normal distributions (4,18,19); for descriptive statistics and statistical analysis the geometric mean platelet volumes (GMPV), equivalent to the median volumes, were employed. However, in the studies of hypotonically swollen platelets (vide infra), platelet volume distributions from cats with marked macrothrombocytosis were electronically truncated by the upper size limit of the Channelyzer (61 fl). For these studies the size distribution modal volumes were used rather than calculating a GMPV from the electronically truncated size distribution. The modal volume, GMPV and variance (s²) of log normal distributions are interrelated, eg. log modal volume (fl) = log GMPV-2.303 log s² (18). Platelet
mass (M) was calculated as follows: 
\[ M (\mu l/ml) = \text{GMPV(\mu l)} \times \text{Platelets/\mu l} \times 10^{-6}. \]

Platelets of EDTA-PRP were diluted (in an isotonic balanced saline solution (IBSS, Isoton, Coulter Electronics, Hialeah, FL) for platelet concentration and volume distribution analysis. Selected platelet samples were then hypotonically swollen using a modification of the method described by Boneu et al. (19). Briefly, 6 ml H\textsubscript{2}O were slowly mixed with 4 ml of the diluted EDTA-PRP remaining after platelet concentration determination. After 1 minute, 10 ml of 40\% IBSS in H\textsubscript{2}O containing 0.16\% formaldehyde was added to prevent additional volume changes and volume distributions of the swollen platelets determined. Platelet surface area (A) of EDTA-sphered platelets before and after hypotonic swelling were calculated from the platelet modal volume (V) assuming spherical shape by the formula:

\[ A = e^{(0.67 \ln 1.9 V + \ln \pi)} \]

Surface connected cannillcular system (SCCS) membrane surface area evaginated by hypotonic swelling was calculated by subtracting the isotonic platelet surface area from the surface area of hypotonically swollen platelets for each of 11 control cats and 10 FeLV-KT-infected cats.

Statistical analyses of the data were done utilizing a NWA STATPAK version 2.1 (Northwest Analytical, Portland, OR) and an Apple II plus computer.
RESULTS

The geometric mean platelet volumes of EDTA-sphered platelets from 8 cats were increased significantly \((P<0.01)\) on weeks 4 through 6 following FeLV-KT inoculation (Table 2.1). All cats were viremic during this period but only 2 (cats 2518, 2524) developed progressive anemia. The trend towards reduced whole blood platelet concentration in FeLV-KT-inoculated cats was significant \((P<0.01)\) only at week 4 (Table 2.2). The late trend of increased platelet mass was not significantly significant (Table 2.3).

Normal platelets in PRP prepared from ACD-A anticoagulated glutaraldehyde-fixed whole blood were primarily discocytes with rare spheroechinocytes by phase contrast microscopy. The same treatment, platelets of FeLV-KT-inoculated cat were discoid but had a tendency to form one or two blunt pseudopods (discoechinocytes). Both ACD-A glutaraldehyde-fixed and EDTA-sphered platelets of FeLV-KT-inoculated cats were significantly larger \((P<0.001)\) than platelets of normal cats but no significant change in the ACD-A/EDTA volume ratio for platelets from infected cats was noted (Table 2.4).

The volume increase in hypotonic diluent was greater \((P<0.05)\) in platelets from FeLV-KT-inoculated cats than platelets of normal cats, however, the percent volume increase in platelets from infected cats was significantly less \((P<0.005)\) than normal (Table 2.5). Thus, when surface areas were calculated, no significant change in SCCS membrane area was noted in FeLV-KT-infected cats despite marked macrothrombocytosis, and the ratio of SCCS area to platelet volume was significantly less than that of normal cats.
DISCUSSION

A marked macrothrombocytosis was noted in EDTA-anticoagulated blood from cats infected with the KT isolate of FeLV. Because in some human hereditary giant platelet syndromes macrothrombocytosis may be due to an in vitro hypervolumetric shape change while circulating discocytes have normal volumes (20,21), the volume distributions of ACD-A anticoagulated glutaraldehyde-fixed platelet samples were also characterized. The glutaraldehyde-fixed samples from FeLV-KT-infected cats, which should approximate circulating platelet populations in terms of shape and volume distributions (22), also were macrocytic, and there was no detectable change in the EDTA-related apparent volume increase as estimated by the ACD-A/EDTA volume ratios. Both platelet shape and volume contribute to the apparent cell volume determined by impedance particle sizing methodology (23). Since the EDTA-induced platelet changes are essentially isovolumetric changes in shape the increase in impedance-determined platelet volume due to EDTA anticoagulation is an estimate of shape change ability (24).

The qualitative observation of discoechinocytes in fixed platelet samples must be interpreted with caution since similar-shaped platelets are described for normal human platelets (25) and are occasionally seen in normal cat PRP (Boyce J.T., personal observations). Even if induced by the collection technique, their frequent occurrence in FeLV-KT-infected cats may imply a predisposition to undergo shape change. As a result, impedance methodology could overestimate circulating platelet volume and underestimate
EDTA-induced shape change if shape change was induced during collection and PRP preparation (23). However, platelets bear membrane Fc receptors (26) and C1q can bind to platelet membrane collagen receptors (27). The qualitative observed shape changes noted could be immune-mediated like those induced by anti-human platelet antibodies in-vitro (28). Since platelets of FeLV-KT-infected cats carry FeLV group-specific antigens (11,12), and circulating immune complexes occur in some FeLV-infected cats (29).

Immune-mediated alteration of circulating platelets cannot be the only mechanism involved in the macrothrombocytosis because the data from hypotonically swollen platelets implied increased total plasma membrane (SCCS surface area + EDTA platelet surface area) without a proportional increase in SCCS. Platelets have little capacity for membrane synthesis; an increase in megakaryocyte cytoplasmic growth and/or a decrease in effective membrane territorial demarcation are the most likely causes of a macrothrombocytosis with increased plasma membrane as seen here. The trend for decreased platelet concentrations in FeLV-KT-infected cats and a shorter than normal platelet survival time (Boyce J.T., Chapter IV) would suggest possible stimulation of thrombopoiesis. Megakaryocyte cytoplasmic growth without the concomitant increase in membrane demarcation required to maintain a normal SCCS surface area to volume ratio could produce the syndrome noted here. Macrocysts without proportionate SCCS area increase is reported for both idiopathic thrombocytopenic purpura (19) and certain human hereditary giant platelet syndromes (30). Either viral-mediated intrinsic megakaryocyte defects or
immune-mediated factors could be important in the pathogenesis of the cat platelet lesion.

Therefore, FeLV-KT-induced macrothrombocytosis appears to be an in vivo animal model of dysthrombopoiesis. The changes in platelet mass implies impaired regulation of platelet size and/or volume (31). Coupled with the observed change in platelet membrane areas, this model should be particularly suited for probing territorial demarcation events as they relate to platelet volume regulation and may allow testing of the Paulus hypothesis wherein the summation of mutually independent cytoplasmic growth and territorial demarcation events predicts platelet volume distribution (4).
SUMMARY

The Kawakami-Theilen strain of feline leukemia virus (FeLV-KT), an exogenous anemia-inducing retrovirus, induced significant macrothrombocytosis during acute infections of cats. The geometric mean platelet volumes of both freshly isolated fixed platelets and EDTA-sphered platelets from infected cats were increased. The ratio of fresh-fixed to EDTA-sphered platelet volumes, an estimate of EDTA-induced isovolumetric shape change, was normal. Total plasma membrane but not estimated surface connected canalicular system surface area increased as the platelet volumes increased in FeLV-KT-infected cats. Significantly reduced platelet concentrations were observed on week 4 post-inoculation. Near normal platelet concentrations with marked macrothrombocytosis resulted in increased platelet mass on week 6 post-inoculation in FeLV-KT-infected cats.

