

ABSTRACT

Ferrets (*Mustela Putorious furo*) have a high incidence of naturally occurring lymphoproliferative diseases. Lymphoma is the most common naturally occurring neoplasm of ferrets. Lymphoma has been documented in ferrets of all ages, and has been recognized to occur in most organ systems. There is speculation that there may be an infectious etiology underlying the high incidence of lymphoma in ferrets. It is speculated that chronic antigenic stimulation may result in a chronic lymphocytic response which could ultimately, given the right stimulus, develop into a lymphoma. To date, the etiology and pathogenesis of ferret lymphoma is not well understood. Immunophenotypic characterization of these syndromes will help in further our understanding of this prevalent disease in ferrets. Furthermore, many of these ferret syndromes have human analogs and therefore the ferret is a potential useful model of human lymphoproliferative diseases. Immunologic tools were identified and characterized in order to study ferret lymphoid tissue in the normal and diseased states. Three syndromes of ferret lymphoma were characterized immunophenotypically to determined the nature of the lymphocytic infiltrate. Young ferrets develop a T-cell lymphoblastic mediastinal lymphoma that has many clinical, pathologic, and phenotypic characteristics similar to certain non-Hodgkin's lymphomas of children. Ocular lymphoma has been described in adult ferrets. Phenotypic analysis determined that this is a T-cell lymphoma. Correlations can be made between this syndrome and virally induced ocular T-cell lymphomas of humans. Lymphocytic gastritis and gastric mucosa associated lymphoid tissue (MALT) lymphoma secondary to chronic *Helicobacter mustelae* infection in ferrets has many shared features with *Helicobacter pylori* associated gastric B-cell MALT lymphoma of people. Chronic lymphocytic gastritis in ferrets is characterized by a T-cell infiltrate initially with subsequent B-cell follicular development over time. These B-cell follicles are believed to be the site of lymphoma development. As with people, ferret gastric lymphoma is of a B-cell phenotype. This study illustrates the usefulness of the ferret as an animal model of certain human lymphoproliferative diseases. These diseases have important implications in comparative medicine both in terms of ferret health and in terms of developing the ferret as a model of human disease. Immunophenotypic characterization of other lymphoproliferative diseases in ferrets may aid in improving our understanding of the ferret immune response to chronic antigenic stimulation and may aid in our understanding of the same phenomenon in humans.

ACKNOWLEDGEMENTS

I would like to thank Dr. James Fox for providing me with the opportunity and the support to complete the Master's degree in Toxicology while simultaneously completing a fellowship in Comparative Medicine.

I wish to thank Dr. Susan Erdman for her guidance, encouragement, and support of my Master's project, as well as for support during the completion of the fellowship in Comparative Medicine.

I would like to thank Dr. Mark Schrenzel for his help in interpretation of histopathologic slides, and more importantly for his enthusiasm and his willingness to teach. I would also like to thank Dr. Keith Reimann and WenYu Lin at Beth Israel's Division of Viral Pathogenesis for their time and advice on running and interpreting flow cytometric data. Finally, I would like to thank Greg Dancer for his technical expertise in creating figures and graphs.

I particularly want to thank my friends and family for putting up with me during these sometimes stressful and tedious three years at MIT.

Last, but most importantly, I want to thank my four-legged buddies, "Barney" and "Ghost" for tolerating periods of benign neglect due to my overly hectic schedule. Just having them around kept me sane at times, and kept me moving forward.

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INTRODUCTION

Lymphoproliferative diseases have been widely described in the ferret (*Mustela putorius furo*). (1-24) These diseases have important implications in comparative medicine both in terms of ferret health and in terms of developing the ferret as a model of human disease. Many of the ferret syndromes have human analogs and therefore the ferret can be a useful model of human lymphoproliferative diseases. (18,22,23)

Ferrets of all ages exhibit a high incidence of naturally occurring lymphoma. Lymphoma has been described in most organ systems. (2-7, 14, 22-24) The etiology and pathogenesis of this syndrome is not well understood. Immunophenotypic characterization of these lymphomas will aid in further elucidating this common syndrome in ferrets. A better understanding of lymphoproliferative disease in ferrets will enable improved treatment of the disease. Furthermore, as the syndrome is better characterized, comparisons of the ferret disease with that seen in other species can be made which will contribute to the overall understanding of lymphoproliferative disease in all species.

There are few immunologic tools commercially available to characterize ferret lymphoid tissue. As a result, little is known about lymphoproliferative disease in ferrets, nor about the normal immune system of ferrets. It is critical to establish the normal distribution of lymphoid cells in healthy adult ferrets prior to assessment of diseased states. This thesis describes an initial assessment of the distribution of lymphoid cells in normal lymphoid tissues and in the peripheral blood of healthy adult ferrets. Human lymphoid cell surface markers which cross react with ferret lymphocytes were employed to establish ratios of T-lymphocytes and B-lymphocytes *in situ*. Subsequently these lymphoid cell surface markers were used to examine several lymphoproliferative syndromes of the ferret.

Young ferrets develop a peracute mediastinal lymphoma similar to that seen in many other species, including humans.(5) Ten cases of juvenile-onset mediastinal lymphoma were examined and characterized immunophenotypically to determine if they were of T-cell or B-cell origin. Genotypic analysis was performed on two of these cases to confirm our phenotypic analysis. In contrast, adult ferrets tend to develop lymphoma that is more variable in presentation, and is often characterized clinically by a chronic course of disease. (2,3) Two subsets of adult lymphoma were characterized immunophenotypically. Ocular lymphoma was identified in two adult ferrets (24) and subsequently immunophenotyped.

Gastric lymphoma has been recognized in a small subset of adult ferrets with chronic lymphocytic gastritis secondary to chronic helicobacter infection. (17-21,23) Thirty cases of lymphocytic gastritis were examined immunophenotypically to identify which subsets of lymphocytes were involved in the gastritis and to attempt to determine which subsets tended to proliferate and contribute to the etiopathogenesis of lymphoma.

This thesis intends to establish the normal distribution of lymphocytes within lymphoid tissue as well as begin to examine certain lymphoproliferative syndromes common to the ferret. Immunophenotypic characterization of lymphoproliferative disease in ferrets will enable us to understand the etiology and progression of the disease in ferrets in the hope to improve the treatment and prognosis for ferrets afflicted with lymphoma. In addition, it will enable us to more fully evaluate and establish the ferret as a useful animal model.

BACKGROUND/SIGNIFICANCE

The European ferret (*Mustela putorius furo*) is a member of the mustelid family which includes the weasel, badger, skunk, otter, and mink. The ferret is a small, easily handled animal that is well suited to domestication, originally believed to have occurred over 2000 years ago. (1) Ferrets were originally domesticated for their adeptness at “rabbiting”. (1) During the past century, the ferret was introduced to the biomedical research community as an important model of Influenza viral infection. (1,25,26,27) Most recently, ferrets have been introduced to the general population as tractable, adaptable, and lovable “exotic” pets.

The ferret is gaining in popularity as both a pet and as a research animal and model of human disease. As a result, knowledge of the basic biology of the ferret and of the common diseases unique to the ferret is becoming increasingly sought after. Elucidation of the pathogenesis of the common diseases of the ferret will aid veterinary practitioners and researchers in developing improved therapies to treat ferrets as well as to contribute to the perpetual search for appropriate animal models of human disease. However, to study disease pathogenesis, it is necessary to understand the host response to inflammation and infection, ie. it is necessary to understand the host immune system. Therefore, it is paramount to characterize the ferret immune system in both the healthy and the compromised state in order to fully understand and treat ferret diseases.

Our knowledge of the ferret immune system is limited. The absolute percentage of lymphocytes in juvenile and adult ferrets is well documented. (1) However, the normal distribution of T and B-lymphocytes in ferret peripheral blood and tissue has not been established nor has the lymphocytic response to inflammation, with respect to T and B lymphocyte subsets, been described. Investigators have attempted to identify immunoglobulin expressing lymphocytes, presumably B-cells, via complement fixation and by an indirect immunofluorescence antibody technique. (28) The percentage of immunoglobulin expressing lymphocytes in peripheral blood ranged from 11-17%, whereas considerable variation was found amongst splenic lymphocytes of which 10-35% expressed immunoglobulin. The reported values for immunoglobulin expressing peripheral blood lymphocytes do fall in accordance with that reported for other domestic species. Generally, B-cells comprise 15-25% of the total lymphocyte population; T-cells comprise about 70%; NK cells comprise 5-8%. (31) The limitations of this study are that it represents an indirect measurement of B-cells, and merely infers that the remaining

lymphocytes must be T-cells. To accurately quantitate T and B-cell subpopulations of lymphocytes, lymphocyte specific antibodies should be employed. Ideally, one would use an antibody specific for ferret B-cells and an antibody specific for ferret T-cells to separate out the respective populations of cells. However, the commercial availability of ferret specific lymphoid antibodies is limited. Therefore creative means of developing specific assays to separate out ferret lymphocytes must be employed.

Lymphocyte proliferation assays have been performed on ferret lymphocytes. (26,27,29,30) As expected, a subset of lymphocytes will proliferate in response to the T-cell mitogens, Concanavalin A and Phytohemagglutinin, and a different subset of lymphocytes will proliferate in response to the B-cell mitogen, Pokeweed mitogen. Furthermore, if lymphocytes are separated on the basis of Fc receptors, which presumably separates B cells from non-B cells, these subpopulations will respond accordingly to the appropriate mitogen. (26) These assays demonstrate that ferret lymphocytes have similar proliferative responses to mitogens as that seen in other domestic species.

Presently, we can infer that the population of B-cells and T-cells in ferrets is comparable to other domestic species in terms of number and basic proliferative capabilities. However, the function and distribution of ferret lymphocyte populations in vivo has not been elucidated which is critical to understanding disease pathogenesis. First, lymphocyte distribution in situ must be established and then function can be elucidated.

It is helpful to distinguish between T-lymphocytes and B-lymphocytes as their functions are quite distinct. B-lymphocytes produce immunoglobulins which are important in the recognition and elimination of extracellular foreign antigen. (31,32) Immunoglobulin binding neutralizes the foreign antigen prior to initiating opsonization of the antigen, and activation of complement which aids in phagocytosis of the foreign antigen. This B-lymphocytic response is termed the humoral response. (31,32) T-lymphocytes are integral players in the cell-mediated immune response as well as the humoral response. T-lymphocytes are important in the recognition and elimination of intracellular foreign antigen. T cells recognize specific peptide sequences that are expressed by the major histocompatibility complex (MHC) on the surface of a cell which contains foreign antigen, ie, viral. Certain T cells, cytotoxic/CD8 T-cells are able to directly kill cells that are infected with foreign antigen. These cytotoxic T-lymphocytes recognize MHC Class I molecules. Other T cells, CD4+ T-lymphocytes, play a crucial role in the activation of other lymphocytes, both B cells and T cells. (31,32) CD4+ T-lymphocytes recognize

MHC Class II molecules expressed on macrophages and on B lymphocytes which have internalized foreign antigen. CD4 T-lymphocytes are further subdivided into T_H 1 and T_H2 subclasses. T_H1 cells activate macrophages to aid in killing intracellular bacteria. T_H2 cells activate B cells to produce antibody and thereby initiate the humoral immune response. Thus, the role of the T-lymphocyte is that of an effector cell. (31) T-lymphocytes and B-lymphocytes work synergistically to protect the host against pathogenic invaders. The interaction of the immune system is carefully regulated. On occasion, this regulation fails due to either external forces (viruses) or due to internal forces (cytokines) and lymphoproliferation ensues. (31)

Lymphoproliferative diseases have been widely described in the ferret. (1-24) Lymphoproliferative diseases include a wide spectrum of benign and malignant syndromes, ranging from reactive lymphocytosis and lymphadenopathy to lymphoma. These diseases have important implications for pet ferrets as well as for research animals. Generally, these syndromes affect all ages, and often have prolonged clinical courses and, potentially, a poor prognosis. The etiology and pathogenesis of many of these disease syndromes is not well understood. Infectious agents have been linked with certain syndromes, and there is speculation of an infectious etiology to others. Determining the nature of the lymphocytic response in these syndromes may aid in further understanding the disease process and the associated pathology.

