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AND 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

Edward Arthur Hoover, D.V.M., M.S.

* * * * *

The Ohio State University
1970

Approved by

Richard A. Greiser
Adviser
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I thank my advisor, Professor Richard A. Griesemer, for his consideration, advice, and continual encouragement during my graduate training. Equally important to me, were his unwavering enthusiasm and keen sense of humor.

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Finally, I thank my wife, Jane, for the vital part she has so unselfishly played in my life and in my work.
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CHAPTER I

FELINE HERPESVIRUS: OSTEOLYTIC LESIONS IN GROWING BONE

Introduction

Reports of virus-induced bone lesions are rare (2). The paucity of reports relating viruses to lesions or diseases of bone merely may indicate that bone is not often examined either histologically or virologically in investigations of viral diseases. Likewise, a viral etiology of inflammatory or destructive bone lesions may not often be considered or investigated. The lability of growing bone in certain viral infections is strikingly demonstrated by the skeletal deformities which follow the cytolytic infection of the bone cells of neonatal hamsters by the H1, H3 and rat parvoviruses (9,16,26). Osteolytic lesions have also been detected in infants congenitally infected with Rubella virus (2) and in children infected with smallpox (9) and vaccinia viruses (8). Osteosarcomas have been reported in hamsters and rats inoculated with murine sarcoma virus (12,25) and viral particles have been demonstrated in the lesions of avian osteopetrosis (24). Here, we present evidence that a virus of the herpes group produces osteolytic lesions in growing bone.

Feline herpesvirus (FHV) is considered the most important cause of upper respiratory disease in the cat. The virus replicates in the upper respiratory mucosa producing extensive necrosis of nasal epithelium associated with resorption of the turbinate bones (10,14,18). This
Investigation was prompted by previous studies (14) in which we detected overt necrosis in the turbinate bones of germfree cats inoculated intranasally with feline herpesvirus (FHV). No report could be found directly relating FHV, or any herpesvirus, to lesions in bone. The objectives of this study were 1) to determine whether FHV has a direct effect on the bones of young cats after intravenous inoculation and 2) to compare the distribution of lesions, virus, and viral antigen in bone with other tissues as an indication of the affinity of the virus for bone.

Materials and Methods

Animals, Inoculation, Necropsy

Ten 1 to 4 day-old (neonatal) and six 8 to 10 week-old (weanling) specific-pathogen-free cats from a breeding colony of caesarian-derived cats maintained in strict isolation were used. The neonatal kittens were removed from the queen after suckling from 1 to 3 days and subsequently fed a sterile liquid diet (Tabbilac, Borden Co.) 6 times daily. Eight neonatal and 4 weanling cats were inoculated intravenously with $10^{7.5}$ TCID$_{50}$ of FHV in 0.5 ml of cell culture medium. Two neonatal and 2 weanling control cats were inoculated intravenously with 0.5 ml of medium from uninoculated cell cultures.

The inoculated neonatal cats were killed on post inoculation days (PID) 2 (2 cats), 3, 4, 5, 6, 7, and 8. The inoculated weanling cats were killed on PID 4, 5, 7, and 8. Blood for virus isolation was collected from the neonatal cats at necropsy and from each weanling cat on PID 2, 3 and at necropsy. Complete necropsies were performed and representative samples of all organ systems collected in either Zenker's
or Bouin's fixative. Paraffin-embedded sections were stained with hematoxylin and eosin.

**Virus**

The C-27 prototype of FHV\(^1\) was used. The virus stock (representing the 21st \textit{in vitro} subpassage) was prepared in a continuous line of feline embryonic kidney (CrFK).\(^2\) The cell culture medium and detached monolayer were harvested at 72 hours postinoculation, frozen and thawed twice, and clarified by centrifugation at 1000 g for 15 minutes.

Because paroviruses are known to affect growing bone the following data were collected to exclude contamination of the inoculum with feline panleukopenia virus (FPV). The FHV stock used had been plaque purified 3 times and did not produce lymphoid or intestinal lesions characteristic of feline panleukopenia in this study or in previous studies (14,15). Negatively-stained pellets of the FHV inoculum revealed only herpesviruses. Ultrastructural examination of bone lesions revealed only herpesviruses. No FPV antigen could be detected by immunofluorescence in sections of bone containing lesions or in FHV-infected or uninoculated CrFK cultures using a fluorescein-conjugated anti-FPV globulin\(^3\). Conversely, CrFK cultures inoculated with FPV exhibited fluorescence only with the anti-FPV conjugate and not with the anti-FHV conjugate described below.

**Virus Isolation and Titration**

Samples of proximal femur, liver, lung, spleen, kidney and small intestine were collected from 7 neonatal cats and proximal femur, spleen,

\(^1\) Obtained from Dr. J. H. Gillespie, New York State Veterinary College, Ithaca, New York, as the 20th passage virus in cell culture. The virus had been plaque purified 3 times.

\(^2\) Originated by Dr. R. A. Crandell and obtained from Dr. F. W. Scott
liver and kidney from each weanling cat. Ten percent weight/volume sus-
pensions were prepared in Hanks' balanced salt solution. Viral isol-
ations were performed using techniques previously described (14) with
the exception that CrFK cells were substituted for primary feline
kidney cells. Parallel titrations in our laboratory indicate that CrFK
and primary kidney cells are equally sensitive to FHV. For virus iso-
lolation, 4 monolayers were each inoculated with 0.2 ml of tissue suspen-
sion, plasma, or blood, incubated at 37° C for 1 hour, and washed once
before media was added. The cultures were examined for 5 days. The
presence of FHV was detected by its characteristic cytopathic effect
associated with eosinophilic intranuclear inclusion bodies. Titrations
were performed using 5 cell cultures per 10-fold dilution of inoculum.
The titers were calculated by the method of Reed and Muench (20).

Electron Microscopy

Blocks of metaphyseal bone from the proximal tibias and ribs of 5
neonatal cats killed from PID 2 to 6 were fixed in 3% glutaraldehyde
with cacodylate buffer, postfixed in osmium tetroxide, dehydrated through
ascending concentrations of ethanol, transferred to propyline oxide, and
embedded in Epon. Thin sections were stained with uranyl acetate and
lead hydroxide and examined with a Philips 200 electron microscope.

Immunofluorescence

Aliquots of the following tissues were collected from 7 neonatal
cats killed between PID 2 and 7: proximal femur, proximal tibia, prox-
imal humerus, rib at costochondral junction, maxilloturbinate, liver,
spleen, lung, adrenal, pharyngeal lymph node and thymus. The tissues
were frozen in liquid nitrogen, stored at -90° C, sectioned on a cryostat
at -20° C, and fixed in cold acetone before staining by the direct method. Globulin from a cat hyperimmunized with FHV (serum neutralizing titer 1:256) was separated as outlined by Cherry et al. (6), conjugated with 12.5 ug of fluorescein isothiocyanate (FITC) per mg of protein, passed through a Sephadex G25 column to remove unconjugated FITC, and absorbed with feline liver homogenate and rabbit liver powder. Rhodamine-conjugated bovine serum albumin was used as a counterstain. The following controls were used: known infected and uninfected cell cultures, tissue sections from the control cats, elimination of specific fluorescence following preincubation with unconjugated anti-FHV serum or globulin but not control serum or globulin, and absence of fluorescence following incubation with FITC-conjugated globulin containing no detectable neutralizing antibody to FHV.

Results

Clinical Signs of Disease

Weak nursing, and a mild serous nasal discharge were evident between PID 4 and 8 in the neonatal cats. Feeding by stomach tube was sometimes necessary when nursing ceased to insure adequate dietary intake.

Clinical signs of illness were minimal in the weanling cats. Fever, mild lethargy, and mild serous nasal discharge occurred between PID 3 and 7. The severe upper respiratory disease which follows intranasal inoculation with FHV (10,14) did not occur after intravenous inoculation.