Thus, FeLV-KT-induced macrocytosis may serve as a model of impaired platelet volume regulation. The platelet volume and platelet membrane surface area abnormalities suggest that this model would allow studies of the megakaryocyte/platelet axis, particularly in the area of membrane formation and territorial demarcation.
Table 2.1 Changes in Geometric Mean Platelet Volume\(^a\) (femtoliters) Associated With FeLV-KT Infection

<table>
<thead>
<tr>
<th>CAT</th>
<th>BASELINE DATA(^b)</th>
<th>POST-INOCULATION WEEK</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>2829</td>
<td>12.5±1.1 (n=4)</td>
<td>ND</td>
</tr>
<tr>
<td>2889</td>
<td>13.2±1.3 (n=4)</td>
<td>ND</td>
</tr>
<tr>
<td>2899</td>
<td>11.7±1.3 (n=4)</td>
<td>ND</td>
</tr>
<tr>
<td>2518</td>
<td>13.5±1.6 (n=4)</td>
<td>15.5</td>
</tr>
<tr>
<td>2524</td>
<td>10.9±1.6 (n=3)</td>
<td>11.9</td>
</tr>
<tr>
<td>2590</td>
<td>13.0±2.1 (n=3)</td>
<td>15.2</td>
</tr>
<tr>
<td>2597</td>
<td>11.4±1.3 (n=3)</td>
<td>11.6</td>
</tr>
<tr>
<td>2627</td>
<td>10.2±1.1 (n=3)</td>
<td>12.7</td>
</tr>
<tr>
<td>(\bar{x})</td>
<td>12.0</td>
<td>13.4</td>
</tr>
<tr>
<td>SD</td>
<td>1.2</td>
<td>1.8</td>
</tr>
</tbody>
</table>

\(^a\) Determined in PRP from EDTA-anticoagulated blood.

\(^b\) Replicate measurements at weekly intervals prior to inoculation.

\(^c\) ND, not done.

\(^d\) Significantly different from baseline data; \(P \leq 0.01\), using Friedman's test for differences between treatments followed by the Mann-Whitney rank-sum test with Bonferroni correction to isolate differences between baseline data and post-inoculation data.
Table 2.2 Changes in Whole Blood Platelet Concentrations\textsuperscript{a} (X \textsuperscript{10\textsuperscript{5}}/\mu l) Associated With FeLV-KT Infection

<table>
<thead>
<tr>
<th>CAT</th>
<th>BASELINE DATA\textsuperscript{b}</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>2829</td>
<td>3.9±1.2 (n=4)</td>
<td>ND\textsuperscript{d}</td>
<td>ND</td>
<td>3.29</td>
<td>3.48</td>
<td>2.93</td>
<td>3.43</td>
</tr>
<tr>
<td>2889</td>
<td>5.2±1.5 (n=4)</td>
<td>ND</td>
<td>ND</td>
<td>6.32</td>
<td>3.32</td>
<td>5.83</td>
<td>4.76</td>
</tr>
<tr>
<td>2899</td>
<td>4.1±2.5 (n=4)</td>
<td>ND</td>
<td>ND</td>
<td>6.96</td>
<td>4.15</td>
<td>7.10</td>
<td>7.56</td>
</tr>
<tr>
<td>2518</td>
<td>6.5±1.1 (n=3)</td>
<td>5.9</td>
<td>4.25</td>
<td>4.42</td>
<td>3.80</td>
<td>4.00</td>
<td>5.21</td>
</tr>
<tr>
<td>2524</td>
<td>6.2±0.3 (n=3)</td>
<td>5.90</td>
<td>4.37</td>
<td>2.15</td>
<td>2.10</td>
<td>3.95</td>
<td>2.28</td>
</tr>
<tr>
<td>2590</td>
<td>5.6±0.1 (n=3)</td>
<td>5.12</td>
<td>4.10</td>
<td>4.72</td>
<td>2.23</td>
<td>2.60</td>
<td>2.80</td>
</tr>
<tr>
<td>2597</td>
<td>6.6±0.6 (n=3)</td>
<td>6.36</td>
<td>5.10</td>
<td>4.20</td>
<td>2.48</td>
<td>5.32</td>
<td>3.85</td>
</tr>
<tr>
<td>2627</td>
<td>6.2±0.3 (n=3)</td>
<td>5.70</td>
<td>4.72</td>
<td>3.28</td>
<td>4.27</td>
<td>6.15</td>
<td>6.25</td>
</tr>
<tr>
<td></td>
<td>(\bar{X})</td>
<td>5.5</td>
<td>5.8</td>
<td>4.5</td>
<td>4.6</td>
<td>3.2\textsuperscript{c}</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>1.1</td>
<td>0.4</td>
<td>0.4</td>
<td>0.2</td>
<td>0.9</td>
<td>1.6</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Determined on PRP from EDTA-anticoagulated blood.
\textsuperscript{b} Replicate measurements at weekly intervals prior to inoculation.
\textsuperscript{c} Significantly different from baseline data; \(P \leq 0.01\), using Friedman's test for differences between treatments followed by the Mann-Whitney rank-sum test with Bonferroni correction to isolate differences between baseline data and post-inoculation data.
\textsuperscript{d} ND, not done.
Table 2.3 Whole Blood Platelet Mass\textsuperscript{a} (μl/ml) Before and After FeLV-KT Infection.

<table>
<thead>
<tr>
<th>CATS</th>
<th>BASELINE\textsuperscript{b}</th>
<th>POST-INOCULATION WEEK</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>2829</td>
<td>5.0</td>
<td>ND</td>
</tr>
<tr>
<td>2889</td>
<td>6.8</td>
<td>ND</td>
</tr>
<tr>
<td>2899</td>
<td>4.8</td>
<td>ND</td>
</tr>
<tr>
<td>2518</td>
<td>8.8</td>
<td>9.1</td>
</tr>
<tr>
<td>2524</td>
<td>6.8</td>
<td>7.0</td>
</tr>
<tr>
<td>2590</td>
<td>7.3</td>
<td>7.8</td>
</tr>
<tr>
<td>2597</td>
<td>7.5</td>
<td>7.4</td>
</tr>
<tr>
<td>2627</td>
<td>6.3</td>
<td>7.2</td>
</tr>
</tbody>
</table>

\(\overline{X}\textsuperscript{d}\) | 6.66 | 7.7 | 6.08 | 6.63 | 5.94 | 8.93 | 9.40 |
\(SD\) | 1.31 | 0.8 | 0.7 | 1.65 | 2.34 | 3.24 | 2.94 |

\textsuperscript{a} Calculated from data of Tables 2.1 and 2.2.
\textsuperscript{b} Calculated from mean baseline data of Tables 2.1 and 2.2.
\textsuperscript{c} ND, not done.
\textsuperscript{d} No significant differences between baseline data and weekly post-inoculation data using Friedman's test for differences between treatments followed by Mann-Whitney rank-sum test with Bonferroni correction to isolate differences between baseline and post-inoculation data.
Table 2.4 ACD-A and EDTA Anticoagulated PRP Platelet Modal Volumes in Control Cats and FeLV-KT-Infected Cats

<table>
<thead>
<tr>
<th>Platelet Rich Plasma</th>
<th>Controls</th>
<th>FeLV-KT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prepared from Blood with</td>
<td>(n = 11)</td>
<td>(n = 7)</td>
</tr>
<tr>
<td>Glutaraldehyde-fixation</td>
<td>6.6±0.6</td>
<td>9.9±2.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>and ACD-A anticoagulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA anticoagulation</td>
<td>9.4±1.2</td>
<td>14.9±2.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ACD-A/EDTA RATIO</td>
<td>0.572</td>
<td>0.537</td>
</tr>
</tbody>
</table>

<sup>a</sup> Comparisons of electronically determined platelet modal volumes of ACD-A anticoagulated blood fixed promptly in glutaraldehyde after phlebotomy and of EDTA anticoagulated blood allow estimation of the isovolumetric EDTA-induced shape change, represented here as the ACD-A/EDTA volume ratios.

<sup>b</sup> Unpaired T test, P<0.001 for FeLV-KT versus control volumes.
Table 2.5 Modal Volumes of EDTA-Sphered Platelets in Isotonic and Hypotonic Diluent and Calculated Membrane Surface Areas for Normal Cats and Cats Infected with FeLV-KT.

<table>
<thead>
<tr>
<th>Platelet Parameter</th>
<th>Units</th>
<th>Controls (n = 11)</th>
<th>PeLV-KT (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotonic Diluent</td>
<td>fl</td>
<td>9.4±1.2</td>
<td>19.2±3.8²</td>
</tr>
<tr>
<td>Hypotonic Diluent</td>
<td>fl</td>
<td>18.0±2.9</td>
<td>30.1±3.7²</td>
</tr>
<tr>
<td>Volume Increase</td>
<td>fl</td>
<td>8.5±2.6</td>
<td>10.9±2.9³</td>
</tr>
<tr>
<td>% Volume Increase</td>
<td>%</td>
<td>92±27</td>
<td>59±20²</td>
</tr>
<tr>
<td>Surface Area in Isotonic Diluent</td>
<td>µ²</td>
<td>21.5±1.8</td>
<td>34.5±4.4³</td>
</tr>
<tr>
<td>Surface Area in Hypotonic Diluent</td>
<td>µ²</td>
<td>35.1±2.9</td>
<td>46.6±3.8³</td>
</tr>
<tr>
<td>Evaginated SCCS Surface Area</td>
<td>µ²</td>
<td>11.6±3.2</td>
<td>12.1±3.3</td>
</tr>
<tr>
<td>SCCS Area to Volume Ratio</td>
<td>µ⁻¹</td>
<td>1.3±0.4</td>
<td>0.7±0.2³</td>
</tr>
</tbody>
</table>

1 Platelet membrane surface areas were calculated from the volumes of EDTA anticoagulated platelets and hypotonically swollen EDTA anticoagulated platelets assuming spherical cells. The increase in surface area of swollen platelets is surface connected cannilicular system (SCCS) membrane evaginated by the swelling.