Aleutian Disease Virus is a parvo virus of mink that can infect ferrets. (1,9-13) It induces a lymphocytic-plasmacytic response in numerous tissues. Clinically, affected ferrets may have vague, non-specific signs of malaise, inappetence, and weight loss. Hypergammaglobulinemia is a salient feature. Posterior paresis and urinary incontinence have been described. Gross lesions include enlargement of the thymus, hepatomegaly, splenomegaly, and lymphadenopathy. Histologically, lesions are characterized by a lymphoplasmacytic infiltrate in many tissues. It has been suggested that ADV may be associated with lymphoma in ferrets. (14) However, this finding has not been substantiated by other studies on ferret lymphoma. (2-5)

Enzootic malignant granulomatosis is a syndrome that was described in the late 1940's. A single outbreak involving a group of young ferrets was described in which the ferrets succumbed to widespread disseminated granulomas affecting the mesenteric and peripheral lymph nodes. Histopathology was suggestive of an infectious etiology although none was identified. Lesions were characterized by granulation tissue surrounded by

lymphocytes, lymphoblasts, plasma cells, neutrophils, fibroblasts and large epitheloid cells. This syndrome has not been reported again. (1)

Epizootic catarrhal enteritis is a highly contagious diarrheal syndrome of unknown etiology. Clinical signs and gross lesions are generally non-specific. However, microscopically, the syndrome is characterized by a lymphocytic infiltrate throughout the small intestine. Lymphocytes are generally found amongst the mucosal epithelial cells. Other histologic features include villus atrophy, fusion, and blunting, and necrosis of apical enterocytes. (15)

Idiopathic hypersplenism has been reported sporadically in ferrets. This syndrome is characterized by splenomegaly, anemia of chronic disease, and leukocytosis. The leukocytosis is secondary to marked extramedullary hematopoiesis which usually reverses following splenectomy. This syndrome can be misdiagnosed as splenic lymphosarcoma due to the large numbers of lymphocytes, both mature and blastic forms which infiltrate the spleen. The etiology is unknown. (1, 16)

Chronic interstitial nephritis occurs with relative frequency in ferrets of all ages. Clinically, ferrets present with signs of renal disease or renal failure. There is suggestion that the disease may be secondary to a high protein commercial cat diet. Gross lesions are consistent with chronic insult with subsequent fibrosis and scarring. Microscopically, fibrosis is widespread, affecting glomerular, tubular, and interstitial tissue. A lymphocytic plasmacytic infiltrate can be found compressing and effacing the interstitium. (15)

Chronic lymphocytic gastritis secondary to *Helicobacter mustelae* infection is well recognized in the ferret. (17-21) *Helicobacter mustelae* was first isolated from the gastric mucosa of ferrets in 1986. (17) The prevalence of *Helicobacter* infection in ferrets is close to 100%. (18) Colonization is believed to occur at weaning age and persists throughout the life of the ferret. Clinical signs are uncommon although both gastric and duodenal ulcers have been reported which could certainly contribute to feelings of malaise, inappetence, and bruxism. *Helicobacter mustelae* colonization is limited largely to the gastric antrum and pylorus. (18,19) Ferrets chronically infected with *Helicobacter* develop histologic changes in the gastric mucosa consistent with that seen in humans that are infected with *Helicobacter pylori*. Superficial gastritis, diffuse antral gastritis, and chronic atrophic gastritis have all been described in ferrets known to be infected with *H. mustelae*. (18,19) Superficial gastritis has been defined as an inflammatory infiltrate and mucus depletion limited to the

superficial gastric mucosa. Diffuse antral gastritis is defined as mucus depletion and an inflammatory infiltrate which involves all layers of the antral mucosa. Chronic atrophic gastritis is defined as glandular atrophy in addition to an inflammatory infiltrate in either the antrum or the body. Chronic atrophic gastritis is considered to be a possible prelude of gastric ulcerogenesis and gastric carcinogenesis. (33) Age matched controls specific pathogen free for *H. mustelae* do not demonstrate histologic changes consistent with gastritis of any severity. (18) The primary difference between *Helicobacter* associated gastritis described in ferrets and that commonly seen in humans is the character of the inflammatory infiltrate. In humans, the inflammation is typically polymononuclear in nature, whereas ferrets characteristically exhibit a lymphocytic-plasmacytic infiltrate in response to *Helicobacter* infection. Interestingly, children tend to exhibit a lymphocytic-plasmacytic infiltrate as well in contrast to that seen typically in adults infected with *H.pylori*. (18)

Lymphoma is the most common naturally occurring neoplasm of ferrets. (1-3) Cluster outbreaks of lymphoma have been described suggesting a potential infectious etiology. (4) Lymphoma has been described in ferrets of all ages and involving all systems. Two distinct syndromes have been described based on the age of the ferret. Young ferrets tend to develop a rapidly progressive syndrome characterized clinically by peracute mortality with few clinical signs or by a clinical syndrome that is characterized by dyspnea, weakness, and anorexia which may progress over days to weeks but results in a mortality rate of close to 100%. (2,3,5) Adult ferrets develop lymphoma which tends to be of a more chronic nature. (2,3) These ferrets may live for several years after a diagnosis of lymphoma is made. Clinical signs tend to wax and wane, and are generally of a non-specific nature. Organ system involvement can be widespread in any ferret with lymphoma. The majority of juveniles tend to develop mediastinal lymphoma, presumably of thymic origin. Adults typically develop generalized lymphadenopathy, and splenomegaly. (2,3) Ocular lymphoma and cutaneous lymphoma have been described. (6,24)

Numerous classification schemes have been developed to categorize lymphomas according to clinicopathologic features. Each scheme has pros and cons. No one classification scheme has been recognized as superior by all pathologists. Ferret lymphomas have historically been classified according to the National Cancer Institute's Working Formulation for classification of non-Hodgkin's lymphomas. (40) The lymphoma seen in young ferrets is consistent with the NCI classification of either a small,

non-cleaved lymphoma or an immunoblastic lymphoma. In adult ferrets, the lymphoma is often more polymorphic. Small, non-cleaved and immunoblastic lymphomas are seen in adult ferrets as well as immunoblastic polymorphic and diffuse small lymphocytic lymphoma. The ocular lymphoma described in two adult ferrets was consistent with the NCI classification of a small, non-cleaved lymphoma.

Immunophenotypic characterization of these syndromes is critical to further understanding of the disease in ferrets and for further evaluation of the ferret as a model of human disease. This thesis focuses on characterizing three syndromes of lymphoma in the ferret which are of considerable importance to ferret biology and disease and which have human analogs and therefore may be of importance in studying certain human disease syndromes.

Mediastinal lymphoma in young ferrets resembles a subset of mediastinal lymphomas common to young children. The disease in ferrets and in children is characterized by an aggressive lymphoma. (41,42,43) The clinical course is rapid and is usually fatal in ferrets. In children, the lymphoma can be treated successfully with chemotherapeutic agents if treatment is begun early in the course of disease. (44) In the ferret, the disease is important as it is not uncommon, and the mortality rate approximates 100%. Treatment of mediastinal lymphoma in ferrets has not been successful due to inadequate early diagnosis of the disease. A better understanding of the syndrome in ferrets may enable an earlier diagnosis, improved therapy, and improved prognosis for ferrets as well as for children with a similar disease syndrome.

Ocular lymphoma in ferrets is aggressive clinically. (24) Many features of the ferret syndrome parallel that seen in other species. Ocular lymphoma is often part of a systemic lymphoma. Ocular lesions have been described in association with many virally-induced lymphomas of several species. In people, the majority of ocular lymphomas are of B-cell origin, however virally induced ocular lymphomas are primarily a T-cell phenotype. (45-49) Determination of the immunophenotype of ferret ocular lymphoma will aid in making correlations between the ferret neoplasm with that of other species. Hopefully, such comparisons will enable us to understand the pathogenesis of the ferret syndrome.

Chronic *Helicobacter* infection has been linked with the development of gastric mucosa associated lymphoid tissue (MALT) lymphoma in people. (34-38) People infected chronically with *H. pylori* develop lymphoid follicles in the gastric antrum. Over time,

chronic antigenic stimulation secondary to *H. pylori* is believed to cause the further development and enlargement of these gastric lymphoid follicles. Eventually, these lymphoid follicles may progress to a B-cell MALT lymphoma. Interestingly, eradication of the *Helicobacter* organisms with antibiotics will cause the MALT lymphoma to regress. (39) It is controversial as to whether these lymphomas can be considered to be truly neoplastic since they are antibiotic responsive. Nonetheless, there does seem to be a correlation between chronic *Helicobacter* infection and the development of MALT lymphoma. Recently, a similar finding was reported in ferrets chronically infected with *H. mustelae*. (23) Four aged (>5 years) ferrets naturally infected with *H. mustelae* developed primary gastric MALT lymphoma. These ferrets had histologic changes consistent with that seen in humans diagnosed with MALT lymphoma. Lymphoid follicles, lymphoepithelial lesions, and B cell light chain clonality were identified in all four ferrets. The lymphoma was limited to the antrum, the site of chronic *Helicobacter* associated gastritis. It can be inferred that the gastric MALT lymphoma arose secondary to chronic lymphoproliferation secondary to chronic *Helicobacter* infection as postulated in humans. *Helicobacter mustelae* induced gastritis and gastric lymphoma in ferrets is a good model of the analogous syndromes in humans associated with *Helicobacter pylori*. (18) There are many similar histologic features shared by ferrets and people with gastric MALT lymphoma. The phenotype of the ferret lymphoma is an important parameter that needs to be evaluated so that further correlations can be made between ferret and human gastric lesions. The ferret could prove to be very useful in the elucidation of the theory that lymphoproliferation secondary to chronic bacterial stimulation could result in the development of lymphoma.

The immunophenotypic characterization of these lymphoproliferative syndromes in ferrets will add one more dimension to the knowledge base of ferret biology and disease. Hopefully this will aid in the meaningful evaluation of the ferret as an animal model of human disease in the future. The more we can learn about a syndrome in one species, the better our ability to learn about analogous syndromes in other species. Drawing on the parallels and on the differences of similar syndromes in different species adds to the base of knowledge in comparative medicine as a whole.

ANTIBODY CHARACTERIZATION

INTRODUCTION

Classification of lymphocytes into functional subsets is important in understanding normal and pathological immune responses, and for characterizing lymphoproliferative diseases. Lymphocytes can be broadly categorized into two distinct populations based on function. (31,32) B lymphocytes produce immunoglobulin and are important in the humoral immune response. T lymphocytes play an important role as effector cells and in the cell-mediated immune response. Microscopically, these two distinct populations of lymphocytes are indistinguishable. However, there are distinct differences between these classes of lymphocytes at the molecular level which can be identified and which are important in cell function. Numerous cell surface-associated lymphoid antigens have been identified in humans and many of the domestic species.(31,32) Many cell surface antigens are uniquely expressed by mature B cells whereas other cell surface antigens are uniquely expressed by mature T cells, or the T cell subsets. These lymphoid antigens can be used as markers of T and B lymphocytes and of their respective subsets to study lymphocyte distribution in normal and diseased states.