Lesions

Bones - Necrosis was present in the growth regions of the long bones, ribs, turbinates, vertebrae, and facial bones of all the cats. The lesions in neonatal and weanling cats were similar in distribution but
much more extensive in the neonatal cats to which the following description pertains. On PID 2 microscopic foci of cellular degeneration and necrosis were present in the primary spongiosa and in the periosteum. Eosinophilic intranuclear inclusion bodies (fig. 1) occurred in endothelial cells, osteoprogenitor cells, osteoclasts, osteoblasts, and periosteal cells. Necrosis of metaphyseal bone cells and capillaries progressed until by PID 6 only necrotic cells and scattered erythrocytes separated most of the trabeculae of spongiosal bone (fig. 2). The necrotic bone was grossly visible in the metaphyses of the long bones as a transverse gray zone 1-2 mm wide below the epiphyseal plate. Inclusion bodies were not detected in hematopoietic cells although these cells also appeared degenerate or necrotic. Usually, little or no inflammatory reaction was evident in the bone lesions. Moderate neutrophilic infiltration sometimes accompanied necrosis of the turbinate bone although the overlying nasal mucosa did not contain lesions.

Necrosis in the periosteum (fig. 3) began near the epiphyseal plate and subsequently extended into the diaphysis. In the nasal turbinals, necrosis was confined to the regions where vascular osteogenic connective tissue was replacing the cartilagenous turbinate scrolls. Likewise, in the facial bones osteolytic lesions were restricted to the zones of osteogenesis. Lesions were not detected in cartilage, compact cortical bone, or odontogenic cells.

Necrosis of small blood vessels occurred in conjunction with necrosis of bone cells as early as PID 2. The vessels entering the metaphysis at its cortical margin were frequently affected and sometimes were barely discernible amidst extravasated erythrocytes and coagulative
necrosis. The vessels in the cartilaginous epiphysis, and in the facial, cranial, and turbinate bones, were also affected. Inclusion bodies were demonstrated in endothelial and perithelial cells (fig. 4).

Other Tissues - Multiple foci of necrosis approximately 0.5 mm in diameter and associated with eosinophilic intranuclear inclusion bodies occurred in the adrenals of all the neonatal cats. A few necrotic foci were present in the adrenals of 1 of the 4 weanling cats. Similar scattered foci of hepatic necrosis were detected in the neonatal cats killed between PID 4 and 8 but did not occur in the weanling cats. Focal necrosis of the nasal epithelium followed the appearance of lesions in the turbinate bones. Lesions were not detected in other tissues including the brain, eye, and genitalia.

Electron Microscopy

Viral infection was demonstrated in osteoprogenitor cells, osteoclasts, osteoblasts, and endothelial cells. Osteoprogenitor cells were identified by the criteria of Scott (22) and Cameron (5) and comprised the majority of the bone cells present in the blocks of primary spongiosa selected for electron microscopy. Cytopathic alterations in all infected bone cells were similar to those produced by FHV in cell cultures (1) and to those produced by herpesviruses in general. The chromatin was clumped and marginated and the nucleoli were often fragmented (figs. 5). The nuclei contained variable amounts of moderately dense, granular, inclusion material (fig. 5) accompanied by discrete aggregates of dense, round, nucleoid-like structures approximately 30 mu in diameter (fig. 6) and numerous, scattered, viral nucleocapsids approximately 100 mu in diameter (fig. 7,8). Viral particles with dense and hollow cores were
present (fig. 8). Mature, enveloped virions approximately 150 μ in diameter were present in cytoplasmic vacuoles and extracellularly (fig. 5). Convolutions of the nuclear membrane and intranuclear bundles of dense, fine, filamentous material arranged in parallel array were also observed. Virtually every degenerate or necrotic cell contained viral particles. Only particles resembling herpesviruses were detected.

Immunofluorescence

Bone - The distribution of virus-specific fluorescence coincided with that of the osteolytic lesions in adjacent frozen sections stained with hematoxylin and eosin. Foci of fluorescent bone cells were present on PID 2 in the primary spongiosa and periosteum of the long bones and ribs. Viral antigen was widespread in the metaphysis and periosteum by PID 7. Scattered cells in the diaphyseal marrow fluoresced in bones examined between PID 5 and 7. Viral antigen was also detected in endothelial and perithelial cells of vessels within the epiphysis (fig. 9). Specific fluorescence in the turbinate bones was restricted to sites of ossification. Fluorescent cells were always in the osteogenic tissue which replaced cartilage and surrounded islands of bone. Large accumulations of viral antigen in the turbinate bones preceded the appearance of foci of fluorescence in the nasal epithelium (fig. 10). No specific fluorescence occurred in cartilage or in compact cortical bone. Fluorescence in tissue sections and in infected cell cultures was both nuclear and cytoplasmic.

Other Tissues - Foci of fluorescence appeared in the adrenals on PID 2 and in the liver on PID 4. These foci corresponded to the foci
of necrosis present in these organs histologically. A few scattered, weakly fluorescing cells occurred in the pharyngeal lymph node, spleen and lung between PID 4 and 7. Discrete foci of fluorescence occurred in the spleen and lung of 1 neonatal cat examined on PID 7. Specific fluorescence was not present in the tissues of the control cats or in sections of brain, small intestine, kidney or thymus from the inoculated cats.

Virus Isolation and Titration

Viremia was demonstrated in neonatal cats from PID 2 through 7 with viral titers ranging from $10^{2.17}$ TCID50 per ml of blood on PID 2 to $10^{2.3}$ on PID 7. Ten per cent suspensions of femur from the same cats contained from $10^{3.62}$/ml on PID 2 to $10^{5.37}$/ml on PID 7 (table 1) indicating that viral replication occurred in the bone. Viral titers in suspensions of liver, spleen, kidney and lung were less than those in blood. In the weanling cats virus was detected in the blood only on PID 2 and 3. FHV was recovered from the femur of all 4 weanling cats killed between PID 4 and 8 but not from the plasma, lung, liver, spleen, kidney or intestine.

Discussion

While the neuroectodermal tropism of herpesviruses is well known, to our knowledge, this is the first report relating a herpesvirus to lesions in bone. FHV exhibited an affinity for the growth regions of bones and for the adrenal cortex following intravenous inoculation. In these sites viral antigen and lesions appeared earliest (PID 2) and became most extensive. The predilection of FHV for growing bone following
Intravenous inoculation could reflect either a specific viral affinity for osteogenic cells or unique properties of the vascular bed in regions of bone formation which favor viral localization. Intravenous inoculation of mature cats, in which appositional bone growth has ceased, does not result in either lesions or viral antigen in the bones (15). Osteoprogenitor cells could be a primary target for the virus when it is introduced into the blood. They are abundant in zones of new bone formation, are closely associated with metaphyseal capillaries, and are the chief bone cells involved in DNA synthesis and mitosis (29). Two main difficulties with this hypothesis exist. FHV also replicates in several other seemingly disparate cell types in vivo viz. upper respiratory epithelium, adrenocortical cells and hepatocytes and, less significantly, in a variety of feline cells in vitro. Secondly, in adult bone, the replacement of osteoblasts and osteoclasts takes place by multiplication of osteoprogenitor cells, yet viral effects on the bones of mature cats are not detectable. Additional factors which may favor the localization of the virus in growing bone, therefore, must be considered.

The vessels invading the epiphyseal cartilage are initially sprouts rather than loops and the endothelium of these capillaries is discontinuous (3,21,26). The open capillary sprouts, discontinuous endothelium and relatively sluggish blood flow (4,13) of the capillary bed may predispose the metaphysis to viral localization. Bacterial osteomyelitis most commonly originates in the metaphysis of rapidly growing bones, possibly also due to the nature of the vascular bed (28). If this is the chief factor determining the affinity of FHV for bone,
similar features must exist in the vascular bed of the intramembranously ossifying cranial and facial bones and in the turbinates, since the virus also affects the growth regions of these bones. The metaphyseal capillary sprouts are morphologically similar to the neocapillaries in healing tissue (7,14). Determination of the affinity of FHV for sites of neovascularization with and without associated bone formation, such as wound healing vs. fracture callus formation or periosteal exotosis in mature animals, might help define the factors responsible for the viral localization in growing bone.