2 PeLV-KT platelets significantly different from controls. Unpaired T test, P<0.005.

3 PeLV-KT platelets significantly different from controls. Unpaired T test, P<0.05.
CHAPTER III
FELINE LEUKEMIA VIRUS-INDUCED PLATELET FUNCTION
DEFICITS IN CATS

INTRODUCTION

Cats infected with feline leukemia virus (FeLV) express feline leukemia virus group specific antigens (FeLV-GSA) in platelets and megakaryocytes, and budding retroviruses are detectable ultrastructurally in these cells. Although bleeding episodes are uncommon, thrombocytopenia and/or bizarre-shaped platelets have been reported in naturally infected cats. Thrombocytopenia was not observed in the initial study of the anemia induced by the Kawakami-Theilen strain of FeLV (FeLV-KT) but transient viremia was reported in cats experimentally infected by contact exposure. FeLV-infection of cats may serve as a model for the human preleukemias and myeloproliferative disorders in which clinically significant episodes of bleeding with bizarre giant platelets, decreased platelet adhesiveness, and impaired in-vitro ADP-, collagen-, or epinephrine-induced aggregation reactions occur. These functional deficits may relate to the abnormal fatty acid metabolism described for human preleukemic platelets. The presence of virus in FeLV-infected cat platelets and the potential

55
value of an animal preleukemic platelet deficit model warranted a prospective study of platelet function in FeLV-infected cats.

We describe herein FeLV-KT-related platelet aggregation and ATP release abnormalities. Platelet function was studied in-vitro before and after FeLV-KT infection in cats utilizing light scattering and chemiluminescence techniques with dilute samples of PRP. The in-vitro effects of purified FeLV-KT on normal cat platelets were also evaluated.
MATERIALS AND METHODS

Eight to 12 month old, 2 to 4.5 kilogram female cats were obtained from a FeLV-free closed breeding colony. Anesthesia (Ketamine HCl, 10 mg/kg IM, Park Davis, Detroit, MI) was used for all procedures. All cats received corticosteroid to abrogate the adult cat's natural resistance to FeLV infection. The corticosteroid (methyl prednisolone acetate, Depomedrol, 5 mg/kg IM, Upjohn, Kalamazoo, MI) was given on days 7, 4 and 0 prior to and day 4 after inoculation with virus. Non-inoculated control cats were randomly selected and given methyl prednisolone as above. Inoculated cats received 1.5 ml intraperitoneally of splenic homogenate (virus passage 5; 7.4 x 10^4 focus forming units/ml) prepared and assayed for infectivity as described elsewhere. Inoculated cats typically are viremic by week 3 post-inoculation. The data included here are derived from normal cats as needed for method development and from 2 sequential studies, the first containing 5 inoculated and 2 control cats, the second containing 3 inoculated cats. Each of the cats in the sequential studies was bled at weekly intervals at least 3 times prior to inoculation to establish baseline values for aggregation assays. All blood samples (4-6 ml volumes) were drawn between 8 and 9 AM, prior to feeding.

Collagen-, adenosine diphosphate (ADP)-, and arachidonic acid (AA)-induced platelet aggregation and adenosine triphosphate (ATP) secretion were studied simultaneously in a whole blood aggregometer (Crono-Log Corp., Haverton, PA) utilizing a modification of the
technique described by Russel-Smith et al. for the assay of aggregation responses in small volumes of whole blood. For the in-vitro assays PRP was prepared by differential centrifugation (110 X g, 25°C, 12 minutes, swinging bucket rotor) from anti-coagulated venous blood (acid citrate dextrose, NIH formula A[ACD-A], 15% in blood). After incubation (37°C, 1 hr, 5% CO₂ in humidified air) the PRP was held tightly capped at room temperature until assayed (up to 3 hours). The platelet diluent, a modified Tyrode's solution with 7.2 mg/dl calcium, included sodium heparin to prevent coagulation of PRP (sodium heparin for injection, Panheparin, 2 units/ml, lot 26-395-AF, Abbott Labs, North Chicago, IL). For each assay 1 ml of appropriately diluted PRP was added to an unsiliconized cuvette and stirred (700 rpm) with a Teflon-coated stirring bar for 1.5 minutes at 37°C before adding the platelet agonist. Firefly lantern extract (FFL, 50 µl, containing the soluble extract of 0.5 mg firefly lanterns, Sigma, St. Louis, MO) was added prior to the platelet agonist. Aggregation responses were monitored by changes in transmitted light (T) and the ATP release reactions were monitored by intensity of chemiluminescence for 6 to 8 minutes after addition of the platelet agonist. Each chemiluminescence curve was calibrated by the addition of 1.25 x 10⁻⁹ moles ATP to the cuvette at the termination of the assay. Each transmitted light curve was automatically calibrated by the aggregometer (0% T, diluted PRP; 100% T, diluent blank).
Two methods of PRP dilution were utilized. In the first, using a standard 20% dilution of PRP, the degree of plasma protein and anticoagulant dilution was held constant in the cuvette. In the second method platelet mass, estimated from the initial light transmittance, was held constant by varying the PRP dilution. To do this the aggregometer's optical channel was calibrated with 0% T for an opaque cuvette and 100% T for diluent and then the PRP dilution was adjusted by trial and error to give a T of 62.5%. All assays on that PRP sample were then run at that dilution with the instrument recalibrated to read 0% T for the diluted sample.

The collagen utilized in these aggregation assays was prepared from the aortas of two specific pathogen-free cats. The aortas were stripped of adherent fat, homogenized and sonicated in 83.5 mM acetic acid, diluted to 23 mM acetic acid in H2O and frozen in aliquots for subsequent use. In pilot assays with 20% normal cat PRP in diluent, 20 µl of a collagen preparation containing 0.60 µg aortic protein induced consistent rapid aggregation. All collagen-induced aggregation assays done with the 20% PRP dilution method utilized 0.6 µg aortic protein. With the constant platelet mass technique, however, the collagen dose-platelet response relationship was more precisely determined. The minimal aggregation time (MAT) was determined with a high concentration of collagen (6.2 µg aortic protein). The quantity of collagen (aortic protein) required to stimulate aggregation at 50% of maximal aggregation rate (2X MAT, assuming that aggregation times and aggregation rates are inversely proportional) was then estimated using dilutions of collagen. Multiple aggregation assays were run
until the 2X MAT time was bracketed. The collagen concentration which would yield 2X MAT was calculated by linear interpolation between the concentrations of the two dilutions which induced aggregation times bracketing 2X MAT.

The aggregation responses to ADP (Sigma, St. Louis, MO) and AA (Bio/Data Corp., Hatboro, PA) were also evaluated. With the 20% PRP dilution method aggregation response to $2 \times 10^{-6}$ M ADP were determined and with the constant platelet mass method the responses to $1 \times 10^{-4}$ M ADP and 500 ug/ml AA were determined. In pilot studies these were the minimum concentrations of agonist which would yield consistent aggregation responses in normal cats.

An Apple II plus computer and the NWA Statpak version 2.1 (Northwest Analytical, Portland OR) program were used to analyze the data. The aggregation curves were compared using the time to 1/2 maximal aggregation ($t_{1/2}$), a parameter reported to be independent of platelet concentration or aggregometer amplifier settings.\(^{19,20,21}\)

Within assay and between assay variation for the constant platelet mass method was assessed by the method of Robard\(^{22}\) with data from 3 replicates each of ADP, collagen, and AA-induced aggregation/secretion assays on each of 2 samples drawn 1 week apart from 3 normal cats. The relationship between platelet mass, initial light transmittance, and $t_{1/2}$ for collagen were characterized using PRP from 5 normal cats. Platelet volumes and concentrations used in mass calculations were determined by standard impedance methodology.