In the ferret, few lymphoid specific markers exist. In fact, the only commercially available ferret specific marker is a polyclonal goat anti-ferret immunoglobulin (heavy & light chain) antibody (Kirkegaard & Perry). This ferret specific immunoglobulin antibody is useful in identifying B cells via flow cytometry. However, this antibody has very limited applications with regards to *in situ* characterization as it only recognizes B-lymphocytes which are actively expressing and secreting immunoglobulin. Furthermore, immunoglobulin antibodies in general tend to demonstrate a high degree of background staining due to non-specific cross-reactivity with other cells which may express Fc receptors (ie. dendritic cells). (50) Due to the scarcity of ferret specific antibodies, ferret lymphoid tissue has not been adequately characterized *in situ* with regards to T-cell and B-cell distribution.

Five commercially available cell surface lymphoid antigen markers were identified which can be used to differentiate ferret T and B lymphocytes. The antibodies were used to detect healthy and pathologic lymphoid tissue *in situ* by immunohistochemistry assays, or to detect classes of circulating peripheral blood lymphocytes via flow cytometric analysis.

All five antibodies are specific for human lymphocytes yet were shown to cross-react with ferret lymphocytes with a high degree of specificity. Specificity of these markers was demonstrated by in situ labeling, flow cytometric analysis, and by molecular techniques.

Polyclonal rabbit anti-human CD3 (DAKO) recognizes ferret T-lymphocytes in situ. Monoclonal mouse anti-human CD79 α (DAKO) recognizes ferret B-lymphocytes in situ. Polyclonal rabbit anti-human kappa immunoglobulin light chain (Biomed) and polyclonal rabbit anti-human lambda immunoglobulin light chain (Biomed) recognize ferret B-cell subsets both in situ and by flow cytometry. OKT8 (Ortho), a human monoclonal antibody specific for the CD8 antigen, will recognize a subset of ferret lymphocytes by flow cytometry. (Table1)

METHODS & MATERIALS

Antibodies:

Polyclonal rabbit anti-human CD3 and monoclonal mouse anti-human CD79 α were used on formalin fixed paraffin-embedded ferret lymphoid tissue. Polyclonal rabbit anti-human lambda light chain immunoglobulin and polyclonal rabbit anti-human kappa light chain immunoglobulin were used on both formalin fixed paraffin-embedded tissue and on fresh ferret peripheral blood lymphocytes. OKT8 was used on fresh ferret peripheral blood lymphocytes.

Antibody Specificity:

Lymph node, spleen, and thymus from clinically normal ferrets were processed for immunohistochemistry to evaluate the affinity of the rabbit anti-human CD3 and the mouse anti-human CD79 α antibodies for ferret lymphoid tissue. Peripheral blood lymphocytes (10^6 - 10^7) from healthy ferret donors were isolated by ficol-hypaque density separation for use in a flow cytometry assay to evaluate the affinity of the kappa, lambda, and OKT8 antibodies for ferret lymphocytes. Fresh lymphocytes (10^8) were isolated from the thymus, spleen, and lymph nodes from healthy ferret donors for use in a Western Blot assay to confirm that the polyclonal CD3 antibody and the monoclonal CD79 α antibody recognize ferret T cells and B cells, respectively, based on molecular weight analysis.

Table 1: Human specific Lymphoid Antibodies which Cross-React with Ferret Lymphoid Tissue

ANTIBODY	ANTIGEN	ASSAY	TISSUE
CD3	T-cells	Immunohistochemistry	Formalin-fixed lymphoid tissue
CD79 α	B-cells	Immunohistochemistry	Formalin-fixed lymphoid tissue
κ Ig	B-cell, subset	Immunohistochemistry Flow Cytometry	Formalin-fixed lymphoid tissue Fresh peripheral blood lymphocytes
λ Ig	B-cell, subset	Immunohistochemistry Flow Cytometry	Formalin-fixed lymphoid tissue Fresh peripheral blood lymphocytes
OKT8	CD8+ T-cell	Flow Cytometry	Fresh Peripheral blood lymphocytes

Immunohistochemistry:

Slides were warmed to 56C for 30 minutes and subsequently deparafinized and rehydrated in a series of xylene and ethanol baths at 25C. All subsequent manipulations were carried out at room temperature. For the CD3, kappa, and lambda antibodies, endogenous peroxidases were inhibited by incubation in Methanol and 3% H₂O₂ for 20 minutes. Slides were washed with Tris-buffered saline, pH 7.4 (TBS), then incubated in pronase (200u/50ml TBS) for 10 minutes, washed extensively in H₂O, and then in TBS.

For the CD79 α antibody, slides were processed by a heat-based antigen retrieval method.

(51) Slides were immersed in Citrate buffer, pH 6.0, and boiled 2X for 5 minutes in a microwave oven. Endogenous peroxidases were inhibited by incubation in Methanol, 0.3% H₂O₂, and 0.1% sodium azide. Slides were subsequently washed in TBS. The primary antibody (CD3, CD79 α , kappa, lambda, non-immune rabbit serum, or non-immune mouse serum) was applied at a 1:100 dilution for 60 minutes. The slides were washed in TBS and the secondary antibody (biotinylated goat anti-rabbit IgG or biotinylated rabbit anti-mouse IgG) was applied at a 1:100 dilution for 30 minutes. Again, the slides were washed in TBS, and streptavidin-horseradish peroxidase applied for 30 minutes. Diaminobenzidine was used as the color substrate. Slides were allowed to air dry and were cover-slipped for subsequent microscopic evaluation. Positive reactions were detected by a brown staining of the cytoplasm of the lymphocyte. (Figure 1)

Flow Cytometry:

Fresh peripheral whole blood (100ul) from adult ferret donors was washed 2X in phosphate buffered saline (PBS) and 0.1% Bovine serum albumin (BSA) to decrease non-specific binding. Samples were incubated with 100ul of the primary antibody (rabbit anti-human kappa light chain immunoglobulin, rabbit anti-lambda light chain immunoglobulin, OKT8, or FITC-conjugated goat anti-ferret heavy and light chain immunoglobulin) for 20 minutes. Samples were washed in PBS, and incubated with a secondary antibody labeled with either FITC, green fluorescence, or phycoerytherin (PE), red fluorescence for 20 minutes. Samples were washed in PBS and subsequently the red blood cells were lysed with Uti-Lyse (DAKO). Samples were washed 2X in PBS and resuspended in PBS/1% formalin and stored at 4C until analysis via the flow cytometer.

(Figure 2)

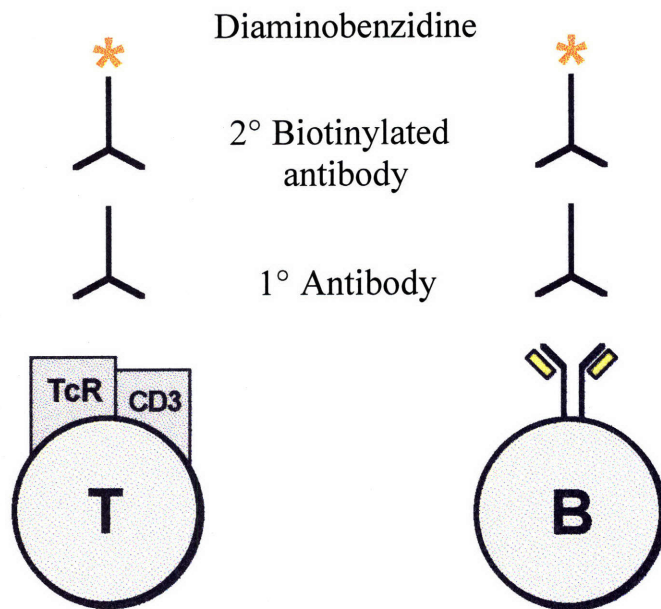
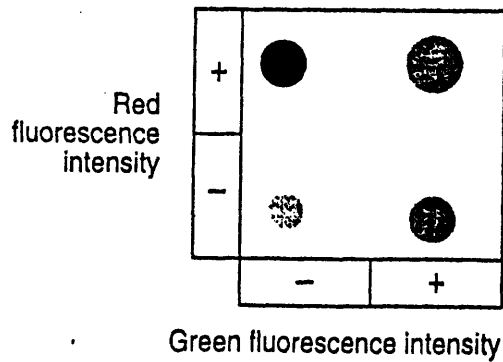
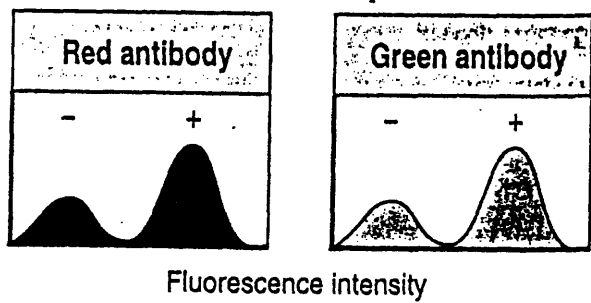
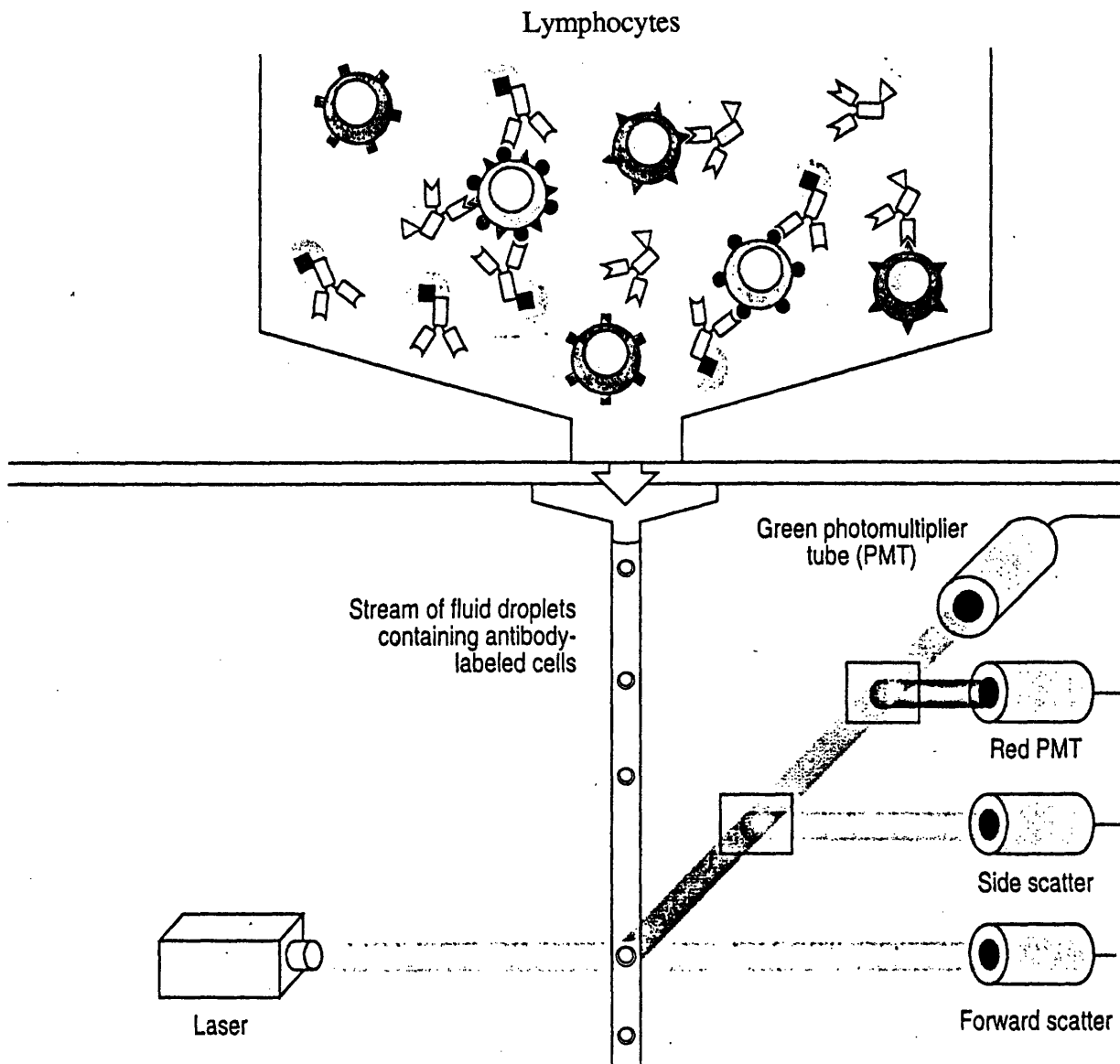


Figure 1. Schematic of immunohistochemistry assay.