Although osteoprogenitor cells and metaphyseal capillary sprouts have a high mitotic activity (13,21,29), the predilection of FHV for sites of osteogenesis is not explicable by an affinity for cells in active DNA synthesis attending mitosis, as appears true for the parvoviruses (19). FHV readily replicates in largely intermitotic cell populations in vivo and in vitro viz. confluent primary cell cultures, nasal epithelium, adrenal cortex and liver. Conversely, viral replication has not been detected in tissues with high mitotic activity in neonatal cats, such as external granular layer of the cerebellum, subependymal plate, lymphoid germinal centers, intestinal crypts, and the postnatally developing renal glomeruli, which are all sites attacked by parvoviruses (15,17,19,26).

Selective viral infection and destruction of vascular endothelium in regions of bone growth may be implicit in the pathogenesis of the FHV-induced bone lesions. In early lesions intranuclear inclusion bodies and viral antigen were demonstrated in endothelial and perithelial cells in addition to bone cells. In other studies (15) we have found that
following intravenous inoculation of pregnant cats FHV selectively localizes in the uterine vessels during the course of placental and fetal infection. Although thrombosis and infarction might be expected to follow virus-induced vascular damage, the regions of bone necrosis were not simply infarcts as indicated by the presence of numerous viral particles in degenerating bone cells as well as rising viral titers and large amounts of viral antigen in the affected bones.

Craniofacial deformities and runting follow the cytolytic infection of the osteogenic cells of neonatal hamsters by the H virus and Rat virus (16,26). The sequele of FHV-induced osteolytic lesions on the bone development of growing kittens are not yet known.

Summary

Feline herpesvirus (FHV) replicates and produces necrosis in the growth regions of the bones of young cats inoculated intravenously. The virus was identified in the lesions by immunofluorescence and viral isolation and titration. Viral infection of osteoprogenitor cells, osteoclasts, osteoblasts and endothelial cells was demonstrated by electron microscopy and by the presence of intranuclear eosinophilic inclusion bodies. Necrosis of growing bone has not previously been associated with a virus of the herpes group.
Table 1. Titration of Feline Herpesvirus in the Tissues of Neonatal Cats Inoculated Intravenously.

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<th>Cat Number</th>
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<td>3.62</td>
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<td>2.3</td>
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Titers expressed as log 10 TCID₅₀ per ml of blood or tissue suspension.
- = no viral infectivity detected.
Fig. 1. Early focus of infected bone cells in the primary spongiosa of the humerus from a kitten 2 days after intravenous inoculation with feline herpesvirus. Eosinophilic intranuclear inclusion bodies (arrows) are present in degenerating osteoprogenitor cells. Hematoxylin and eosin (H & E) X 315.
Fig. 2. Necrotic primary spongiosal bone in the tibia of a kitten 6 days after intravenous inoculation with feline herpesvirus. There is widespread pyknosis and karyorrhexis of bone cells (arrows) lining the trabeculae of spongiosal bone. H & E X 315.
Fig. 3. Periosteal and subperiosteal necrosis (arrows) in the femur of a kitten 6 days after intravenous inoculation with feline herpesvirus. H & E X 315.
Fig. 3
Fig. 4. Necrosis of an epiphyseal vessel in the femur of a kitten 6 days after intravenous inoculation with feline herpesvirus. Eosinophilic intranuclear inclusion bodies are present in endothelial and perithelial cells (arrows). H & E X 315.
Fig. 5. Osteoclast infected with feline herpesvirus. The nuclei contain margined chromatin, fragmented nucleoli (nu), finely granular, moderately dense inclusion material (im), and intranuclear viral nucleocapsids (vc). Enveloped virions (ev) are present within an invagination of the nuclear membrane, in cytoplasmic vacuoles, and extracellularly. X 10,650.
Fig. 6. Degenerating osteoprogenitor cell infected with feline herpesvirus. Intranuclear aggregates of dense particles (dp) approximately 30 µm in diameter, inclusion material (im), and viral nucleocapsids (arrow) are present within the nucleus. X 19,500.
Fig. 7. Osteoblast infected with feline herpesvirus. Arrow indicates nonenveloped intranuclear viral particles. X 33,400.
Fig. 8. Nonenveloped feline herpesvirus in the nucleus of an osteoblast. The particles range from 99-105 µm in diameter; nucleocapsids with dense and lucent cores are present. X 82,000.
Fig. 9. Viral antigen in the endothelial cells of an epiphyseal vessel of a kitten 3 days after intravenous inoculation with feline herpesvirus. Immunofluorescence X 315.
Fig. 10. Large accumulation of fluorescent viral antigen in turbinate bone of a kitten 5 days after intravenous inoculation with feline herpesvirus. The nasal epithelium (arrow) is not involved. Immunofluorescence X 125.
CHAPTER 2
EXPERIMENTAL FELINE HERPESVIRUS INFECTION IN PREGNANT CATS

Introduction
While infection by the herpesviruses indigenous to several species is usually restricted to the upper respiratory or digestive tracts, infection during pregnancy may result in abortion or congenital fetal infection (1-9). Generalized infection of newborn infants by herpes simplex and varicella viruses has been recognized for over 30 years (10,11) and evidence for transplacental infection by these agents has subsequently been reported (1-4). Genital and transplacental infection by the herpesviruses indigenous to the equine (6), bovine (7,8), and canine (9) species have also been recognized, suggesting that common pathogenetic mechanisms exist by which all herpesviruses infect the gravid uterus. Yet, relatively little information is available regarding the pathogenesis of abortion, fetal death, or fetal infection caused by herpesviruses. Descriptions of lesions in the placenta are limited (12-15). No studies in a natural host system which attempt to correlate the distribution of viral antigen in the uterus, placenta, and fetus during maternal infection have been reported. Transplacental infection by herpes simplex virus has been demonstrated in the rabbit (15,16) and mouse (17). Correlation of placental lesions with the distribution of viral antigen in the uterus and placenta has been attempted only in the mouse (17).
Feline herpesvirus (feline rhinotracheitis virus) is usually associated with acute, upper respiratory disease in the cat (19,20). We have observed that pregnant cats spontaneously infected with feline herpesvirus (FHV) frequently abort (21). No studies of the effects of FHV on pregnant cats have been reported. This investigation was undertaken so that the role of FHV in feline abortion as well as its potential value as a model to study the pathogenesis of transplacental infection by indigenous herpesviruses might be evaluated. The specific objectives of this study were - 1) to determine whether FHV produces abortion, fetal death, or fetal infection following intranasal or intravenous inoculation of pregnant cats, 2) to characterize the lesions produced by the virus in the uterus, placenta, and fetuses, and 3) to correlate the presence of virus and viral antigen with lesions in these tissues.

Materials and Methods

Animals and Inoculation

Ten pregnant, specific-pathogen-free cats between the 40th and 50th day of gestation were used. The cats were selected from a breeding colony of caesarian-derived cats maintained in strict isolation. Preinoculation serum samples were collected from all the cats. Four cats were inoculated intranasally and five intravenously with $10^8$ TCID$_{50}$ of feline herpesvirus (FHV) in 1.5 ml of cell culture medium. One control cat was inoculated intravenously with 4.0 ml of medium from uninfected cell cultures.