The in-vitro effects of FeLV-KT virus on normal cat platelets was evaluated. Serial dilutions of an ultraviolet light-inactivated
FeLV-KT virus concentrate prepared by molecular filtration and sucrose density gradient purification of tissue culture fluids were incubated with normal cat PRP (30 minutes, 37°C, 5% CO₂ in humidified air). The platelet aggregation and ATP secretion responses to collagen were then determined.
RESULTS

When normal cat PRP was diluted in modified Tyrode's solution aggregation could readily be elicited at dilution levels (5-20%) where aggregation could not be induced if autologous platelet-poor plasma was the diluent. Normal aggregation times and ATP release levels, within assay variation, and between assay variation for the constant platelet mass technique were estimated in 3 normal cats (Table 3.1). The coefficients of variation (CV) ranged between 5 and 22%, with the exception of the between assay CV of 41.6% for collagen-induced peak ATP release. No significant differences in aggregation or secretion were observed in paired samples collected 1 week apart from these normal cats. The relationship between initial light transmittance and platelet mass was characterized using normal PRP from 5 cats at several dilutions (Fig. 3.1). When the transmittance readings (T) were transformed to initial light extinction (Eo) values by the formula: (24)

\[ E_o = -\log T_o \]

there was a direct linear correlation (r = 0.9552) between \( E_o \) and platelet mass which was highly significant (p < 0.01). With the same 5 cats there was a significant (p < 0.01) non-linear inverse correlation between mass and collagen-induced aggregation times (Fig. 3.2). Typical aggregation response and ATP secretion curves for pre- and post-inoculation assays are given in Figures 3.3, 3.4 and 3.5.

In cats infected with FeLV-KT, significant impairments of collagen-induced aggregation were noted with both PRP dilution techniques (Tables 3.2, 3.3). With the 20% PRP dilution method, infected
cat but not control cat platelet aggregation times increased significantly with time after infection (Table 3.2, linear regression analysis, $P<0.01$). When examined with the constant platelet mass technique, no significant changes were noted in aggregation using high levels of collagen, but the concentration of collagen required to induce 2X MAT was significantly increased ($p<0.05$) at both post-inoculation assay times (Table 3.3).

No consistent changes in ADP-induced aggregation responses were noted with either dilution technique (Table 3.5). However, there was a marked decrease in the peak ATP released with ADP stimulation at all assay times after inoculation (Table 3.4).

Arachidonic acid-induced aggregation responses were markedly impaired in the virus infected cats (Table 3.6). The AA used in this study inhibited the chemiluminescence reaction when added to the cuvette during normal ADP-induced release reactions and, therefore, ATP release curves induced by AA could not be observed with the methods used herein.

In-vitro incubation of normal cat PRP with purified FeLV-KT virus, when compared with media controls, did not significantly alter platelet aggregation or ATP secretion (Table 7). Greater than 65 µg viral protein per ml PRP induced platelet aggregation during incubation.
DISCUSSION

Dilution of PRP for aggregation assays was done to minimize the volumes of blood necessary for sequential studies. The platelet concentrations (5 to 20% PRP) used here were less than those typical for standard aggregation techniques \(^{(24,25)}\). The low platelet concentration may have been offset in part by the stimulatory effects of near physiologic levels of diluent calcium. \(^{(17)}\) Heparin was included to prevent fibrin formation in the presence of these calcium levels. Heparin can permit shape change and affect platelet aggregation, \(^{(26)}\) but the one lot used here did not appear to have adverse effects, although this was not specifically tested for. The collagen and ADP responses noted here were similar to those described for cat platelets with undiluted PRP, however, some normal cat platelets may release ATP in response to AA with standard PRP aggregometry. \(^{(27-30)}\) The ATP release reaction is not required for cat platelet AA-induced aggregation since dense granule-deficient cat platelets aggregate with AA. \(^{(30)}\)

The \(t_{1/2}\) time was chosen for comparison of aggregation responses because it is a kinetic parameter relatively unaffected by platelet concentration, \(^{(19)}\) however, with the PRP dilution techniques used herein there was a significant correlation between cat PRP \(t_{1/2}\) and platelet mass. The coefficients of variation for the different parameters studied, with the exception of collagen-induced ATP release, are comparable with more standard techniques although quantitative data in the literature is limited. As estimated from their data, Meyers et al. had a 20.6% CV for ATP release using an
isotopic technique\textsuperscript{(30)} and, estimated from figures, Tschopp reported 21-28\% CV for maximal ADP-induced aggregation rates.\textsuperscript{(27)}

The data shows FeLV-induced functional deficits involving 3 classes of aggregating agents: collagen, ADP, and AA. Significant platelet volume increases occur in FeLV infected cats, but volume change is unlikely the primary cause of the dysfunction. Large but otherwise normal platelets of other species typically aggregate well\textsuperscript{(32,33)} and we noted impaired aggregation while controlling platelet mass in the cuvette. Data in other species also suggest that as platelets circulate they lose portions of membrane and cytoplasm in reversible aggregation events, with young large platelets losing volume and functional capacity as they age.\textsuperscript{(34)} If FeLV-KT-infected platelets did not participate in these reversible aggregation events, then senescent FeLV-KT-infected platelets could remain large but less functional than normal. Platelet survival data, however, imply increased platelet turnover in infected cats (see Chapter IV).

The initial event in aggregation is binding of agonist to specific platelet membrane receptors. The FeLV antigens are unlikely to be specific blockers for all 3 receptors involved here, but a specific effect on collagen receptors is possible via immune complexes and complement activation, because the CIq of immune complexes can bind to collagen receptors\textsuperscript{(34,35)} and circulating immune complexes which activate complement have been reported in some FeLV-infected cats.\textsuperscript{(36)} Since human myeloproliferative\textsuperscript{(38)} and possibly FeLV-infected cat platelets have increased F\textsubscript{c} receptors, large
amounts of complexes, if present, could be bound to the feline platelets, specifically blocking collagen receptors and interfering indirectly with other receptors. FeLV antigens also impair con A receptor capping of lymphocytes and could alter platelet membrane fluidity, but the functional deficit is not like that induced by agents known to alter fluidity. Ethanol, for example, increases fluidity and impairs collagen- or ADP-induced aggregation and release phenomena but markedly stimulates AA metabolism.\(^{(40,41)}\)

While involving different receptors, all 3 aggregating agents probably utilize a common "second messenger" to affect intracellular metabolism.\(^{(42,43)}\) The putative second messenger in platelets is calcium mobilized from intracellular sites, most probably the areas of closely associated dense tubule/surface connected cannalicular system aggregates (SCCS).\(^{(41,44)}\) Since large FeLV-KT-infected platelets have less SCCS/unit volume (see Chapter II) they may have an impaired capacity to mobilize calcium which could affect all aggregation and release reactions. The AA deficit may specifically involve these membrane aggregates since platelet prostaglandin biosynthesis is localized in the dense tubular system.\(^{(45)}\)

Holmsen's unifying concept for the "basic platelet reaction" incorporates agonists with different stimulatory abilities, calcium as the second messenger, and the two independent but synergistic stimulatory feed back loops of dense granule components (ADP, serotonin) and prostaglandins/thromboxanes.\(^{(43)}\) The feedback loops are critical for aggregation induced by ADP, an agonist of intermediate potency; the abnormal ADP-induced aggregation and secretion
noted here may be secondary to impaired AA metabolism. Since normal cat platelet AA-induced aggregation correlates with the ability to generate thromboxane \( B_2 \), the impaired AA aggregation of FeLV cats implies abnormal AA metabolism.\(^{29}\) High levels of collagen, but not low levels, are relatively independent of feedback aggregation amplification.\(^{43}\) Thus, the impaired aggregation at low but not high levels of collagen may relate to metabolic deficits rather than membrane receptor factors and the collagen, ADP and AA lesions may have a common metabolic basis.

The final steps of aggregation, eg. actual adhesion and aggregation of cells, are dependent upon specific membrane glycoproteins.\(^{46,47}\) In human thrombasthenia (eg. Glanzmann's thrombasthenia) there are abnormally low concentrations of membrane glycoproteins which correlate with functional deficits similar to the FeLV-infected platelet deficits.\(^{48}\) Functional deficits similar to those of Glanzmann's thrombasthenia are inducible in normal platelets by alloantibodies against the glycoproteins.\(^{49}\) The FeLV infected cells could have impaired synthesis, membrane insertion, or immune blocking of these glycoproteins, such that they are less effective in aggregation. The use of dilute platelet suspensions for aggregation studies should maximize the importance of adhesion efficiency since fewer collisions between platelets would occur and subtle alterations in membrane structure could be more critical than with standard aggregation techniques.

During the time platelet function was being monitored in this study, the FeLV-KT virus was replicating concomitant with the host's
responses to the infection which include humoral and cellular immune
responses.\(^{(1,2)}\) While no data are available, it is probable that the
host responses include increased acute phase reactant proteins. One
human acute phase reactant, C-reactive protein, impairs in vitro
platelet aggregation and release reactions and presumably has a
regulatory function in vivo.\(^{(50,51)}\) Considering the sensitivity of
the aggregation technique used herein, the abnormalities found could
reflect either non-specific acute phase host responses to infection
in general, a specific host response, or specific virus mediated
platelet deficits similar to those noted in human myeloproliferative
diseases.