Figure 2: Schematic of Flow Cytometry



Adapted from Janeway & Travers, 1994

Determination of Ferret Kappa:Lambda Ratio:

The ferret kappa: lambda ratio was determined by two different means - immunohistochemistry and flow cytometry. Semi-quantitative analysis was performed by counting positively stained lymphocytes in sections of normal formalin-fixed paraffin-embedded ferret small intestine stained by immunohistochemistry. 100 positively stained lymphocytes in 3 fields at 100X magnification were counted in sections of small intestine from 10 healthy ferrets. The percentage of kappa+ cells versus lambda+ cells was determined and subsequently, the ratio of kappa:lambda positively stained cells was calculated. To verify the immunohistochemical results, fresh peripheral blood lymphocytes were stained with the kappa and lambda antibodies and evaluated by flow cytometry. Two experiments were performed. A double labeling experiment to verify that the antibodies were labeling mutually exclusive populations of cells was performed first. Each sample was labeled first with FITC kappa, and subsequently labeled with RPE lambda. These samples were then compared to samples of lymphocytes labeled with FITC goat anti-ferret IgG. The second experiment entailed the labeling of peripheral blood lymphocytes from several ferrets every four weeks to evaluate the consistency of the antibody specificity. Percentage of positive cells from both experiments was determined to obtain a ferret kappa:lambda ratio.

Western Blot Analysis:

To confirm that the rabbit anti-human CD3 antibody recognizes ferret T cells and the mouse anti-human CD79 α antibody recognizes ferret B cells, western blot analysis was performed. Fresh lymphocytes (10^8) were isolated from the thymus, spleen, and lymph nodes from healthy ferret donors. Cells were lysed in RIPA Lysis Buffer and run on a 12.5% acrylamide gel. The proteins were electrophoretically transferred to a nitrocellulose filter over 4 hours. The filter was blocked in 10% nonfat milk and 1% bovine serum albumin (Sigma) overnight at 4C, washed 3X in PBS/Tween and then incubated with the primary antibody (CD3 or CD79 α) for 1 hour at room temperature. The filter was washed 3X in PBS/Tween prior to incubation with the secondary antibody (peroxidase labeled anti-rabbit IgG or peroxidase labeled anti-mouse IgG) for 1 hour at room temperature. The filter was then washed 6X in PBS/Tween prior to detection via ECL system (Amersham Life Science). (Figure 3)

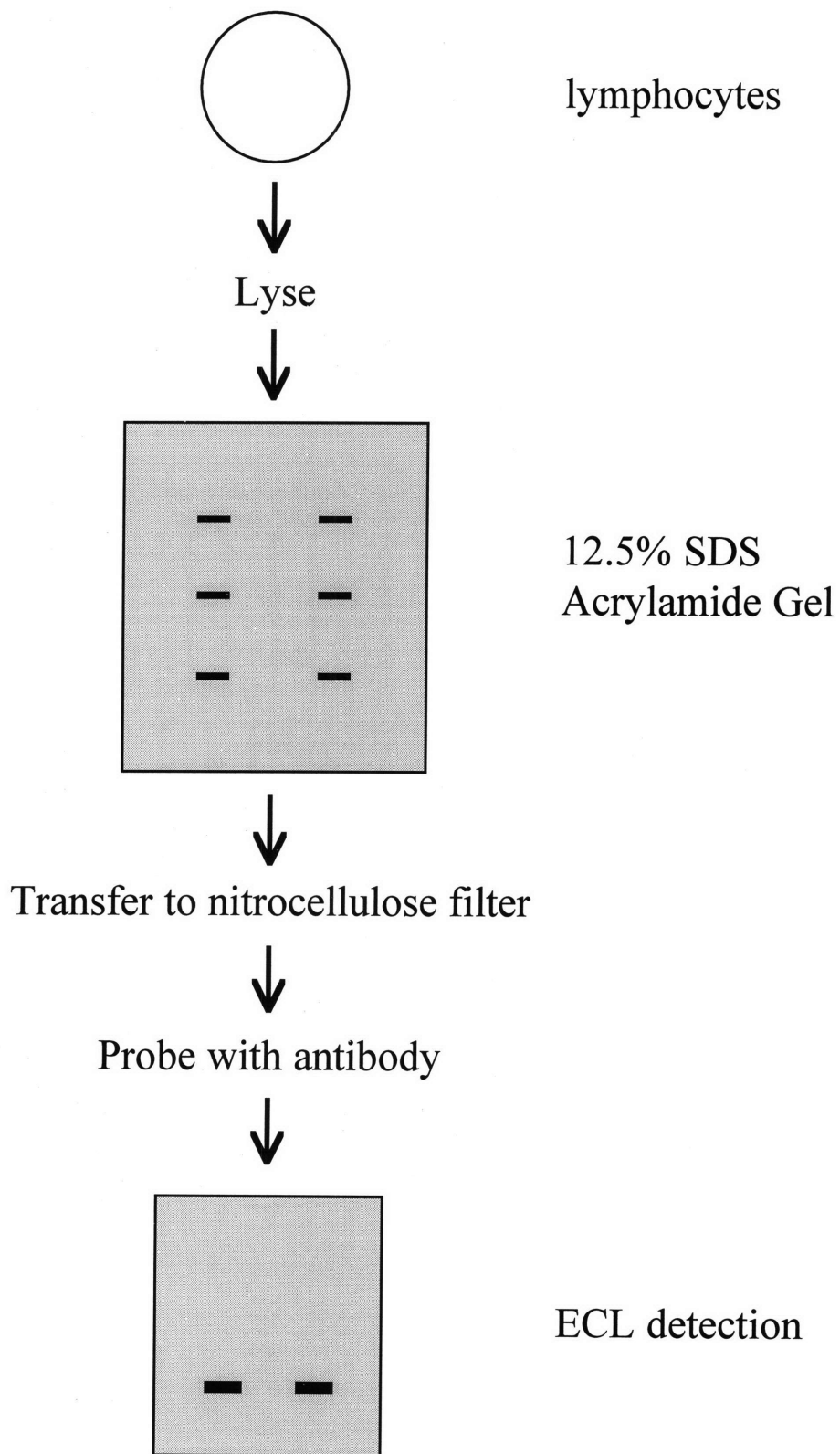


Figure 3. Schematic of Western Blot Analysis

RESULTS

Immunohistochemistry:

The rabbit anti-human CD3 antibody and the mouse anti-human CD79 α antibody recognize ferret T and B lymphocytes, respectively. A similar staining pattern was observed in both a human lymph node and in the ferret lymph node. CD3 positive cells were identified in the cortical interfollicular region, the site of T lymphocytes. Few CD3 positive cells could be found in the follicles, the site of B lymphocytes. CD79 α positive cells were identified in the lymph node follicles, but not in the cortical interfollicular region. In the ferret spleen, CD3 positive cells were identified in the peri-arteriolar lymphoid sheath (PALS), a T lymphocyte rich zone. CD79 α positive cells were identified in the splenic follicles, a B lymphocyte rich zone. In the thymus, the majority of the cells were CD3 positive, and CD79 α negative. (Table 2) (Figure 4)

Flow Cytometry:

Four antibodies (goat anti-ferret IgG, OKT8, rabbit anti-human kappa/lambda light chain immunoglobulin) were identified which were capable of detecting viable ferret peripheral blood lymphocytes by flow cytometry. Blood samples from a group of 10 ferrets were analyzed numerous times over a twelve month period. Percentages of lymphocyte subsets remained consistent within an individual ferret over time. Ferret specific IgG will label 25-40% of ferret peripheral blood lymphocytes. OKT8 will label 15-25% of ferret peripheral blood lymphocytes which correlates with the percent of CD8+ lymphocytes seen in other species. Rabbit anti-human kappa light chain immunoglobulin and rabbit anti-human lambda light chain immunoglobulin label 10-20 % of ferret peripheral blood lymphocytes, respectively. (Figure 5&6)

Kappa:Lambda Ratio:

The number of lymphocytes positively labeled with kappa Ig in lymphoid tissue from normal healthy ferrets approximated the number of lymphocytes positively labeled with lambda Ig. The lamina propria of the ferret small intestine contains a diffuse population of lymphocytes which were detected by the kappa and lambda antibodies in equal numbers. Semi-quantitative analysis of the lymphocyte staining by immunohistochemistry resulted in a kappa:lambda ratio of 1.2:1. Quantitative analysis by flow cytometry resulted in a comparable kappa:lambda ratio of 1:1 which remained

Table 2: REACTIVITY OF CD3 AND CD79 α ANTIBODIES IN FERRET LYMPHOID TISSUE

	<u>LYMPH NODE</u>	<u>SPLEEN</u>	<u>THYMUS</u>
<u>CD3</u>	Cortical interfollicular zone	Peri-Arteriolar Lymphoid Sheath	Cortex & Medulla
<u>CD79α</u>	Follicles	Follicles	negative