Postinoculation Procedures

Following inoculation clinical signs and rectal temperature were recorded daily. Plasma and blood for virus isolation was collected from
post inoculation day (PID) 1 through 6 from 2 cats inoculated intranasally and two inoculated intravenously. Pre-moistened dacron vaginal swabs were collected in 2 ml of balanced salt solution plus 5% fetal bovine serum between PID 2 and 7 from 6 cats. The cats were necropsied the day of abortion except for two which were killed when signs of impending abortion were evident on PID 6 and 26 (table 1). Representative sections of all organ systems from the cats and fetuses were fixed in either Zenker's or Bouin's fixative and paraffin-embedded histologic sections were stained with hematoxylin and eosin. Serum for determination of neutralizing antibody was collected on the day of necropsy.

**Virus**

The C-27 prototype isolate of FHV\(^1\) was used. A viral stock (representing the 21st *in vitro* subpassage) was prepared in a continuous line of feline embryonic kidney (CrFK)\(^2\). Cell culture medium was harvested at 72 hours post inoculation, frozen and thawed twice, and clarified by centrifugation at 1000 g for 15 minutes.

**Virus isolation and Serum Neutralizing Antibody**

Previously standardized techniques (20) were used except CrFK cells were substituted for primary feline kidney cells. Parallel titrations in our laboratory have indicated that CrFK and primary feline kidney cells are equally sensitive to FHV. Ten percent weight/volume suspensions of placentas, uterus, liver and spleen from each queen and of

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1 Obtained from Dr. J. H. Gillespie, New York State Veterinary College, Ithaca, New York, as the 20th cell culture passage. The virus had been plaque purified 3 times.

2 Originated by Dr. R. A. Crandell and obtained from Dr. F. W. Scott,
liver, spleen and adrenal from each fetus were prepared in Hanks' balanced salt solution. Amniotic fluid was collected from fetuses of 3 cats (PID 6, 6, and 26) inoculated intravenously. Four cell culture monolayers were inoculated with 0.2 ml of tissue suspension, fluid in which vaginal swabs had been expressed, amniotic fluid, or plasma. The monolayers were incubated for 1 hour at 37°C and washed once before media was added. The cultures were examined for 5 days. The presence of FHV was identified by its characteristic cytopathic effect and the presence of eosinophilic intranuclear inclusion bodies.

Serum neutralizing antibody was assayed using 2-fold serial dilutions of heat-inactivated serum added to 100 TCID₅₀ of virus. The mixture was incubated for 1 hour at room temperature before inoculation of 0.2 ml onto each of 5 CrFK cultures. After 5 days the 50% neutralizing titer was computed by the method of Reed and Muench (22).

**Immunofluorescence**

Sections of placenta, uterus, liver, spleen, maxillary turbinate, and costochondral junction from each cat along with liver, spleen, adrenal and turbinate from at least 2 fetuses of each litter were frozen in liquid nitrogen and stored at -90°C. The tissues were sectioned on a cryostat at -20°C and fixed in cold acetone before staining by the direct method. Globulin from a cat hyperimmunized with FHV (serum neutralizing antibody titer of 1:256) was separated as outlined by Cherry _et al._ (23), conjugated with 12.5 µg of fluorescein isothiocyanate (FITC) per mg of protein, passed through a Sephadex G-25 column to remove unconjugated FITC, and absorbed with feline liver homogenate and rabbit liver powder. Rhodamine-conjugated bovine serum albumin was used as a
counterstain. The following controls were used: known infected and uninfected cell cultures, tissue sections from the control cat, absence of fluorescence following preincubation with unconjugated anti-FHV serum or globulin but not with control serum or globulin, and absence of fluorescence following incubation with FITC-conjugated globulin containing no detectable neutralizing antibody to FHV.

**Results**

1. **Cats Inoculated Intravenously**

**Clinical Signs**

Two cats aborted on PID 6 and one on PID 9. The remaining 2 cats were killed on PID 6 and 26 when a sanguinous vaginal discharge was present suggesting impending abortion. Other signs of illness were minimal. Mild lethargy, transient fever on PID 2 or 3, and mild serous nasal discharge were detected in all five inoculated cats. The control cat inoculated with media from uninoculated CrFK cultures delivered normal, term kittens with normal placentas.

**Gross Lesions**

Macroscopic lesions were present in the placentas of 2 of 4 cats inoculated intravenously. The placentas of the remaining cat which aborted on PID 9 were not recovered for examination. Multiple, well delineated, 0.5 to 1.0 mm, white areas were present which contrasted sharply with interspersed regions of grossly normal placental tissue (fig. 1). The lesions involved the entire thickness of the placenta and were firm and dry on cross section. Three of 5 fetuses present in utero on PID 6 were dead and autolyzed while the remaining two were grossly unaffected and viable. All 4 fetuses present in utero on
PID 26 were dead and two of these were severely autolyzed (fig. 2). The autolyzed fetuses were red-brown, friable, and surrounded by brown, odorless, clear to slightly turbid fetal fluids. The placentas with the most severe lesions were those of the autolyzed fetuses. The autolyzed fetuses in both cases were those nearest the body of the uterus. None of the 8 fetuses aborted by the 3 remaining cats were autolytic and, where determinable, were alive when aborted. Since the fetal membranes were frequently not removed by the dam and because the aborted fetuses were sometimes not discovered immediately, fetal viability at the time of abortion could not always be determined.

Gross lesions in fetuses were detected in only 1 fetus from the cat killed on PID 26. Several grey foci, approximately 0.5 mm in diameter, were present beneath the capsule of the liver. No other gross lesions were detected in this or the other fetuses.

**Microscopic Lesions**

**Placenta and Uterus** - The following changes occurred in varying proportion in the placentas and uteri of all the cats inoculated intravenously: 1) multiple regions of infarction in the placental labyrinth, 2) necrosis and thrombosis of maternal vessels in the placenta and uterus, 3) multifocal degeneration and necrosis of the giant-cell trophoblast and endometrial epithelium in the junctional zone of the placenta associated with the presence of eosinophilic intranuclear inclusion bodies, 4) separation of the placental villi from the endometrium at the junctional zone.

The multiple pale areas demonstrated grossly in the placentas of 2 cats necropsied on PID 6 and 26 consisted of coagulative necrosis
which in most places traversed the entire thickness of the placenta but
did not involve endometrium (fig. 3). The uniform coagulative necrosis
of all labyrinthine structures could only be interpreted as infarction.
The deeper regions of the placenta near the endometrial junction appear-
ed to be the first affected. Thrombosis of maternal vessels entering
the junctional zone of the placenta was associated with overlying
labyrinthine infarction and separation. The walls of some of the small
arteries in the uterus were deeply eosinophilic, and contained pyknotic
and karyorrhetic nuclei (fig. 4).

Degeneration and necrosis of the multinucleated syncytial tropho-
blast in the junctional zone occurred in all the placentas. The giant
cell trophoblast in the placentas of the 3 cats examined on PID 6 con-
tained intranuclear eosinophilic inclusion bodies (fig. 5). Inclusion
bodies were also detected in the endometrial epithelium subjacent to
infected giant cell trophoblast. These changes were frequently associ-
ated with areas of coagulative necrosis in the overlying labyrinth and
thrombosis of the maternal vessels entering the placenta from the endo-
metrium. Inclusion bodies were not present in cytotrophoblast,
syntial trophoblast, decidual cells, endothelial cells, or connective
tissue cells in or adjacent to the zones of necrotic placental laby-
rinth. The endometrium of 2 cats which aborted on PID 6 and one on
PID 9 contained neutrophilic infiltration surrounding the endometrial
glands and in association with cellular detritus in the lumen.

On PID 26 in addition to the placental changes already described
there was necrosis of the choioallantois covering the fetal margin of
the placenta. The choioallantois was composed only of cells containing
pyknotic or karyorrhetic nuclei and retracted, deeply eosinophilic cytoplasm. Inclusion bodies could not be demonstrated in uterine or placental cells on PID 26.

Other Tissues - Focal necrosis of vaginal epithelium and intranuclear inclusion body formation were present in the 3 cats necropsied on PID 6. A few scattered 0.2 to 0.5 mm foci of necrosis were present in the adrenal cortex of all the cats. Focal necrosis of nasal epithelium and intranuclear inclusion bodies were evident on PID 6 and 9. Massive nasal epithelial necrosis, which follows intranasal inoculation with FHV (9,12), did not occur.