Ultra-violet light-inactivated purified virus did not induce
detectable platelet deficits, when incubated with normal platelets
in vitro, in contrast with the ability to induce immunologic deficits
in-vitro with similar preparations.\(^{(39)}\) This could imply that host
responses are more critical in the platelet lesion, or that the
mechanism may involve viral replication, perhaps in the megakaryo-
cytes. This experimentally inducible model of platelet dysfunction
offers the opportunity to investigate the relative roles which
platelet metabolism, retroviral infection, and host responses to
infection play in the genesis of the lesions.
SUMMARY

In vitro platelet function was studied in normal cats and cats infected with the Kawakami-Theilen strain of feline leukemia virus (FeLV-KT), an anemia-inducing exogenous feline retrovirus. Because collecting large volumes of blood would alter platelet kinetics, a method was developed to simultaneously assay platelet aggregation by light scattering techniques and ATP release by chemiluminescence on small volumes of platelet rich plasma (PRP) diluted in a modified Tyrode's solution. Aggregation responses and ATP release reactions were similar to those described for cat platelets using conventional techniques and undiluted PRP.

In FeLV-KT-infected cats, significant increases in aggregation times were noted for collagen and arachidonic acid (AA) but not ADP-induced aggregation. The release of ATP was significantly reduced for ADP but not collagen-induced aggregation. Incubation of purified FeLV-KT virus with normal platelets in-vitro did not induce similar changes. Since hemostatic deficits were not noted clinically, this in vitro aggregation technique appeared highly sensitive to subclinical changes in platelet function. The FeLV-KT-induced abnormalities offer an opportunity to study the effects of retroviral infection and associated host responses on platelet function.
### TABLE 3.1

**NORMAL PLATELET REACTION TIMES WITH ESTIMATION OF THE SOURCES OF ASSAY VARIATION**

<table>
<thead>
<tr>
<th></th>
<th>$\bar{X}<em>{t</em>{\max}}$</th>
<th>Within Assay</th>
<th>Between Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$S_w$</td>
<td>$CV_w$</td>
</tr>
<tr>
<td>Collagen (6.2 ug/ml)</td>
<td>0.875 minutes</td>
<td>0.06</td>
<td>5.2%</td>
</tr>
<tr>
<td>ADP (100 um)</td>
<td>1.50 minutes</td>
<td>0.19</td>
<td>12.7%</td>
</tr>
<tr>
<td>AA (500 ug/ml)</td>
<td>1.77 minutes</td>
<td>0.12</td>
<td>6.8%</td>
</tr>
</tbody>
</table>

**ATP release**

|                | ATP release         | $S_w$        | $CV_w$        | $S_b$ | $CV_b$ |
|----------------|---------------------|--------------|---------------|
| Collagen (6.2 ug/ml) | $2.4 \times 10^{-9}$ Moles | 0.5         | 20.8%         | 1.0   | 41.6%  |
| ADP (100 um)    | $6.4 \times 10^{-9}$ Moles | 0.9         | 13.6%         | 1.3   | 20.5%  |

---

$S_w$ (within assay standard deviation) and $S_b$ (between assay standard deviation) calculated as described by Robard. The term $S_b$ includes both between assay and between individual cat components of variation, since the data was derived from 3 cats, sampled twice each, with 3 replicates run for each sample and agonist combination. $CV_w$ and $CV_b$ are the coefficients of variation for within assay and between assay variation, respectively.
TABLE 3.2
SEQUENTIAL STUDY OF COLLAGEN-INDUCED\(^a\) AGGREGATION
IN FeLV-KT INOCULATED AND CONTROL CATS
USING 20% PRP IN DILUENT

<table>
<thead>
<tr>
<th>Cat</th>
<th>Time to 1/2 Maximal Aggregation (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-Inoculation</td>
</tr>
<tr>
<td>Control</td>
<td>1.9±0.2</td>
</tr>
<tr>
<td>Control</td>
<td>2.4±0.4</td>
</tr>
<tr>
<td>FeLV#1</td>
<td>1.9±0.1</td>
</tr>
<tr>
<td>FeLV#2</td>
<td>1.6±0.2</td>
</tr>
<tr>
<td>FeLV#3</td>
<td>1.6±0.2</td>
</tr>
<tr>
<td>FeLV#4</td>
<td>2.0±0.1</td>
</tr>
<tr>
<td>FeLV#5</td>
<td>1.4±0.2</td>
</tr>
</tbody>
</table>

FeLV-KT inoculated but not control group aggregation times increased significantly during the post-inoculation observation period (linear regression analysis, \(P<0.01\).

\(^a\) 0.6 µg cat aortic protein used as collagen source.
\(^b\) \(\bar{X} \pm S.D.\) for 3 weekly replicates.
\(^c\) PI, post-inoculation week.
TABLE 3.3

SEQUENTIAL STUDY OF COLLAGEN-INDUCED AGGREGATION IN FeLV-KT INOCULATED CATS USING A CONSTANT PLATELET MASS TECHNIQUE

A. Minimum Time (minutes) to 1/2 Max Aggregation

<table>
<thead>
<tr>
<th>Cat</th>
<th>Pre-Inoculation</th>
<th>PI 3</th>
<th>PI 4</th>
<th>PI 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeLV#6</td>
<td>0.9 ± 0.3</td>
<td>0.6</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>FeLV#7</td>
<td>0.9 ± 0.1</td>
<td>0.8</td>
<td>1.1</td>
<td>0.9</td>
</tr>
<tr>
<td>FeLV#8</td>
<td>0.7 ± 0.1</td>
<td>0.7</td>
<td>0.9</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Friedman's test

- NS
- NS
- NS

B. Collagen Required to Double the Minimum Time

<table>
<thead>
<tr>
<th>Cat</th>
<th>Pre-Inoculation</th>
<th>PI 3</th>
<th>PI 4</th>
<th>PI 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeLV#6</td>
<td>0.59±0.15 µg</td>
<td>1.02 µg</td>
<td>1.7 µg</td>
<td>1.9 µg</td>
</tr>
<tr>
<td>FeLV#7</td>
<td>0.46±0.14 µg</td>
<td>1.5 µg</td>
<td>0.9 µg</td>
<td>0.9 µg</td>
</tr>
<tr>
<td>FeLV#8</td>
<td>0.68±0.26 µg</td>
<td>1.02 µg</td>
<td>1.4 µg</td>
<td>1.5 µg</td>
</tr>
</tbody>
</table>

Friedman's test

- p<0.05
- p<0.05
- p<0.05

---

a 6.2 µg aortic protein/ml used to stimulate rapid aggregation.
b Collagen concentration in µg aortic protein/ml.
c PI = post-inoculation week after inoculation with FeLV-KT.
d Friedman's test comparing pre-inoculation means with post-inoculation data; NS, not significant.
<table>
<thead>
<tr>
<th>Cat</th>
<th>Agonist</th>
<th>Pre-Inoculation</th>
<th>PI 3</th>
<th>PI 4</th>
<th>PI 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeLV#6</td>
<td>6 µg Collagen</td>
<td>1.6</td>
<td>ND</td>
<td>1.4</td>
<td>0.4</td>
</tr>
<tr>
<td>FeLV#7</td>
<td>6 µg Collagen</td>
<td>1.1</td>
<td>ND</td>
<td>1.3</td>
<td>0.8</td>
</tr>
<tr>
<td>FeLV#8</td>
<td>6 µg Collagen</td>
<td>1.3</td>
<td>ND</td>
<td>1.0</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Friedman's test\(^1\)  

<table>
<thead>
<tr>
<th>Cat</th>
<th>Agonist</th>
<th>Pre-Inoculation</th>
<th>PI 3</th>
<th>PI 4</th>
<th>PI 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeLV#6</td>
<td>100 µM ADP</td>
<td>7.5</td>
<td>2.5</td>
<td>1.9</td>
<td>1.4</td>
</tr>
<tr>
<td>FeLV#7</td>
<td>100 µM ADP</td>
<td>4.8</td>
<td>1.7</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>FeLV#8</td>
<td>100 µM ADP</td>
<td>5.2</td>
<td>1.5</td>
<td>1.5</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Friedman's test\(^1\)  

\(^1\) Friedman's test for differences between groups followed by paired t tests with Bonferroni corrections to identify differences between pre-inoculation and post-inoculation data. ND, not done; NS, not significant (P>0.05).
TABLE 3.5