**Figure 4: Normal Ferret Spleen labeled with CD3 (*top*)
Normal Ferret Lymph Node labeled with CD79 α (*bottom*)**

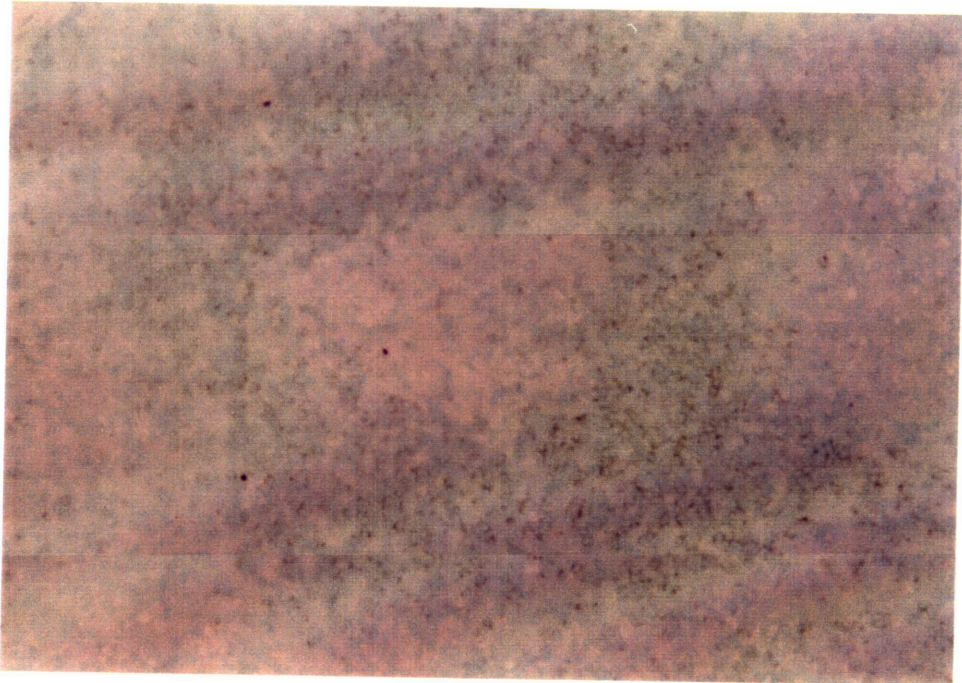
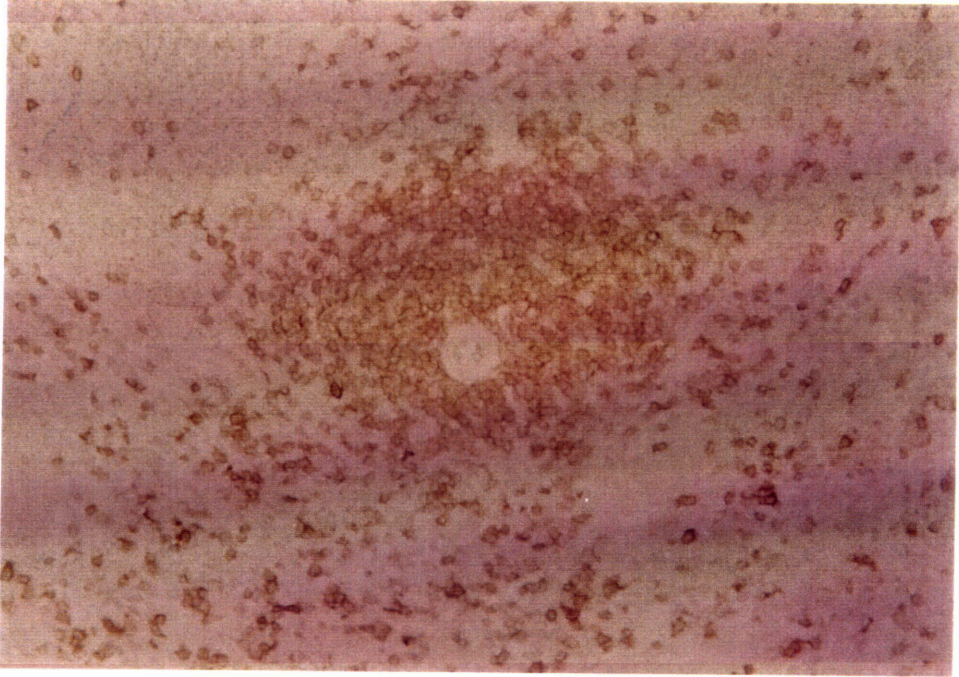
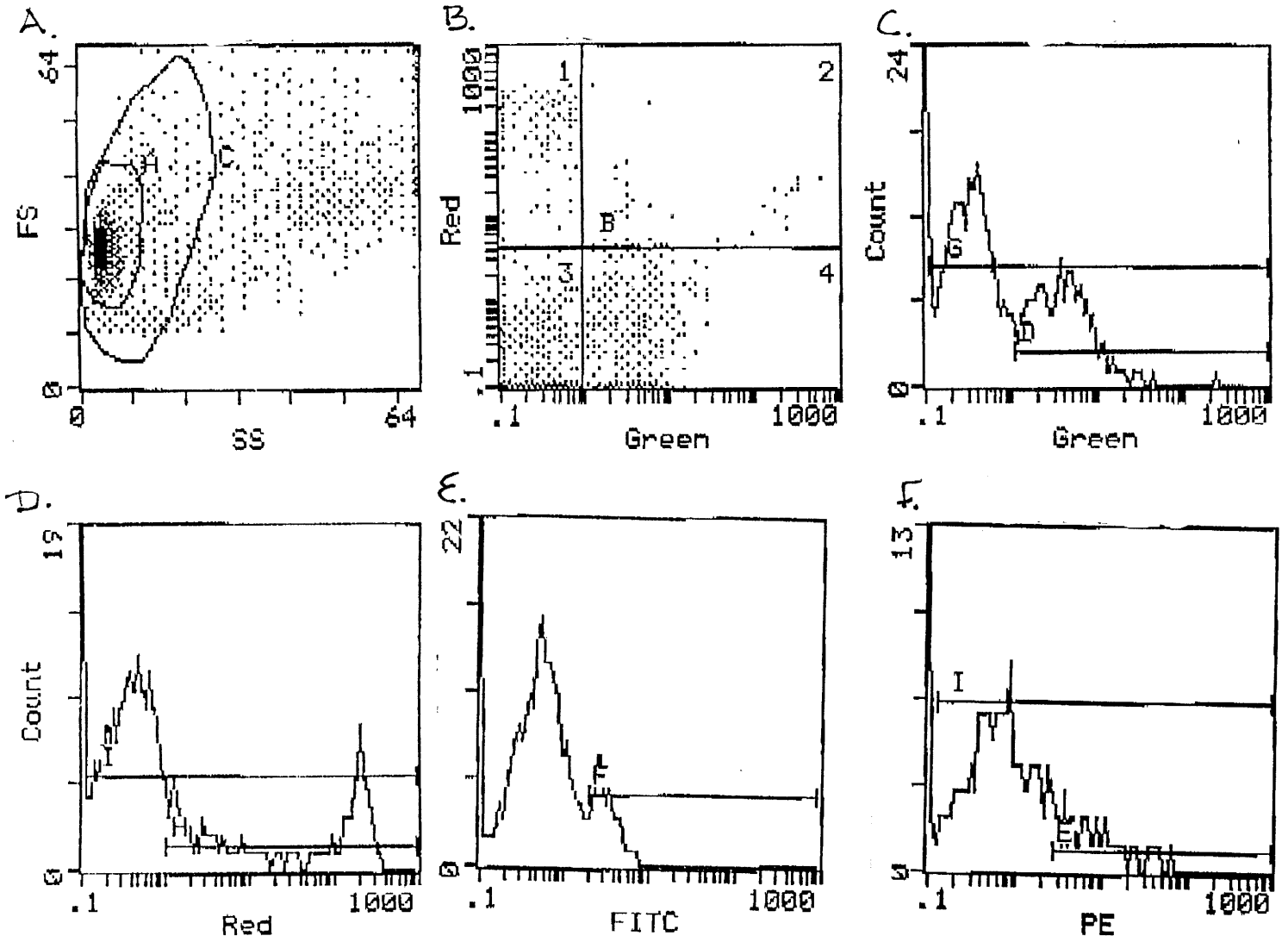


Figure 5: Flow Cytometric Results of IgG, OKT8, kappa Ig, and lambda Ig Reactivity with Ferret Peripheral Blood Lymphocytes



A:FS=forward scatter indicative of viability/SS=side scatter indicative of size & granularity
 B:Red/Green = cells labeled with either PE or FITC antibodies
 C:Lymphocytes labeled with FITC ferret IgG (34%)
 D:Lymphocytes labeled with PE OKT8 (18%)
 E:Lymphocytes labeled with FITC lambda (12%)
 F:Lymphocytes labeled with PE kappa (11%)

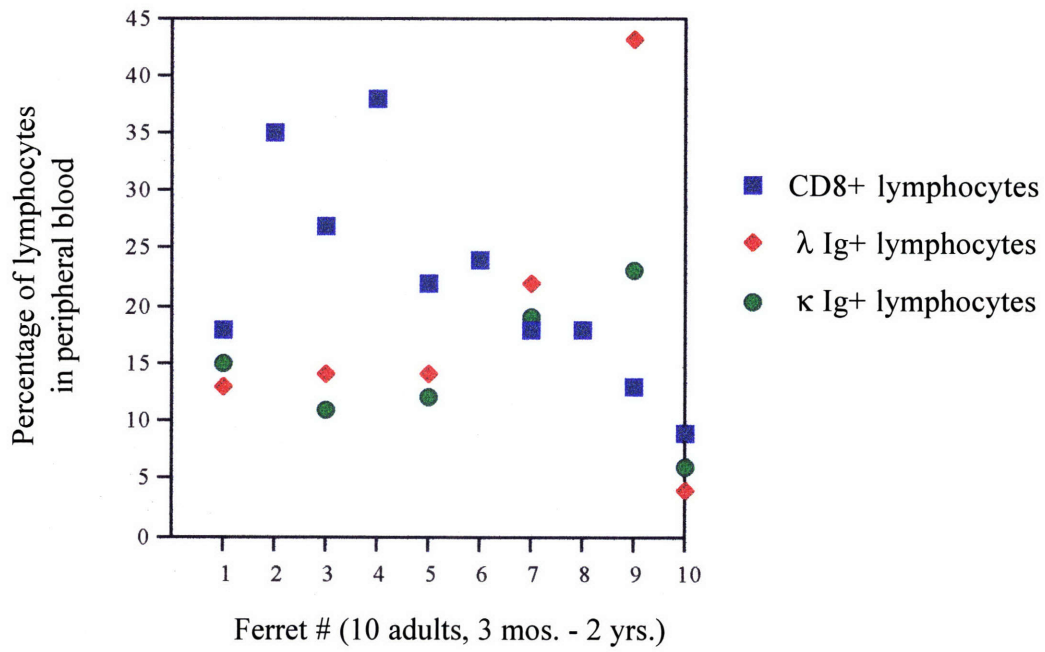
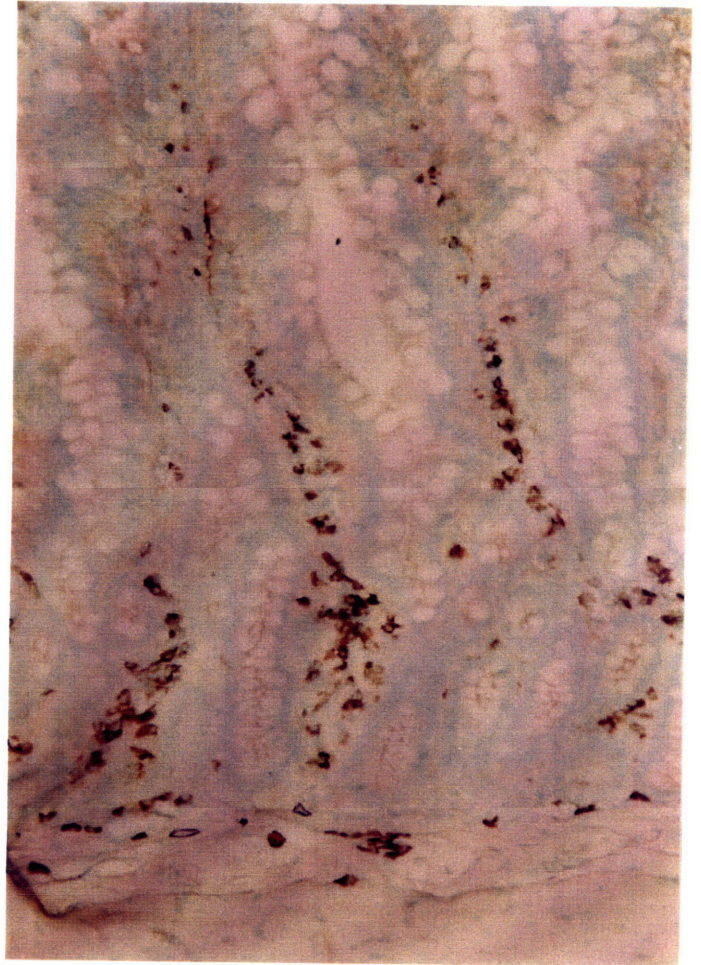
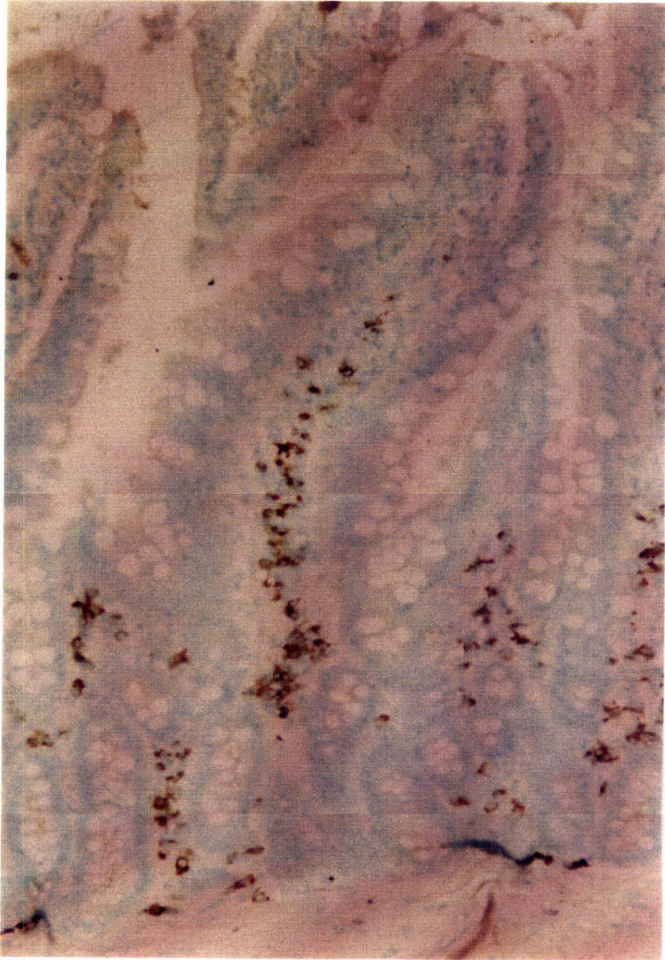


Figure 6. Mean percentage of lymphocyte subsets in individual ferrets sampled monthly.