Fetal Lesions - No significant microscopic lesions were present in 10 fetuses from the 4 cats examined between 6 and 9 days after maternal inoculation. One of the 2 fetuses which was not severely autolyzed in utero on PID 26 had scattered foci of hepatocellular necrosis (fig. 6). A few peripheral hepatocytes contained eosinophilic intranuclear inclusion bodies. Lesions were not detected in other organs of this fetus.

Virus Isolation

FHV was isolated from all of 9 suspensions of placenta from the cats inoculated intravenously. The virus was recovered from each of the 4 uteri examined from PID 6 through 9 but not from the uterus of the cat killed on PID 26 (table 1). The virus was also recovered from the amniotic fluid of each of 3 fetuses on PID 6, 6 and 26 respectively. Vaginal swabs collected between PID 4 and 6 all yielded virus. The virus was not isolated from the blood, plasma, spleen, or liver of any of the cats on the day of necropsy (table 1). However, viremia was
present between PID 1 and 3 in both of the cats from which blood was collected during this interval. Suspensions of lung, liver and spleen of 8 fetuses examined between PID 4 and 9 did not yield virus. The only viral isolation from fetal tissues was made from the liver of the fetus examined on PID 26 that had gross and microscopic lesions.

**Serum Neutralizing Antibody**

None of the preinoculation sera had neutralizing activity at 1:2 dilution. Neutralizing antibody was detected only in the cat killed on PID 26 at a titer of 1:20.

**Immunofluorescence**

Viral antigen was demonstrated in the walls of uterine blood vessels of the 3 cats necropsied on PID 6 (fig. 7). Fluorescence occurred in endothelial, intramural, and perithelial cells. Viral antigen was also detected in the multinucleated trophoblast and the endometrial epithelium in the junctional zone of 4 placentas from 2 cats killed on PID 6. Neither infarcted nor viable regions of the placental labyrinth contained viral antigen.

On PID 26 a large amount of viral antigen was localized in the chorioallantois covering the fetal margin of each of 3 placentas examined. The fluorescence was confined to the chorioallantois, sometimes extending a short distance into the fetal labyrinthine villi (fig. 8).

**II. Cats Inoculated Intranasally**

**Clinical Signs**

In all 4 cats, severe upper respiratory disease characterized by fever, anorexia, copious nasal and conjunctival exudate, paroxysmal
sneezing, dyspnea, and weight loss was followed by abortion between PID 7 and 14. No vaginal discharge was detected prior to abortion.

Lesions

Placenta, Uterus, Fetuses - No significant lesions could be detected in the 11 placentas and fetuses examined from the 4 cats. The only changes present in the uteri were edema, congestion and hemorrhage at the site of placentation. Degeneration of the giant-cell trophoblast, vascular lesions, and placental infarction were absent.

Other Tissues - Healing foci of necrosis in the vaginal epithelium were detected in the cats killed on PID 9 and 13. Extensive necrosis of nasal epithelium similar to that previously described following intranasal inoculation of FHV (19,20) was evident in all the cats inoculated intranasally.

Virus Isolation

FHV was isolated from vaginal swabs of 2 cats on PID 6 and 9. The virus was not detected in the blood, placentas, or fetuses of the cats inoculated intranasally (table 1).

Immunofluorescence

No specific fluorescence was observed in sections of placenta, uterus, liver or spleen of the cats. Neither was viral antigen detected in liver, spleen, adrenal, or turbinate from any of the 11 fetuses.

Discussion

The results of this study indicate that feline herpesvirus has an affinity for the gravid uterus when introduced into the blood and can produce placental lesions, fetal death, and fetal infection. Abortion
which follows intravenous inoculation appears to be the result of virus-induced lesions in the uterus and placenta while the signs of disease in the dam are minimal. Abortion following intranasal inoculation appears to be a non-specific reaction related to the severe, debilitating upper respiratory disease which followed intranasal inoculation of FHV rather than a direct effect of the virus on the gravid uterus. Neither virus, viral antigen, nor lesions were detected in the uterus, placenta or fetuses of the cats inoculated intranasally. Several examples of abortion accompanying viral infection but not associated with placental or fetal infection have been cited by Kibrick (24) and Mims (25). The specific exciting factors and pathogenesis of such abortions are unknown (24,25).

The presence of viremia is probably the critical factor which permits localization of FHV in the uterus and placenta of the pregnant cats inoculated intravenously but not in those inoculated intranasally. Although extensive viral replication and necrosis occurs in the nasal mucosa following intranasal inoculation (19,20), virus was apparently confined to the upper respiratory tract and was not detected in the blood or plasma. In previous studies (20) FHV was isolated from only 1 of 21 blood samples collected between PID 2 and 13 from weanling cats inoculated intranasally. The presence of virus and lesions in the vaginas of 2 of 4 cats inoculated intranasally suggests either that undetected viremia resulted in vaginal but not placental infection or external spread of virus occurred from the nares to the vagina. In contrast to our findings with FHV, abortion following intranasal inoculation of infectious bovine rhinotracheitis (IBR) virus and equine rhinopneumo-
nitis virus is associated with fetal infection (26,27). A significant feature of equine rhinopneumonitis which could explain this difference is leukocyte-associated viremia which has been demonstrated for as long as 33 days following intranasal inoculation (27). IBR virus, however, is not often detectable in the blood during upper respiratory infection (26). It should be emphasized that the effects of maternal infection with FHV at stages of gestation other than 40-50 days were not evaluated. Placental and fetal infection may be more readily induced earlier in pregnancy by transient viremia of low magnitude which likely follows intranasal inoculation.

FHV antigen was demonstrated in the vessels in the endometrium 6 days after intravenous inoculation when 2 cats aborted and a third had signs of impending abortion. Viral localization in maternal vessels has not been previously described in the course of spontaneous or experimental infection by herpesviruses. Virus-induced necrosis in the maternal vessels might be expected to activate the intrinsic coagulation mechanism and cause adherence of platelets (28). This suggests the pathogenesis of the thrombosis and infarction of the placenta which followed intravenous inoculation of FHV. The zones of necrotic placental labyrinth interpreted as infarcts did not contain viral antigen. Inclusion bodies and viral antigen were restricted to the endometrial epithelium and adjacent giant-cell trophoblast in the junctional zone of the placenta. McKay (29) in his study of the pathogenesis of experimental pregnancy toxemia in the rat found that degeneration and necrosis of the giant-cell trophoblast in the junctional zone was followed by fibrin thrombi in the maternal vascular spaces suggesting that procoagulant
factors were liberated by the degenerating giant-cell trophoblast. Localization and proliferation of FHV in the endometrial vessels, giant-cell trophoblast, and endometrial epithelium, therefore, may alter the coagulative homeostasis of the placenta, resulting in thrombosis, infarction, placental separation and abortion. If placental lesions are not severe enough to cause early abortion or fetal death, fetal infection subsequently occurs.

The coagulative necrosis of the placental villi which followed intravenous inoculation of FHV is similar to the placental lesions reported in conjunction with congenital infection by herpes simplex virus (13) and IBR virus (12). The relationship of placental lesions to the pathogenesis of abortion or congenital infection by herpesviruses has not been established. Molello et al. (12) concluded that the placental necrosis which followed parenteral inoculation of IBR virus followed fetal infection and death. However, coagulative necrosis of placental villi has also been reported in the placentas of premature but viable infants congenitally infected with herpes simplex (13) and varicella (14) viruses. Our observations indicate that the placental lesions induced by FHV precede fetal infection and fetal death. Six days after intravenous maternal inoculation virus, viral antigen, and lesions were present in all the placentas but in none of the fetuses examined. The placentas of several viable aborted fetuses contained degeneration and necrosis in the junctional zone as well as necrosis of labyrinthine villi. Thus, following introduction of feline herpesvirus into the maternal blood, virus-induced placental lesions result in abortion the etiology of which would not be identified if only fetal tissues were
examined. Placental infection independent of fetal infection has also
been observed in herpes simplex and mumps infection in hamsters
(18,31), murine cytomegalovirus infection (32) and rubella infection in
man (33,34).