IN VITRO OF ADP-INDUCED AGGREGATION IN CONTROL AND FeLV-KT INOCULATED CATS

TIMES TO 1/2 MAX AGGREGATION (minutes)\(^{a}\)

<table>
<thead>
<tr>
<th>Cat</th>
<th>Dilution Method</th>
<th>Baseline Data (n=3)(^{b})</th>
<th>Post-Inoculation Week</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Control</td>
<td>20%PRP</td>
<td>1.1 ± 0.4</td>
<td>0.9</td>
</tr>
<tr>
<td>Control</td>
<td>20%PRP</td>
<td>1.4 ± 0.5</td>
<td>1.6</td>
</tr>
<tr>
<td>FeLV#1</td>
<td>20%PRP</td>
<td>1.2 ± 0.3</td>
<td>1.8</td>
</tr>
<tr>
<td>FeLV#2</td>
<td>20%PRP</td>
<td>1.0 ± 0.4</td>
<td>ND</td>
</tr>
<tr>
<td>FeLV#3</td>
<td>20%PRP</td>
<td>1.0 ± 0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>FeLV#4</td>
<td>20%PRP</td>
<td>0.8 ± 0.4</td>
<td>1.0</td>
</tr>
<tr>
<td>FeLV#5</td>
<td>20%PRP</td>
<td>0.8 ± 0.3</td>
<td>0.8</td>
</tr>
<tr>
<td>FeLV#6</td>
<td>62.5% T</td>
<td>0.8 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td>FeLV#7</td>
<td>62.5% T</td>
<td>1.5 ± 0.3</td>
<td>ND</td>
</tr>
<tr>
<td>FeLV#8</td>
<td>62.5% T</td>
<td>1.3 ± 0.3</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^{a}\) No significant differences between groups were identified.

\(^{b}\) Baseline data derived from 3 weekly samples prior to viral inoculation, \(\bar{X} \pm SD\).
**TABLE 3.6**

**CHANGES IN AA-STIMULATED PLATELET AGGREGATION TIMES ASSOCIATED WITH FeLV-KT INFECTION**

<table>
<thead>
<tr>
<th>CATS</th>
<th>BASELINE DATA (n=3)</th>
<th>PI 3&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PI 4&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PI 6&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeLV#6</td>
<td>1.75 ± 0.2</td>
<td>4.0</td>
<td>4.5</td>
<td>2.9</td>
</tr>
<tr>
<td>FeLV#7</td>
<td>1.40 ± 0.4</td>
<td>4.0</td>
<td>4.2</td>
<td>3.2</td>
</tr>
<tr>
<td>FeLV#8</td>
<td>1.60 ± 0.3</td>
<td>3.0</td>
<td>4.5</td>
<td>5.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>Significantly (p<0.01) increased T<sub>a</sub> by one way analysis of variance and Duncan's multiple range procedures, compared to pre-inoculation baseline data derived from 3 weekly samples prior to inoculation. PI; Post-inoculation week.
### TABLE 3.7

**EFFECTS OF PURIFIED FeLV-KT VIRUS ON NORMAL PLATELETS$^1$ IN-VITRO**

<table>
<thead>
<tr>
<th>Viral Protein in PRP (ug/ml)</th>
<th>Aggregation Times (t$_{1/2}$)$^2$</th>
<th>ATP Secretion$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Collagen</td>
<td>ADP</td>
</tr>
<tr>
<td>65</td>
<td>1.9 ± 0.2</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>32.5</td>
<td>1.8 ± 0.1</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>16.3</td>
<td>1.8 ± 0.3</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>8</td>
<td>1.9 ± 0.2</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>4</td>
<td>1.8 ± 0.3</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>Ø</td>
<td>2.0 ± 0.2</td>
<td>1.4 ± 0.4</td>
</tr>
</tbody>
</table>

$^1$ Data from 2 cats.

$^2$ $t_{1/2}$ in minutes for either 0.6 collagen or 100 µM ADP-induced aggregation using a constant platelet mass dilution technique (X $10^{-9}$ Moles, X ± SD for 2 cats).

$^3$ Peak ATP release (X $10^{-9}$ Moles, X ± SD for 2 cats).
Fig. 3.1 Relationship between platelet mass in cuvette and initial light transmittance: When the initial light transmittance $T_0$ was converted to extinction $E_0$; eq, $E_0 = -\log T_0$; there was a direct linear correlation between mass and light extinction.
PLATELET MASS (μl·ml)

INITIAL LIGHT EXTINCTION

Fig. 3.1
Fig. 3.2 Relationship of collagen-stimulated platelet aggregation times to platelet mass: A non-linear inverse correlation was noted between mass and the time to 1/2 maximal aggregation (T%).
Fig. 3.2
Fig. 3.3 Typical platelet aggregation curves and ATP secretion curves. When stimulated by high concentrations of collagen (6 μg/ml aortic protein) aggregation curves of normal cat platelets (D) and platelets from FeLV-KT-infected cats (C) are similar but with low concentrations of collagen (0.6 μg/ml aortic protein), platelets from FeLV-KT-infected cats aggregate slower (curve A) than platelets from control cats (curve B). Peak ATP release (curves E and F) were not different.
Fig. 3.4 Typical ADP-induced aggregation and ATP secretion reaction by platelets from normal and FeLV-KT-infected cats had less ATP release (curve D) than normal cat platelets (curve C) and tended to aggregate less rapidly (curve A) than normal (curve B) platelets. The reduced ATP secretion but not reduced aggregation rate of platelets from FeLV-KT-infected cats was statistically significant.
Fig. 3.5 Typical platelet aggregation curves for AA-induced aggregation by normal cat platelets (curve A) and platelets from FeLV-KT-infected cats (curves B and C).
Fig. 3.5
CHAPTER IV

FLOW CYTOMETRIC AND RADIOISOTOPIC DETERMINATIONS OF PLATELET
SURVIVAL TIME IN NORMAL CATS AND FELINE LEUKEMIA
VIRUS-INFECTED CATS

INTRODUCTION

Although well-established radioisotopic methods are available for estimating platelet survival time all have technical limitations, stimulating a search for suitable non-isotopic techniques.\(^{(1,2)}\) Alternative methods have emphasized assay of platelet cyclo-oxygenase function recovery after irreversible inhibition; an assay of platelet production rather than survival time.\(^{(2)}\) The success of FITC as a lymphocyte label\(^{(3,4)}\) and advent of flow cytometric technology suggested new methodology for blood cell survival times. Herein we describe a novel flow-cytometric method for assessing platelet survival times, compare it with a \(^{51}\)Cr method, and apply it in normal and FeLV-KT-infected cats.

Feline leukemia viruses induce a spectrum of hematopoietic disease in naturally infected cats including a preleukemic syndrome complete with clinical episodes of anemia, leukopenia, and/or thrombocytopenia.\(^{(5)}\) Nonregenerative anemia due to erythroid aplasia can be reproduced experimentally with the Kawakami-Theilen strain of
feline leukemia virus (FeLV-KT) in neonatal or steroid-treated adult cats. (6–8) No consistent changes in platelet concentrations were observed in the initial studies of FeLV-KT-induced anemia, (2) but a transient thrombocytopenia was noted in contact-exposed viremic cats by Pederson et al. (9) Platelets from infected cats could be affected by immune-mediated cell clearance mechanisms since platelets (in some species) express Fc and Clq receptors (10) and the FeLV-infected cats can have both circulating immune complexes (11) and platelet-associated FeLV group-specific antigens. (12) Thus, platelet life-span data could contribute to our understanding of the pathogenesis of FeLV-KT-induced disease.
MATERIALS AND METHODS

All cats were 2 to 4 kg young adult females from a FeLV-free closed breeding colony of gnotobiotic origin. Ketamine HCl (Vetalar, 10 mg/kg IM, Parke Davis, Morris Plains, NJ) anesthesia was used for all procedures. Three cats were given 1.5 ml of a splenic homogenate containing $7.5 \times 10^4$ focus forming units of FeLV-KT virus intraperitoneally in conjunction with corticosteroid to abrogate the natural adult resistance to infection. The corticosteroid (methyl prednisolone acetate, Depomedrol, 5 mg/kg IM, Kalamazoo, MI) was given 7, 4, and 0 days prior to and 4 days post-inoculation. Platelet survival times were determined 8 weeks after inoculation in these 3 cats and in 10 normal cats.