**Figure 7: Normal Ferret Small Intestine labeled with Kappa Ig (*left*)
and labeled with Lambda Ig (*right*)**



consistent in several ferrets evaluated over a several month period. The double labeling flow cytometric experiment demonstrated that the antibodies are labeling consistently mutually exclusive populations of lymphocytes. Comparison of the kappa/lambda labeling with the ferret specific IgG illustrates that the combined positive kappa and lambda lymphocytes equals the percentage of lymphocytes labeled with IgG as expected. (Figure 7)

Western Blot Analysis:

Western blot analysis confirmed that the rabbit anti-human CD3 antibody is recognizing an antigen, approximately 20kD, on ferret lymphocytes which corresponds with the known molecular weight of the CD3 molecule (Figure 8) and the mouse anti-human CD79 α is recognizing an antigen of 47kD corresponding with the known molecular weight of the mb-1 antigen of the B cell receptor. (Figure 9)

DISCUSSION

The CD3 antigen is a complex of 5 polypeptides ($\gamma, \delta, \epsilon, \xi, \eta$), of approximately 20 kDa. (52) The CD3 molecule is structurally and functionally associated with the T-cell receptor (TcR), a 90 kD disulfide linked heterodimer (α/β) or (γ/δ). (Figure 10) The CD3/TcR complex is important in signal transduction, and recognition of foreign antigen. The CD3 molecule is a transmembrane molecule with long cytoplasmic extensions that are able to interact with cytosolic protein tyrosine kinases. A cascade of intracellular events is initiated resulting in cellular activation. (31) Expression of the CD3 antigen is unique to T-lymphocytes, although weak expression by purkinje cells in the cerebellum has been described. (53) Cytoplasmic expression of the CD3 antigen early on in T-cell maturation has been described (54). Mature T-lymphocytes express CD3 on their surface. It is well documented that CD3 continues to be expressed in many T-cell neoplasms. (55,56) Dako's polyclonal rabbit anti-human CD3 antibody recognizes a conserved intracytoplasmic epitope on the ϵ subunit, believed to be the most immunogenic of the 5 associated polypeptides. (57)

All mature T cells express CD3 in conjunction with the T cell receptor. Additionally, all mature T cells express co-receptor molecules, either CD4 or CD8. These co-receptor molecules aid in the affinity of binding between an antigen and the CD3/TcR molecules. (52) CD4 and CD8 play an important role in the recognition of the major histocompatibility complex (MHC). (31) CD4 is expressed by a subset of T cells, termed

Figure 8: Western Blot - Ferret Lymph Node labeled with anti-CD3

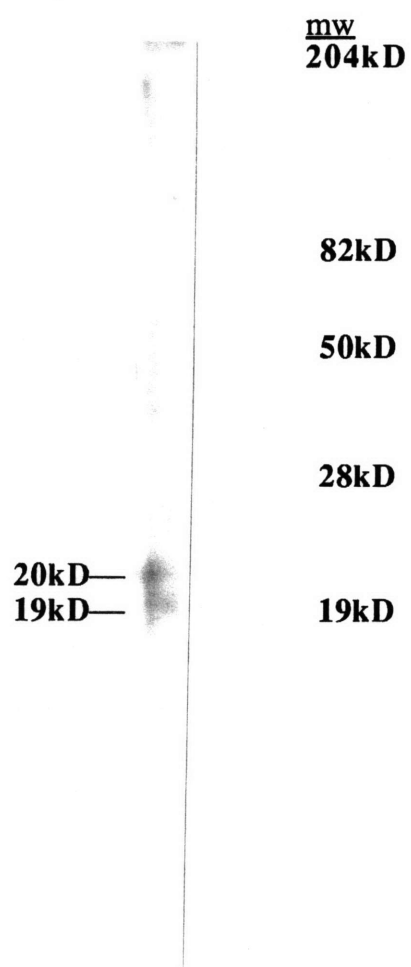
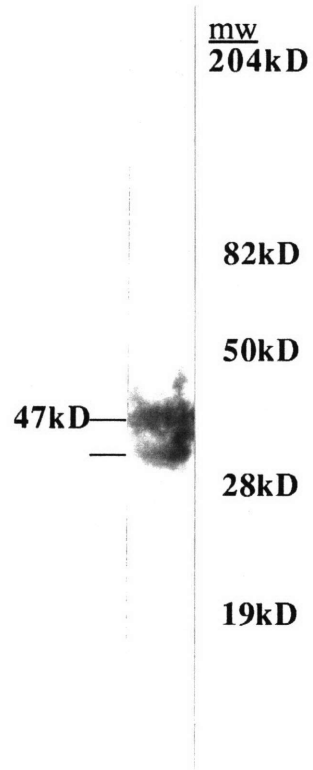


Figure 9: Western Blot - Ferret Lymph Node labeled with anti-CD79 α



“helper” T cells. CD4 is MHC, class II restricted. The expression of MHC class II molecules is generally limited to B cells and macrophages. The function of CD4 is to

activate B cells and macrophages to either produce immunoglobulin or to induce phagocytosis and killing, respectively. Structurally, CD4 is a 55 kDa membrane glycoprotein composed of four immunoglobulin-like domains. The cytoplasmic domain plays a role in signal transduction by interacting with intracellular tyrosine kinases. CD4 binds the MHC molecule at a site distinct from the site bound by the CD3/TcR complex, thereby increasing the binding specificity and avidity for a specific antigen. (31)

CD8 is expressed by a subset of T cells, termed either “suppressor” T cells or “cytotoxic” T cells. CD8 is MHC, class I restricted. MHC class I expression is more widespread than that of class II expression. The majority of nucleated cells express MHC class I molecules, although differential expression of MHC I exists dependent on the cell type. MHC I molecules process and present short peptides from cytosolic pathogens on their cell surface. The function of CD8 is to kill any cell which is expressing these foreign peptides. Structurally, CD8 is a 32 kDa membrane glycoprotein consisting of a disulfide linked heterodimer. Each chain of the heterodimer contains a single immunoglobulin-like domain. Analogous to the CD4, the cytoplasmic domain of the CD8 molecule plays a role in signal transduction by interacting with intracellular tyrosine kinases. CD8 binding increases the affinity of binding with viral or other intracytosolic antigens that are MHC, class I restricted. (31,52)

OKT8, a human monoclonal antibody specific for the CD8 antigen, has been shown to recognize the CD8 antigen in mink. (58) We have done preliminary work with this antibody in ferrets. OKT8 recognizes a subset of ferret peripheral blood lymphocytes by flow cytometry. The antibody consistently recognizes 15-25% of circulating ferret peripheral blood lymphocytes which correlates with the percent of CD8+ lymphocytes seen in other species. (31) The OKT8 antibody was not used in any of the retrospective studies on ferret lymphoproliferative disease because it can not be used on formalin-fixed paraffin-embedded tissues. This antibody will be useful on any prospective or ongoing studies that evaluate lymphocyte subsets in the ferret. To date, however, we do not have an antibody which will recognize the complementary CD4+ T-lymphocyte subset in ferrets.

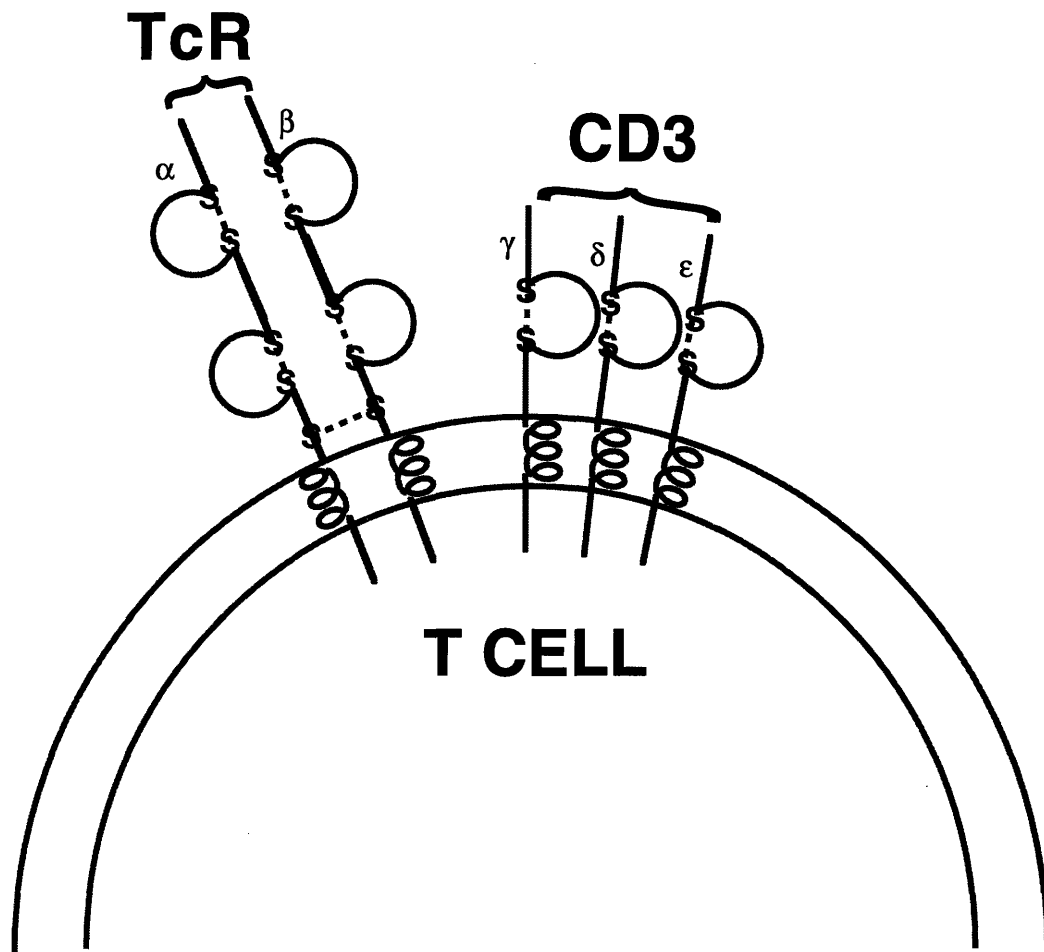


Figure 10. Structural model of CD3/T-cell receptor complex.
(Adapted from Kuby, 1992)

B lymphocytes express cell-surface immunoglobulins. Surface immunoglobulins are associated with a heterodimer comprised of an alpha and beta chain that are the products of the mb-1 gene and the B29 gene respectively. (59-62) (Figure 11) The mb-1 and B29 molecules comprise the B cell antigen receptor complex which is involved in signal transduction analogous to the CD3/TcR complex of T cells. (63) The CD79 α , or mb-1, antigen is a polypeptide of approximately 47 kDa and its expression is unique to all B cells. (60) Mb-1 is expressed early on in B cell development and continues to be expressed through the terminally differentiated plasma cell stage. (61,62) It has been reported that mb-1 expression persists in the plasma cell stage, when surface immunoglobulins can no longer be detected. Mb-1 plasma cell expression is typically cytoplasmic. (60) Dako's mouse anti-human CD79 α antibody recognizes a conserved intracytoplasmic epitope on the C-terminus of the mb-1 polypeptide of the B cell receptor.