Vaginal infection by FHV is significant in light of the association
of the canine (35) and bovine (36) herpesviruses and type 2 herpes sim­
plex virus (37) with vaginal or cervical infection. Bittle and Peckham
(38) have recently reported experimental induction of vaginal infection
with FHV. The possibility of respiratory and genital types of FHV
differing in neutralization kinetics, cytopathic effect, or DNA density
as do the type 1 and 2 viruses (37) has not been examined. Thus far
neither spontaneous genital infection by FHV nor viral isolates differ­
ing in cytopathic effect or other characteristics have been reported.
Bowing et al. (39) did not find significant differences in cytopathic
effect, neutralization slope, or DNA density between respiratory and
genital isolates of IBR virus.

The persistence of virus on the fetal side of the placenta on
PID 26 despite the presence of neutralizing antibody in the maternal
serum is consistent with evidence indicating that transfer of antibody
through the endothelial-chorial placenta of the cat is minimal (40).
Focal hepatic necrosis in the fetus congenitally infected with FHV is
similar to that associated with congenital infection by the equine
(42), bovine (8,42) and human (10) herpesviruses. Although a direct
viral effect on the gravid uterus could not be demonstrated in the cats
which aborted after intranasal inoculation, the tropism of FHV for the
uterus and placenta demonstrated after intravenous inoculation warrants
further studies of the role of the virus in feline abortion and genital infection. Yet to be explored are the possible teratogenic effects of maternal infection by FHV in early pregnancy. The results of this study indicate that experimental feline herpesvirus infection in pregnant cats constitutes a promising model in which to investigate the interaction of indigenous herpesviruses with the uterus, placenta, and fetus.
Summary

Feline herpesvirus (FHV) localized in the uterus and placentas of 5 pregnant cats inoculated intravenously resulting in abortion, intrauterine fetal death, and fetal infection. Placental lesions included: multiple regions of coagulative necrosis in the placental labyrinth, degeneration and thrombosis of maternal arterioles in the uterus and placenta, and degeneration and necrosis of the giant-cell trophoblast and endometrial epithelium in the junctional zone of the placenta associated with the presence of intranuclear inclusion bodies. FHV was isolated from the placentas of all the cats inoculated intravenously. Viral antigen was demonstrated in the uterine vessels, the giant-cell trophoblast, and the endometrium but not in fetuses on postinoculation day (PID) 6. On PID 26 the virus was restricted to the chorioallantoic membrane covering the fetal border of the placenta. Congenital fetal infection was also demonstrated on PID 26. Although all 4 pregnant cats inoculated intranasally aborted, neither virus, viral antigen, nor significant lesions could be detected in the uteruses, placentas, or fetuses. Abortion in these cats was interpreted as a non-specific reaction related to the severe, debilitating upper respiratory disease which followed intranasal, but not intravenous, inoculation of FHV.
Table 1. Effects of Intravenous and Intranasal Inoculation of Pregnant Queens with Feline Herpesvirus.

<table>
<thead>
<tr>
<th>Cat No.</th>
<th>Route of Inoc.</th>
<th>Abortion &amp; Necropsy PID</th>
<th>Lesions</th>
<th>Virus Isolation</th>
<th>Viral Antigen</th>
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<td>Uterus/Placenta/Fetuses</td>
<td>Uterus/Placenta/Blood/Liver/Spleen/Vaginal Organs</td>
<td>Uterus/Placenta/Fetal Organs</td>
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<td>2</td>
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<td>3</td>
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<td>10</td>
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NA = placentas not available
^1 FHV detected in plasma collected on PID 2 and 3 but no on PID 6.
^2 FHV isolated on PID 6 but not PID 13.
Fig. 1. Placenta and fetus from a pregnant cat 6 days after intravenous inoculation of feline herpesvirus. Multiple, pale, well defined areas of necrosis (arrow) are present in the placenta. The fetus was dead in utero.
Fig. 2. Placentas and four fetuses from a pregnant cat 26 days after intravenous inoculation with feline herpesvirus. None of the fetuses was alive in utero and two are severely autolyzed. Placental lesions are similar to those in Fig. 1, but more advanced.
Fig. 3. Microscopic appearance of one of the placental lesions shown grossly in fig. 1. There is uniform, coagulative necrosis of the entire placental labyrinth with no inflammatory reaction. The endometrial epithelium at the base of the placenta is not involved. The lesion was interpreted as placental infarction. H & E X 125.
Fig. 4. Medial necrosis (arrows) in an endometrial vessel from a cat which aborted 9 days after intravenous inoculation with feline herpesvirus. H & E X 315.
Fig. 5. Multinucleated trophoblast in the junctional zone of the placenta containing eosinophilic intranuclear inclusion bodies (arrow) 6 days after intravenous inoculation of feline herpesvirus. H & E X 315.
Fig. 6. Focus of hepatic necrosis (arrows) in the liver of the fetus shown at the extreme left in Fig. 2. The fetus was dead in utero 26 days after maternal inoculation with feline herpesvirus. H & E X 315.
Fig. 7. Viral antigen (arrow) in a necrotic endometrial vessel of a cat which aborted 6 days after intravenous inoculation with feline herpesvirus. Immunofluorescence X 105.
Fig. 8. Accumulation of viral antigen in the cells of the chorioallantoic membrane (arrows) covering the fetal border of the placenta 26 days after intravenous inoculation with feline herpesvirus. L = placental labyrinth. Immunofluorescence X 50.
CHAPTER 3

INTRANASAL AND INTRAPERITONEAL TRANSMISSION OF FELINE LEUKEMIA

Introduction

Evidence for horizontal transmission of feline leukemia virus (FeLV) has been reported (1-3) but the disease has only been successfully transmitted by parenteral inoculation (1,4,5). Rickard et al. (1) reported that 2 of 25 control kittens in contact with inoculated littermates died by 136 days of age with leukemic lesions. Intranasal inoculation of 7 other kittens from 3 litters with FeLV, however, did not induce leukemia after 7 months. Household clusters of feline leukemia have been reported (3) and FeLV antigen has been detected in unrelated cats involved in such clusters.

The objective of this study was to determine whether feline leukemia can be transmitted by intranasal exposure, a route which might be of importance in natural transmission. Gnotobiotic cats were used to permit characterization of the disease and lesions produced by FeLV in the absence of intercurrent microbial infection and to permit protection of personnel from exposure to FeLV and FeLV-infected cats.

Materials and Methods

Cats

Fifteen gnotobiotic cats representing 5 litters and 6 specific-pathogen-free (SPF) cats from 2 litters were used. The gnotobiotic kittens were obtained by hysterectomy and raised in sterile flexible
plastic isolators according to methods developed by Rohovsky et al. (6). The kittens were fed a commercially-available sterile liquid diet (Tabbilac, Borden) 6 times daily until they were weaned onto sterile, canned food (C/D, Hill Packing Co.). Sterility was monitored by the methods of Wagner (7) as modified by Griesemer and Gibson (8). The SPF cats were obtained from a caesarian-derived, disease-free breeding colony which has been maintained in strict isolation for 6 years. The kittens and their dams were placed in isolators on the day of birth.