For the FITC technique, 34 ml of venous blood was drawn through a 20-gauge needle into 6 ml ACD-A and processed using a modification (Appendix 1) of Butcher, Scollay, and Weisman's lymphocyte labelling techniques. Primary modifications were the omission of cell washing steps and using ACD-A to maintain a pH of approximately 6.5 in order to minimize platelet losses and maintain viability. Suspension in PBS was necessary because plasma proteins blocked labelling. The stock FITC concentration, concentration of FITC/platelet, and platelet FITC in-vitro elution rates at 37°C were determined by absorbance spectroscopy using an estimated molar extinction coefficient of 68,000 at 495 nm (Gilford 240 spectrophotometer, Gilford Inst., Oberlin, OH). The stock FITC solution prepared as described contained 480 ug/ml FITC. The in-vitro elution of FITC from labelled platelets in autologous plasma at 37°C was determined.
on platelets from 2 normal cats. The labelled platelets were divided into 1 ml aliquots. At 2, 4, 9, 24, and 48 hours one aliquot of each cat's platelets was centrifuged. After a PBS wash the platelets were lysed by sonification while suspended in 1% Triton X. FITC in the lysate was determined by absorbance spectroscopy.

In 3 normal and 3 FeLV-KT-infected cats, FITC and $^{51}$Cr platelet survival techniques (Appendix 2) were compared. Platelet survival times were determined by the FITC method alone in 7 additional normal cats. Venous blood was drawn at 2, 24, 48, 72, and 96 hours after return of labelled platelets to the cats for assay of platelet survival. To establish platelet survival curves using the FITC technique, PRP was prepared from 2 ml of ACD-A anticoagulated blood (15% v/v) by centrifugation (100 g, 25°C, 12 minutes), diluted in a balanced electrolyte solution (Isotonic, Coulter Electronics, Hialeah, FL), and analyzed on a flow cytometer (Model TPS-ITC, Coulter Electronics, Hialeah, FL). The argon-ion laser output was set at 1 watt. Other instrument settings included a photocathode fluorescence detector amplification of 20, the high voltage at 600, and the light scatter detector at 20. A 515 nanometer long wavelength pass filter was used. Total platelets were counted by light scatter and the sorting function counted particles that both fluoresced and scattered light above threshold values. The fluorescent platelet fraction was the mean of 6 counts totalling 600,000 platelets for each sample without correction for autofluorescent platelets.
For $^{51}$Cr platelet survival curves, 1 ml of ACD-A anticoagulated blood (15% v/v) was mixed with 1 ml water containing a lysing agent (Ultralyse, Clay Adams, Parsippany, NJ). Radioactivity was measured with a gamma spectrometer (Beckman Gamma 5500, Beckman, Fullerton, CA). Platelet concentrations and volume distributions were determined by impedance methodology (Coulter ZBI, Coulter Electronics, Hialeah, FL). The platelet aggregation responses of FITC-labelled, $^{51}$Cr-labelled, and placebo-treated (PBS without FITC) samples from 3 normal cats to cat aortic collagen were assayed by light transmission techniques (Whole Blood Aggregometer, Chronolog Corp., Haverton, PA).

To facilitate data analysis and comparisons between curves, the following transformations were performed for each point, where $t_x$ is time of sampling after returning labelled platelets:

A) for FITC platelet survival curves

$$\frac{\log_{10} \text{Fluorescent platelets } @ t_x}{\text{Fluorescent platelets } @ 2 \text{ hr}} \times 1000$$

B) for $^{51}$Cr platelet survival curves, where CPM is counts/minute

$$\frac{\log_{10} \frac{\text{CPM } @ t_x}{\text{CPM } @ 2 \text{ hr}}}{\text{CPM } @ 2 \text{ hr}} \times 1000$$

After transformation the curves were linear with a common 2 hour dependent variable value of 3, but had the same slopes as the raw data after simple log transformation.

Statistical analyses of the transformed data were done utilizing a NWA STATPAK version 2.1 (Northwest Analytical, Portland, OR) and an
Apple II plus computer. A survival curve was fitted to each data set by linear regression analysis, with t-testing for differences in slope used to compare the fitted curves.\(^{(14)}\) Platelet half-life was estimated by calculating the time to reach 2.6990 for each curve (equivalent to 1/2 initial label) and mean lifespan as 1.443 x platelet half-life.
RESULTS

Utilizing the FITC-labelling protocol, $5.4 \times 10^9 \pm 2.6 \times 10^9$ (x ± SD, n=10) platelets were isolated and labelled from 34 ml of blood. Based upon initial platelet counts this was $67 \pm 19\%$ (x ± SD, n=10) of the platelets harvested. Two hours after injection $3.1 \pm 1.9\%$ (x ± 1 SD, n=10) of the total circulating platelets were FITC-positive. Technique improved during the study and 3 to 5.5% labelling rates were typical in the later assays as centrifugation and washing procedures were adjusted for maximum yield. As determined by absorbance spectroscopy, approximately $3 \times 10^7$ molecules FITC initially were bound to each platelet. The in-vitro elution of FITC from platelets was exponential with rapid initial elution (Fig. 4.1). At 8 hours the platelet FITC concentration was only 31% of initial (10 min) levels, but by linear regression analysis between 8 and 48 hours the in-vitro elution rate was constant with a half-life of 38 hours (Fig. 4.1). FITC-labelled platelets were detectable by flow cytometry as long as 120 hours after return to the cats. By phase contrast examination, FITC-labelled platelet preparations consisted of spherocinocytes which could adhere to and spread on glass slides. In-vitro aggregation responses to cat aortic collagen by FITC-labelled platelets from 3 normal cats did not differ significantly from placebo-treated platelets (Table 4.1).

The FITC procedure was compared with the $^{51}$Cr procedure in 3 normal and 3 FeLV-KT-infected cats. The two procedures yielded identical slopes of the survival curves in both groups of cats (Fig. 4.2). Although neither procedure significantly suppressed
collagen-induced aggregation when compared with placebo-treated PRP (Table 4.1), normal cat PRP not pelleted and resuspended in PBS had a normal collagen-induced aggregation time of 1.6 ± 0.4 minutes to 1/2 maximal aggregation, which was significantly less (P<0.01) than that noted for either FITC, $^{51}$Cr, or PBS-treated platelets. No qualitative differences in shape were noted comparing FITC with $^{51}$Cr-labelled platelet populations by phase contrast microscopy. Impedence-determined geometric mean platelet volumes of FITC but not $^{51}$Cr-labelled platelet samples had significant increases in volume compared with the platelet volume prior to labelling (Table 4.2, P<0.05, student's t-test).

The FITC platelet survival curves for 10 normal (Fig. 4.3) and 3 FeLV-infected cats were linear after the logarithmic transformation. Platelet survival curves from 2 of 3 FeLV-infected cats and the curve of the pooled FeLV cat data had significantly greater slopes (P<0.05) than the mean slope of the 10 normal cat platelet survival curves (Fig. 4.4). The average platelet half-life and platelet mean survival time by flow cytometry were, respectively, 21.5 and 31.0 hours for normal platelets, in contrast to 11.9 hours and 17.2 hours for platelets from cats infected with FeLV-KT.
DISCUSSION

The FITC cell label was first utilized in studies of lymphocyte migration and maturation. The observations of labelled lymphocytes in thoracic duct fluid implied a potential for determining survival times for cells circulating in the blood.\(^{3,4}\) The lymphocyte labelling technique proved readily adaptable for use in platelet survival studies; the cat platelets bound enough FITC to be detectable in PRP by flow cytometry up to 5 days after the return of autologous labelled platelets to cats. The platelet survival curves generated by flow cytometric techniques were identical to those obtained from \(^{51}\)Cr-labelled platelet studies. The two techniques induced similar changes in platelet volume, shape, and function, although the impedance-determined volumes of FITC-labelled platelets but not \(^{51}\)Cr-labelled platelets were significantly increased when compared with platelet volumes prior to labelling. This fluorescent-labelling technique should be widely applicable to platelet survival studies since FITC binds nonspecifically to cell proteins and there is little species-dependent variation in platelet total protein content.\(^{15}\)

Both isotopic and non-isotopic methods are available for assessing platelet survival but all have technical limitations. Non-isotopic techniques monitor the return to normal of cyclooxygenase function after acetylsalicylic acid inhibition; however, there is wide variation both between individuals and for repeated studies within individuals.\(^{2}\) Cohort in-vivo isotopic labels, including \(^{32}\)P, \(^{75}\)Se-methionine, and \(^{35}\)S-methionine have a long period.
of availability compared with the platelet lifespan complicating calculations of survival times. The in-vivo isotopic random platelet label, $^{32}$P-orthophosphate also labels leukocytes and RBCs and undergoes rapid elution or exchange. $^{1,2}$ Although $^{11}$In-hydroxyquinolone methods allow gamma camera imaging of platelet sequestration, the $^{51}$Cr in-vitro platelet labelling technique remains the recommended method for platelet survival. $^{1}$ The FITC technique yields similar data to the $^{51}$Cr technique but eliminates the radiation hazard. Re-utilization of eluted label is not a problem because eluted plasma protein-bound FITC is not measured by the flow cytometer. Although gamma-camera imaging is not applicable to the FITC technique, in experimental applications FITC-labelled lymphocytes were demonstrable either in frozen sections or by flow cytometric analysis of cell suspensions from organs. $^{4}$

The cat platelet survival curves for either the FITC or $^{51}$Cr labels were exponential in nature, whereas survival curves of $^{51}$Cr-labelled human platelets are essentially linear in most recent studies. $^{2}$ The linearity implies a predominantly age-related platelet loss while the exponential platelet curves from cats could imply either age-related platelet loss with a great variation in intrinsic feline platelet lifespan or non-age-related random platelet losses. The similar effects of PBS treatment, FITC, or $^{51}$Cr labelling on shape change and in-vitro aggregation suggest that the platelet preparation technique may damage cat platelets and alter survival. Considering the difficulties in preparing cat PRP by
centrifugation without shape change and frequent spontaneous aggregation,\textsuperscript{(16)} normal cat platelet survival curves may not approach the age-related senescence pattern obtained with optimum human $^{51}$Cr techniques.