Surface immunoglobulins are comprised of four polypeptides, two heavy chains and two light chains. There are two types of light chains, kappa and lambda. Each immunoglobulin molecule expresses only one type of light chain, either kappa or lambda. (Figure 12) (31, 64) Expression of either kappa or lambda is determined early on in B cell differentiation. There is no known functional difference between the two light chains. Expression of the light chains is variable between species but consistent within a species. (65,66) Antibodies have been developed which are specific for either the kappa or lambda light chains. These antibodies are very useful in the differentiation of lymphoproliferation and lymphoma. (67-71) In most species, a given population of B cells will randomly express a mix of kappa and lambda immunoglobulins. Overexpression of one light chain is indicative of a clonal expansion of one immunoglobulin suggestive of malignancy.

Immunoglobulin diversity is achieved via a sequence of gene rearrangements which begin in the immature B cell stage. (31, 72) (Figure 13) Gene rearrangement of both the heavy and light immunoglobulin chains occurs. The heavy chain consists of a variable region (V_H), a diversity region (D), and joining region (J_H) all of which will recombine to form a complete variable region which will bind with the constant region. There are 200-1000 variable gene segments, fifteen diversity gene segments, and four joining gene segments which can yield an extraordinary number of unique immunoglobulin heavy chain gene rearrangements. In the pro-B cell stage one D segment will pair with one J_H segment on both chromosomes. Subsequently, one V_H segment will join with the DJ_H complex on

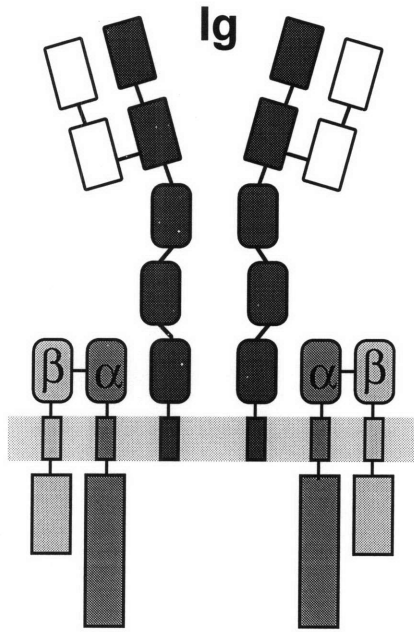


Figure 11. Structural model of B-Cell antigen receptor complex.
(Adapted from Reth, 1995)

B Lymphocytes

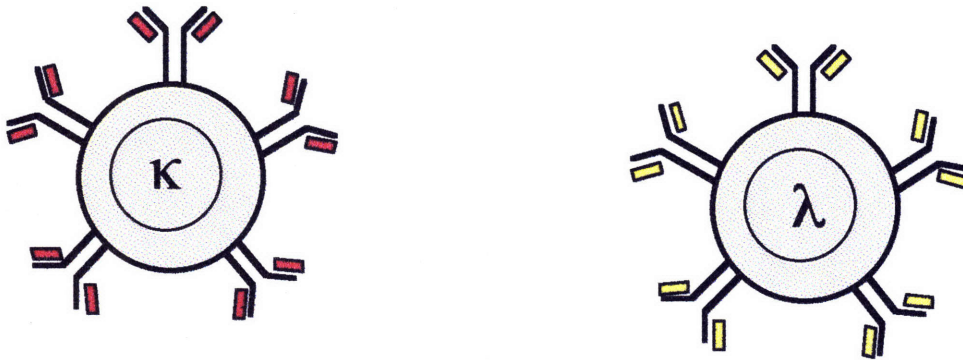


Figure 12. Selective expression of Kappa and Lambda light chain immunoglobulins.

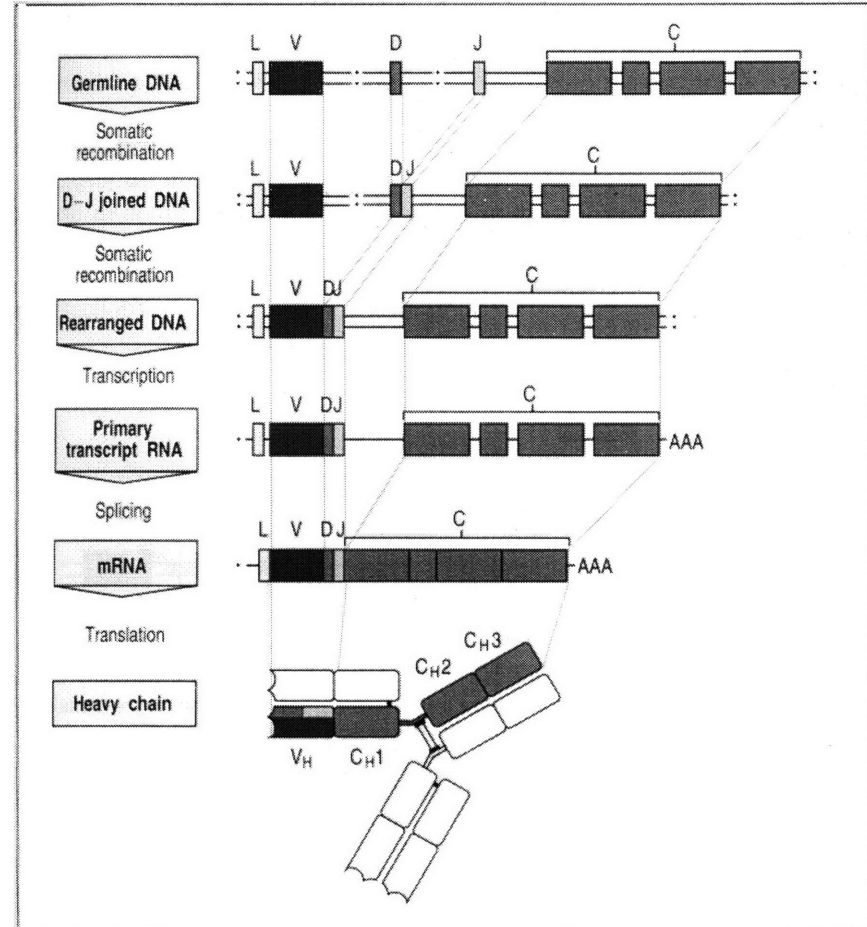
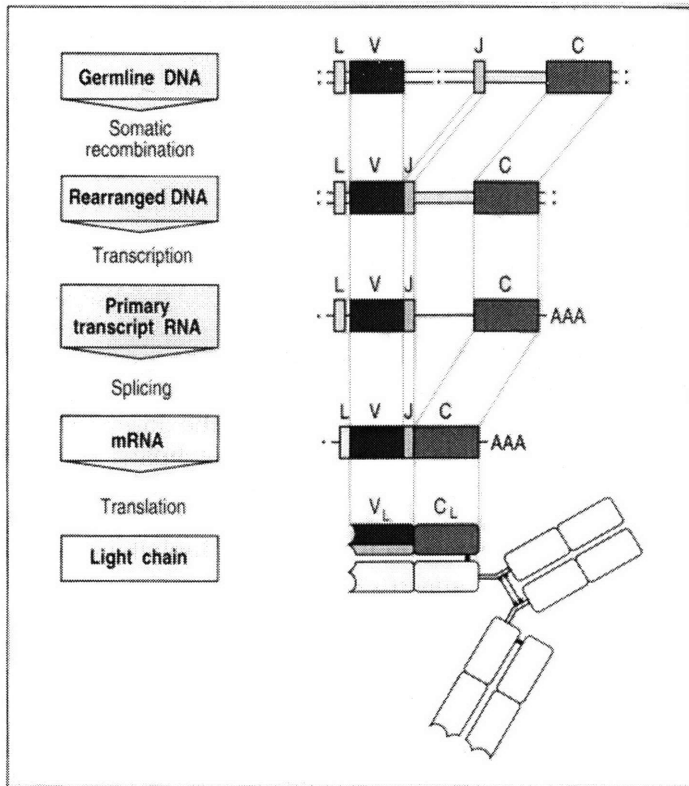


Figure 13. Schematic of light chain Ig and heavy chain Ig gene. Rearrangement to achieve Ig diversity. (Janeway and Travers, 1994)

one chromosome. Therefore, by the pre-B cell stage heavy chain rearrangement has occurred resulting in a unique V_HDJ_HC complex. Light chain rearrangement occurs in the pre-B cell stage. The light chain consists of a variable region (V_L), and a joining region (J_L) which recombine to form the complete variable region which binds with the constant region. In the mouse, the kappa light chain consists of 250 variable gene segments and four joining segments. The lambda light chain consists of only two variable gene segments and three joining segments. In general, the kappa light chain will rearrange first. If unsuccessful, the lambda light chain will rearrange. Final rearrangement of the light chains is antigen dependent. The immature B cell will express IgM. Further changes in immunoglobulin specificity can occur once an antibody recognizes an antigen via a mechanism called somatic hypermutation. Eventually a B cell will differentiate into a plasma cell capable of secreting large amounts of specific antibody.

Immunoglobulin diversification results in numerous B cells that can recognize a vast array of antigens. The gene rearrangement of a B cell should be unique. In an individual, B cell genomic expression tends to be polyclonal. B-cell tumors however, are the result of the clonal expansion of a single neoplastic B cell. This results in numerous copies of a single rearranged B cell which can be detected. As a result, a normal B cell response should be polyclonal, ie. a mix of B cells with different gene rearrangements. An abnormal B cell proliferation, or a B cell neoplasm, will be monoclonal, ie. all B cells will have the identical gene rearrangement.

Kappa and lambda light chain antibodies are a very useful tool for the study of lymphoproliferative diseases in humans. (67-71) These antibodies take advantage of the concept that an individual should normally express roughly equal numbers of kappa and lambda B cells. If a clonal shift is detected, ie. an excess of one light chain over the other, then the lymphoproliferation can be considered to be monoclonal which is suggestive of a neoplasm. This kappa/lambda tool is only useful in those species that normally express an equal number of kappa and lambda B cells. Expression of kappa and lambda is highly variable between species. In the mouse, kappa expression far exceeds lambda expression at a ratio of $20\kappa+:1\lambda+$ B cells in the circulation and in the tissues. In cows, the opposite is true. Lambda expression far exceeds kappa expression. (65) As a result, kappa/lambda analysis is not very useful in these species. Normal lymphoproliferative responses will appear monoclonal. In order to determine if these tools would be useful in the ferret, the

normal kappa:lambda ratio had to be determined. This ratio was determined by analyzing circulating peripheral blood lymphocytes by flow cytometry and by analyzing the distribution of kappa and lambda lymphocytes in situ by immunohistochemistry assays. The ferret kappa:lambda ratio was determined to be 1.2:1 which is similar to that seen in humans. Thus, kappa/lambda analysis in the ferret is potentially a very useful diagnostic tool. (Figure 14)

The phenotypic characterization of cell populations can be accomplished by numerous techniques. There are advantages and disadvantages to all techniques. No single technique can be employed to assess the characteristics of a cell population. Certain techniques allow one to study populations of cells in situ which provides important information with regards to the interactions of different cell populations. One can also isolate cells and study suspensions of cells to evaluate molecular parameters as well as phenotypic expression of cellular markers.