**Virus and Inoculation**

Three newborn gnotobiotic cats (cats 6, 7, and 11 of Table 1) were inoculated intraperitoneally with 0.25 ml each of a 20% suspension of the tumor tissue\(^1\) from which the FeLV isolate of Rickard et al. (1) had been obtained. The inoculum had previously been frozen and thawed 3 times. A 20% weight/volume suspension of spleen was prepared from the first cat which became moribund with lymphoma on postinoculation day (PID) 96 (cat 7 of Table 1). The suspension was clarified by low-speed centrifugation and filtered through a 0.45 μm Millipore filter. Subsequent inoculations employed one ml of this first subpassage inoculum administered to newborn kittens. Six kittens were inoculated intranasally and nine intraperitoneally to serve as positive controls. Two cats of 1 litter received 4 ml instead of 1 ml of inoculum intranasally. Three uninoculated kittens served as contact exposed controls.

\(^1\) Provided by Dr. C. G. Rickard.
Postinoculation Procedures and Necropsy

All cats were weighed daily for the first 4 months postinoculation and twice weekly thereafter. The cats were fed 2 to 4 times daily, depending on age, and food consumption was estimated at each feeding. Periodic blood samples for hematologic evaluation were collected under Cl634 (9) sedation. The inoculated cats were killed and necropsied when moribund. Representative portions of all organ systems were fixed in Bouin's or Shein's fixatives and paraffin-embedded, hematoxylin and eosin sections were prepared. Imprints of lymphoid tumors and smears of bone marrow and blood were fixed in methanol for Giemsa staining.

For electron microscopy, bone marrow, lymphoid tumor or mesenteric lymph node, and spleen were fixed in buffered 3% glutaraldehyde, post-fixed in osmium tetroxide, dehydrated through increasing concentrations of ethanol, transferred to propylene oxide and embedded in maraglas or Epon (Shell Chemical Co.). Thin sections were stained with uranyl acetate and lead hydroxide and examined with a Philips 200 electron microscope.

Results

Transmission of Disease

Three of 6 cats inoculated intranasally became moribund with malignant lymphoma on PID 170, 304, and 329 respectively (Table 2). The cat with terminal leukemia on PID 170 received 4 times the amount of inoculum administered to the other cats. Seven of 12 gnotobiotic cats inoculated intraperitoneally became moribund or died with malignant lymphoma from PID 82 to 120 (Table 2). Two cats inoculated intraperitoneally
died before the minimum latent period from urethral obstruction. Three of 7 cats inoculated intranasally and 3 of 12 cats inoculated intraperitoneally died or were killed when moribund between PID 67 and 96 with a non-neoplastic syndrome characterized by thymic atrophy and generalized lymphoid depletion. One cat inoculated intranasally has not developed signs of disease at 500 PID.

Nature of Disease Produced

Three manifestations of disease were observed following inoculation with FeLV by either route: I) abdominal and thoracic malignant lymphoma, II) malignant lymphoma involving only the bone marrow and associated with depletion of lymphoid tissues, and III) thymic atrophy and lymphoid depletion without concurrent evidence of neoplasia. The clinical signs of these 3 syndromes were virtually identical. Anorexia and weight loss were the first indications of disease. Progressive lethargy, weakness, and prostration followed in 7 to 40 days.

1. Generalized Malignant Lymphoma

Seven of 12 cats inoculated intraperitoneally and 1 of five inoculated intranasally developed abdominal and thoracic malignant lymphoma (Table 1). The sites involved, in decreasing order of frequency were the abdominal lymph nodes, kidneys, bone marrow, heart, thymus and thoracic lymph nodes, intestine and spleen (figs. 1-2). The liver and peripheral lymph nodes were not involved. The thymus was either severely atrophic and undetectable grossly (4 cats) or only nodules of lymphoma could be detected in the thymic region (3 cats).

The neoplastic cells were principally lymphoblastic in type. The large, round nuclei had a fine chromatin pattern and a sparse amount
of lightly eosinophilic cytoplasm (fig. 3). In Giemsa-stained impression smears, one or two large nucleoli were prominent and the cytoplasm was pale to deep blue and often vacuolated (fig. 4). Areas of necrosis and hemorrhage were common in the large thoracic and mesenteric tumors. Histocytes with pale cytoplasm containing phagocytized basophilic material were usually interspersed among the lymphoblastic cells. Infiltration of neoplastic cells in the renal cortex and heart was sometimes extensive, producing isolated, degenerate tubules or muscle fibers surrounded by masses of lymphoblastic cells. Germinal centers, parafollicular zones, and sinusoids of involved lymph nodes were replaced by masses of large lymphoblastic cells. Twenty to sixty per cent of the bone marrow cells in 4 of the 7 cats were lymphoblasts and lymphocytes. Lymphoblastic cells with or without absolute lymphocytosis were detected in the peripheral blood only in the terminal 1 to 2 weeks of the disease.

II. Malignant Lymphoma of the Bone Marrow

Two cats (cats 5 and 7, Table 2) inoculated intranasally developed lymphoma of the bone marrow on PID 170 and 329. Thymic atrophy, generalized lymphoid depletion, and pancytopenia accompanied a diffuse proliferation of lymphoid cells which replaced the normal marrow. One of the 2 cats had previously been afflicted with a wasting disease characterized by subnormal growth, rough hair coat, and hard, brittle bones. Lymphoid cells comprised over 75% of all the marrow cells. A continuous spectrum from blast cells through mature small lymphocytes was present (fig. 4). Erythrocytic and granulocytic precursors were rare.
Pancytopenia in both cats was manifested by packed cell volume of 4% or less, hemoglobin less than 2 gm/100 ml, total erythrocytes under 900,000/cu. mm, total leukocytes below 5000 cu. mm and lymphocytes under 1000/cu. mm. Only approximately 20% of the lymphocytes were immature forms. The involvement of the marrow in the 2 cats was similar to that which often accompanied widespread abdominal and thoracic lymphoma but much more extensive. The involvement of bone marrow and pancytopenia distinguished this form of lymphoma from the condition described below.

III. Thymic Atrophy and Lymphoid Depletion

Four of the 18 cats inoculated with FeLV became moribund between PID 67 and 96 with a non-neoplastic condition characterized by generalized lymphoid depletion. The thymus was not detectable grossly. The remnants of thymic lobules of thymic tissue detected microscopically (fig. 5) were composed principally of connective tissue and reticular cells with interspersed small lymphocytes and occasional Hassal's corpuscles separated by wide bands of loose connective tissue. Cortical and medullary regions could not be discerned in contrast with the thymuses of control cats (fig. 6). The lymph nodes were small and frequently had a diffuse red-brown discoloration. A narrow cortical mantle of small lymphocytes was present containing only primary follicles. The medullary cords were sparsely populated with small lymphocytes. The paracortical regions were notably depleted of lymphocytes. Extravasated erythrocytes and erythrophagia were prominent in the nodes of all the affected cats.

A contact control littermate (cat 4, Table 2) of 2 intranasally inoculated cats (cats 2 and 3, Table 2) which became moribund with this
condition on PID 79 remained free of clinical disease and when killed on PID 82 the thymus and lymph nodes were of normal size and histologic appearance. The lymph nodes of the affected cats were approximately 1/3 the size of the littermate control. The cause of the clinical signs in the cats which developed lymphoid atrophy was not established. Two other kittens (cats 1 and 2, Table 1) inoculated intraperitoneally were killed on PID 42 and 59 while asymptomatic. Striking atrophy of the thymus was present in both kittens. The inoculated gnotobiotic kittens which died acutely of urethral obstruction on PID 53 and 71 (before the minimum observed latent period for lymphoma induction) also had severe thymic atrophy.

Electron Microscopy

Budding and extracellular C-type particles (fig. 7) were present in the bone marrow and less frequently in the lymphoid tumors of all of 6 cats with generalized lymphoma examined and in both cats with lymphoma of the bone marrow. One of 2 cats with lymphoid atrophy also had budding C-type virus in the bone marrow on PID 79. No virus could be demonstrated in the uninoculated littermate control killed on PID 82. The viral particles in the marrow were most frequently associated with the membranes of megakaryocytes but were also present in lymphoma cells.