The FITC technique, when applied to FeLV-KT-infected cats, disclosed significantly shortened platelet lifespan with no significant differences between the FITC and $^{51}$Cr techniques. When estimated by $^{51}$Cr labelling, FeLV-infected cat RBCs have shortened survival times (Kociba GJ, Weiser G; unpublished observations), and, while the mechanism is unknown, a similar effect on platelet survival is not surprising. Although FeLV-KT cat platelets are larger than normal, large but otherwise normal platelets should have normal circulation times.\textsuperscript{(2)} However, the increased size in concert with FeLV viral glycoproteins in the platelet membranes of infected cats could result in increased RE clearance with or without immune system-mediated opsonization.
SUMMARY

A flow cytometric technique was developed to measure platelet survival time in cats utilizing autologous cat platelets labelled in-vitro with fluorescein isothiocyanate. The initial FITC elution rate was rapid, but after 8 hours was constant with a half-life of 38 hours. Circulating autologous FITC-labelled platelets were detectable by flow cytometry up to 120 hours after labeling. When compared with a $^{51}$Cr method, no significant differences in estimated survival times were found. Both the $^{51}$Cr and FITC labelling procedures induced similar changes in platelet shape, volume, and aggregation responses. These changes were primarily related to the platelet centrifugation and washing procedures rather than the labels themselves.

In a preliminary study of the platelet survival time in feline leukemia virus-infected cats, 2 of 3 cats had significantly reduced survival times using either flow cytometric or radioisotopic methods. This novel technique should have wide applicability to cell circulation time studies as flow cytometry equipment becomes more readily available.
Appendix 1: FITC Platelet Labeling

1. Mix 40 ml whole blood (15% ACD-A) with 40 ml PBS (pH 6.5, containing 1 g glucose/l and 15% ACD-A).
2. Centrifuge (120xg, 20 minutes, 25°C).
3. Collect the PRP and add ACD-A (0.2 ml/20 ml PRP).
5. Decant and save PPP.
6. Suspend the platelet pellet in 3 ml PBS.
7. Add 0.3 ml FITC stock (prepared as described [11]), and incubate (10 minutes, 25°C).
8. Dilute to 10 ml with PPP.
9. Return to cat via jugular vein.

Appendix 2: $^{51}$Cr/FITC Platelet Labeling

1-5. Identical to FITC except divide PRP into 2 equal portions in step 3.
6. Process one sample for FITC. Suspend the second platelet pellet in 1 ml PPP.
7. Add 30 μCi of $^{51}$Cr (specific activity 0.3 Cr/mg Cr) and incubate (25°C, 30 minutes).
8. Add 10 ml PPP and centrifuge (240xg, 25°C, 30 minutes).
9. Decant supernatant, overlay pellet with 1 ml PPP, and decant carefully.
10. Resuspend pellet with the 10 ml of FITC-labelled platelets.
11. Return to cat via jugular vein.
TABLE 4.1  

Comparison of Collagen-Induced Aggregation Times* for Placebo-Treated and Labelled Platelets.

<table>
<thead>
<tr>
<th>Cat</th>
<th>FITC</th>
<th>FeCr</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>2629</td>
<td>2.9</td>
<td>3.4</td>
<td>2.5</td>
</tr>
<tr>
<td>2621</td>
<td>3.0</td>
<td>2.8</td>
<td>3.3</td>
</tr>
<tr>
<td>2146</td>
<td>2.4</td>
<td>2.6</td>
<td>2.2</td>
</tr>
</tbody>
</table>

Mean ± SD 2.8±0.3 2.9±0.4 2.7±0.6

*Aggregation responses to 6.4 μg cat aortic collagen of labelled or placebo-treated platelets in 1 ml of a modified Tyrodes solution containing 20% PRP(17) were assayed. Data presented is the time to 1/2 maximal aggregation in minutes. No significant differences were noted.
TABLE 4.2

Geometric Mean Platelet Volumes (fl) of Unlabelled Fixed Platelets, FITC-Labelled and $^{51}$Cr-Labelled Platelets.

<table>
<thead>
<tr>
<th>Cat</th>
<th>Fixed ACD-A</th>
<th>FITC Label</th>
<th>$^{51}$Cr Label</th>
</tr>
</thead>
<tbody>
<tr>
<td>2829</td>
<td>12.29</td>
<td>16.81</td>
<td>14.68</td>
</tr>
<tr>
<td>2889</td>
<td>11.88</td>
<td>18.86</td>
<td>15.65</td>
</tr>
<tr>
<td>2899</td>
<td>8.98</td>
<td>13.07</td>
<td>11.43</td>
</tr>
<tr>
<td>X</td>
<td>11.03</td>
<td>16.24*</td>
<td>13.92</td>
</tr>
<tr>
<td>SD</td>
<td>1.83</td>
<td>2.93</td>
<td>2.21</td>
</tr>
</tbody>
</table>

Comparison of Coulter $Z_{BI}$ determined platelet volumes before and after the FITC and $^{51}$Cr labelling procedures. An ACD-A anticoagulated blood sample was fixed in glutaraldehyde immediately after phlebotomy to prevent volume or shape changes prior to volume analysis. *Significantly different from fixed ACD-A platelets (P<0.05, one-way analysis of variance and least significant difference tests. With these tests the volumes of $^{51}$Cr labelled platelets were not significantly different from either fixed ACD-A anticoagulated or FITC-labelled platelets).
Fig. 4.1 In-vitro elution of FITC from labelled platelets of 2 normal cats. Absorbance due to FITC at 495 nm was determined on aliquots of platelets at intervals between 2 and 48 hours. FITC absorbance = 0.218-0.06 ln time, with r=0.9588 for the entire period but for 9 through 48 hours FITC absorbance = 0.08-(0.001 x time), with r=0.9229.
Fig. 4.1
Fig. 4.2 Comparison of FITC and $^{51}$Cr survival times in 3 normal and 3 FeLV-KT-infected cats. □, normal FITC; ○, normal $^{51}$Cr, ■, FeLV-KT FITC; ●, FeLV-KT $^{51}$Cr. Symbol and brackets represent $\bar{x} \pm SD$. 
$\log_{10} \left( \frac{\text{OBSERVED LABELED CELLS}}{\text{INITIAL LABELED CELLS}} \times 10^3 \right)$
Fig. 4.3 Platelet survival curves for autologous FITC-labelled platelets in 10 normal cats.
Fig. 4.3

\[ \log_{10} \left( \frac{\text{OBSERVED LABELED CELLS}}{\text{INITIAL LABELED CELLS}} \times 10^3 \right) \]

Y-axis: Logarithmic scale
X-axis: Time (hours)

Data points plotted over time, showing growth patterns.
Fig. 4.4 Comparison of FeLV-infected and normal cat platelet survival curves after linear regression analysis of the log transformed data. $X_{B1}$ is the slope for pooled data from 10 normal cats with 95% confidence limits indicated by the shaded area. b1, b2, and b3 are FeLV-infected cat curves with $X_{B2}$ the slope for the pooled FeLV survival data. Platelet 1/2 lives indicated for $X_{B1}$ (B) and $X_{B2}$ (A) were 21.5 and 11.9 hours, respectively.
Fig. 4.4

\[
\log_{10} \left( \frac{\text{OBSERVED LABELED CELLS}}{\text{INITIAL LABELED CELLS}} \times 10^3 \right)
\]

TIME (hours)

- A
- B
- C
- D
- E

0 2 4 6
REFERENCES

CHAPTER I


CHAPTER II


CHAPTER III


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CHAPTER IV