Discussion

The transmission of leukemia after a single intranasal exposure to FeLV lends support to the evidence (2,3) indicating that horizontal spread may account in part for the transmission feline leukemia in nature. The latent period following intranasal exposure to FeLV was 2 to 3 times
that following intraperitoneal inoculation with an equal amount of virus (mean 270 vs 104 days). The prolonged latent period following intranasal inoculation presumably reflects a smaller retained viral dose. Although the presence of C-type virus in feline embryos and fetuses (10) suggests that the primary mode of FeLV transmission may be vertical, horizontal transmission is now proven feasible and warrants further study. In view of the capacity of FeLV to replicate in human, porcine, and canine cells (11), the production of lymphoma in an experimentally infected dog (1), and the transmission of feline leukemia by a single intranasal inoculation exposure, precautions to protect personnel handling FeLV or infected kittens are indicated. Aerosol exposure has proven effective in transmitting Rauscher murine leukemia (12), Yaba virus histocytomas (13), Marek's disease (14) and avian leukosis (15). Successful oral transmission ofmurine (16) and cavian (17) leukemia has been reported. Oral washings (18), urine (17-20), feces (18), and milk (19,20) from mice infected with various murine leukemia viruses have been shown to be leukemogenic. FeLV antigen has been detected in the salivary gland of a leukemic cat. Experiments to determine the presence of FeLV in the saliva, urine, and feces of infected kittens are in progress.

The consistent occurrence of thymic atrophy prior to and in conjunction with lymphoma and as part of a generalized lymphoid depletion in cats inoculated with FeLV suggests that thymic atrophy is preleukemic change and that the thymus may be a target organ for FeLV. Thymic atrophy occurs in mice inoculated with the Gross (20), Moloney (22) and Rich (23) murine leukemia viruses and precedes the onset of spontaneous
leukemia in AKR mice (24). Thymic atrophy in kittens infected with FeLV has been previously observed by Jarrett (26). Lymphoid depletion in the germinal centers and paracortical regions of the lymph nodes was associated with thymic atrophy in some cats. This may indicate a deficiency in the thymic-dependent lymphoid cell population and associated increased susceptibility to intercurrent infection, as suggested by Jarrett and colleagues (26). Deficiency in the cell-mediated immune response has been demonstrated during preleukemia in mice (27). The cats which developed lymphoid atrophy, however, had no clinical signs or lesions to indicate intercurrent bacterial or viral infection and were housed under gnotobiotic conditions in isolators since birth. A contact control littermate had neither signs of disease nor lymphoid depletion when necropsied at the same time as 2 affected littermates. The cause of death in these cats was not determined. No similar disease syndrome has been observed in over 150 litters of SPF cats or 150 litters of gnotobiotic cats raised in our laboratories.

Generalized lymphoid depletion and pancytopenia were associated with lymphoma of the bone marrow in 2 of the cats inoculated intranasally. Jarrett et al. (4) have also reported lymphoma involving only the bone marrow following inoculation of FeLV. Myeloproliferative disorders in cats have also been associated with the presence of C-type viral particles in the bone marrow (28). Since most of the lymphocytes are produced in the bone marrow (28), the interaction of FeLV with bone marrow stem cells might result in either lymphoid leukemia or various other myeloproliferative disorders.
Summary

Feline leukemia was transmitted by intranasal inoculation in 3 of 6 newborn gnotobiotic or specific-pathogen-free cats. The intranasally inoculated cats died or became moribund with lymphoma on postinoculation days 170, 304, and 329. By contrast, the latent period in 7 cats inoculated intraperitoneally was 82 to 120 days. Three manifestations of disease were recognized following inoculation with FeLV: 1) thoracic and abdominal lymphoma, 2) lymphoma restricted to the bone marrow and associated with depletion of lymphoid tissues and pancytopenia, and 3) a non-neoplastic condition characterized by thymic atrophy and generalized lymphoid depletion. Thymic atrophy was consistently associated with inoculation of FeLV and was considered a preleukemic lesion.
Table 1. Intraperitoneal Transmission of Feline Leukemia

<table>
<thead>
<tr>
<th>Cat Number</th>
<th>Flora</th>
<th>PID until Dead or Moribund</th>
<th>Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SPF</td>
<td>42*</td>
<td>Thymic atrophy</td>
</tr>
<tr>
<td>2</td>
<td>''</td>
<td>59*</td>
<td>''</td>
</tr>
<tr>
<td>3</td>
<td>Gnotobiotic</td>
<td>53</td>
<td>Urethral obstruction, thymic atrophy</td>
</tr>
<tr>
<td>4</td>
<td>''</td>
<td>71</td>
<td>Urethral obstruction, thymic atrophy</td>
</tr>
<tr>
<td>5</td>
<td>''</td>
<td>82</td>
<td>Malignant lymphoma</td>
</tr>
<tr>
<td>6</td>
<td>''</td>
<td>96</td>
<td>Thymic atrophy/lymphoid depletion</td>
</tr>
<tr>
<td>7</td>
<td>''</td>
<td>96</td>
<td>Malignant lymphoma</td>
</tr>
<tr>
<td>8</td>
<td>''</td>
<td>96</td>
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<td>120</td>
<td>''</td>
</tr>
<tr>
<td>13</td>
<td>''</td>
<td>250</td>
<td>alive contact control</td>
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</table>

* = no signs of disease when euthanized
Table 2. Intranasal Transmission of Feline Leukemia

<table>
<thead>
<tr>
<th>Cat Number</th>
<th>Flora</th>
<th>PID until Dead or Moribund</th>
<th>Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gnotobiotic</td>
<td>67</td>
<td>Thymic atrophy/lymphoid depletion</td>
</tr>
<tr>
<td>2</td>
<td>SPF</td>
<td>79</td>
<td>&quot;</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>80</td>
<td>&quot;</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>82*</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>Gnotobiotic</td>
<td>170**</td>
<td>Malignant lymphoma of bone marrow</td>
</tr>
<tr>
<td>6</td>
<td>&quot;</td>
<td>304</td>
<td>Malignant lymphoma</td>
</tr>
<tr>
<td>7</td>
<td>&quot;</td>
<td>329</td>
<td>Malignant lymphoma of bone marrow</td>
</tr>
<tr>
<td>8</td>
<td>&quot;</td>
<td>500 alive</td>
<td>-</td>
</tr>
</tbody>
</table>

* = no signs of disease when euthanized

** = 4 ml instead of the 1 ml of inoculum administered to all other cats.
Fig. 1. Lymphoma (delineated by arrows) in thymic region of a cat that died 304 days after intranasal inoculation with feline leukemia virus.
Fig. 2. Malignant lymphoma of the mesenteric lymph nodes of a cat that died 304 days after intranasal inoculation with feline leukemia virus.
Fig. 3. Photomicrograph of the kidney of a gnotobiotic cat, 119 days following intraperitoneal inoculation with feline leukemia virus. The glomeruli and tubules are surrounded by masses of neoplastic lymphoid cells with occasional mitotic figures (arrow). H & E X 125.
Fig. 4. Neoplastic lymphoid cells in the bone marrow of gnotobiotic cats 329 days after intranasal inoculation with feline leukemia virus. Giemsa X 1250.
Fig. 5. Severe thymic atrophy in a cat inoculated intranasally with feline leukemia virus 79 days previously. This is a section of the entire thymus illustrated at the same magnification as the thymus of the littermate control in fig. 6. The thymic lobules are composed principally of small lymphocytes separated by wide bands of loose connective tissue. Cortical and medullary regions are not discernible. The arrow indicates a Hassal's corpuscle. H & E X 50.
Fig. 6. Photomicrograph of the thymus from an uninoculated control cat killed when 82 days of age. Compare with fig. 5. H & E X 50.
Fig. 7. C-type viral particle budding from the plasma membrane of a lymphoblastic cell in the bone marrow of a gnotobiotic cat 329 days after intranasal inoculation with feline leukemia virus. X 74,500.
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CHAPTER 3