Immunohistochemistry is an effective means to phenotype cells in situ. The technique is based on the premise that antibodies are specific for an antigen, and that the antigen-antibody binding is stable with relatively high affinity for its target. This affinity allows the antibody to find its target amongst many hundreds of antigens. (31) The binding is stable enough to survive repeated washings which enables one to utilize a sandwich technique common to many immunologic methods. The general principle entails a primary antibody which recognizes the antigen of interest. A secondary antibody is used which is capable of recognizing the primary antibody. The secondary antibody is usually labeled with a tag that can be recognized by a substrate which can undergo a color change thereby allowing the visual detection of the cell of interest. In these studies, formalin-fixed, paraffin-embedded tissue was studied by markers capable of recognizing broad classes of lymphocytes.

Flow cytometry is an effective means to phenotype viable suspensions of cells and to sort cells into specific populations. Flow cytometry allows one to study the properties of individual cells (31). The basic premise behind flow cytometry is that cells which have been labeled with a fluorescence marker are passed in single file in front of a laser beam. The cells can be sorted based on several different parameters. The flow cytometer detects fluorescence of several different wavelengths so that a population of cells can be labeled with different colored antibodies (ie. red vs. green). The flow cytometer is capable of detecting the forward light scatter given off by the cell as it passes in front of the laser

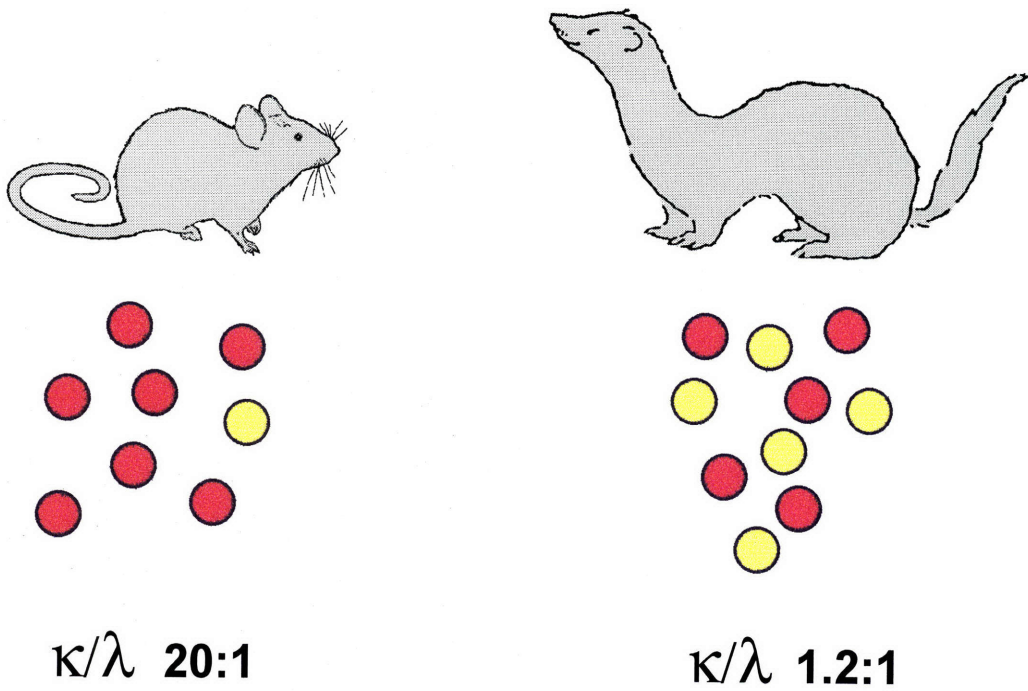


Figure 14. Variable $\kappa:\lambda$ ratios between species.

beam. Forward light scatter allows one to distinguish between dead and viable cells. Dead cells will scatter less light than a viable cell. Cells can be sorted into different populations based on cell size and cell granularity which is important when analyzing leukocytes. Polymononuclear cells are medium in size and are very granular. Macrophages and monocytes are large with low granularity. Lymphocytes are medium in size with low granularity. Therefore, the primary advantage of flow cytometry is that a population of cells, ie. leukocytes can be labeled with one or several monoclonal antibodies specific to a subset of the cell population, ie. B lymphocytes vs. T lymphocytes and then sorted based on viability, size, and antibody labeling. (73) Flow cytometry is extremely sensitive as it can identify as few as 5-10% of the cells in a given population. (73) The advantage of flow cytometry is a rapid analysis of individual cells. The disadvantage is the lack of information with regards to the interaction of that cell with other cells, which is critical to understanding disease pathogenesis.

Not all antibodies are created equal! Not all antibodies can be used for all immunologic techniques. More often than not, an antibody which is useful in an immunohistochemistry assay has limited applications in flow cytometry and vice-versa. The location of the epitope recognized by the antibody is critical to the type of immunoassay used. For example, an antibody which recognizes an intracellular epitope can not be used on living cell suspensions, and therefore is not a good choice for use by conventional flow cytometry. (74) In contrast, many of the antibodies we use on formalin fixed tissue for immunohistochemistry do recognize an intracellular epitope. In this case, an intracellular epitope is critical as it is able to survive the fixation process and remain in its native conformation.

The antibodies described were selected for evaluation against ferret lymphoid tissue for several reasons. All five antibodies recognize conserved epitopes which increases the likelihood of species cross-reactivity. The CD3 and CD79 α antibodies have been demonstrated to recognize the appropriate antigen in normal and neoplastic tissue in numerous species. The four antibodies (CD3, CD79 α , kappa, and lambda) which have demonstrated ability to recognize the correct antigen in formalin-fixed paraffin-embedded ferret tissues are extremely useful as tissue architecture is preserved enabling accurate *in situ* assessment of lymphoid distributions, as well as enabling one to perform retrospective studies and evaluate archival tissues. (75)

A retrospective analysis of three permutations of lymphoma in the ferret was performed with the use of these T-cell and B-cell markers. Rabbit anti-human CD3, mouse anti-human CD79 α , and rabbit anti-human kappa and lambda light chain immunoglobulin antibodies were used to characterize the lymphoid infiltrate in a peracute syndrome of mediastinal lymphoma in young ferrets; an aggressive form of ocular lymphoma in two adult ferrets; *Helicobacter mustelae* associated chronic lymphocytic gastritis and gastric MALT lymphoma in adult ferrets.

Immunophenotypic Characterization of Spontaneous Lymphoma in Young Ferrets

INTRODUCTION

Naturally occurring lymphoma is a well documented and common neoplasm of ferrets (*Mustela putorius furo*). (2-5) Two distinct syndromes have been described. Young ferrets tend to exhibit a peracute disease course, characterized by respiratory distress secondary to thymic enlargement. Microscopic evaluation of the mediastinal mass is consistent with the National Cancer Institute (NCI) classification of either an immunoblastic or a small, non-cleaved lymphoma. (2,3,5) Clinically, this syndrome is consistent with that seen in children and in young cats with mediastinal lymphoblastic lymphoma (41-43) In contrast, aged ferrets tend to develop lymphoma that is more variable in its presentation, and is often characterized clinically by a chronic course of disease. These ferrets develop generalized lymphadenopathy and splenomegaly. (2,3) Histologically, many of these neoplasms are more pleomorphic than those seen in the young ferrets. (3)

Immunophenotypic characterization of the lymphomas in young ferrets is critical to further understanding of the disease in ferrets, and for further evaluation of the ferret as a model of human disease. In cats, which have been suggested as a model of juvenile-onset lymphoma, and in children, primary alimentary lymphomas are predominantly of a B-cell phenotype, whereas those which originate in the mediastinum are generally of T-cell origin. (42,44,76-78) Lymphoblastic lymphoma and small non-cleaved lymphoma, common in children and young cats, are typically very aggressive clinically and histologically. (42,43,76) There is speculation that many of the juvenile onset lymphomas are virally-induced. In cats, Feline Leukemia Virus (FELV) is linked with mediastinal T-cell lymphoma. (76,77) In children, Epstein-Barr Virus (EBV) has been linked with Burkitt's lymphoma, a B-cell tumor of the abdomen, head and neck. (41,42,79) Similarly, a viral etiology to spontaneous lymphoma in ferrets has been proposed. (22)

Classification of the ferret lymphomas as either T-cell or B-cell in origin is helpful in understanding pathogenesis, and possible etiologies of the disease. Numerous cell surface-associated lymphoid antigens have been identified and subsequently exploited as markers of T and B lymphocytes. Polyclonal and monoclonal antibodies specific for these lymphoid antigens have been developed in order to study lymphocyte distribution in normal and diseased conditions of humans and of several animal species. In the ferret, however,

few lymphoid specific markers exist. As a result, ferret lymphoma has not been adequately characterized with regards to T-cell and B-cell distribution.

Two commercially available cell surface lymphoid antigen markers, polyclonal rabbit anti-human CD3 and monoclonal mouse anti-human CD79 α , were found to be useful in differentiating T and B lymphocytes, respectively, in ferret lymphoid tissue, in situ. The markers were characterized initially on normal ferret lymphoid tissue to determine specificity and to delineate normal T and B lymphocyte distribution. Subsequently, tissues from 10 young ferrets with a histologic diagnosis of lymphoma were typed by immunohistochemistry and classified as T-cell or B-cell in origin.

MATERIALS & METHODS

Animals:

Tissue from ten young ferrets (<18 months) with clinical and pathologic evidence of spontaneously occurring lymphoma was evaluated immunophenotypically. Four ferrets (3 pets and 1 research animal) were referred to MIT's Division of Comparative Medicine for a complete diagnostic evaluation. Four ferrets were pets which were diagnosed by the referring veterinarian and had representative post-mortem tissue samples sent to the MIT Division of Comparative Medicine's diagnostic lab for histopathologic evaluation. Two ferrets were part of the MIT research colony specific pathogen-free for *Helicobacter mustelae*, *Campylobacter* spp., *Salmonella* spp., *Giardia* sp., *Coccidia*, and *Aleutian Disease Virus* and were maintained in an AAALAC approved facility. Clinical pathology was determined on eight ferrets. ADV status based on serology was determined on eight ferrets by immunofluorescent antibody (MIT) and by counter-immunoelectrophoresis (United Vaccines, Harlan Sprague Dawley, Inc., Madison, Wisconsin) as previously described. (22) Result were confirmed by a polymerase chain reaction based assay. (4) Eight ferrets were tested for FELV by a p27 antigen enzyme-linked immunosorbent assay (Leukassay FII, Pitman-Moore, Washington Crossing, New Jersey). FELV status was confirmed by PCR. (4) All ferrets were humanely euthanized due to poor clinical prognosis. Complete necropsy was done on all ferrets either by the referring veterinarian or by the MIT pathology group.

Tissue:

Tissue from all ferrets was trimmed, thinly sectioned (5 μ m) and processed for haematoxylin & eosin (H&E) staining and for immunohistochemical staining. Tissue for

immunohistochemical staining was placed on coated slides (Fisherbrand Colorfrost/Plus), and stored at room temperature until processed. A histopathologic diagnosis of lymphoma was made based on H&E staining. Lymphomas were classified according to the NCI's working formulation of non-Hodgkin's lymphoma (40). The lymphomas were graded based on the size of the neoplastic cell and on the mitotic index as previously described (4). Briefly, the nuclear diameter of the neoplastic cell is compared to the diameter of a red blood cell (RBC). A small cell lymphoma consists of neoplastic cells with a nuclear diameter equivalent to one RBC; a medium cell lymphoma consists of cells with a nuclear diameter equivalent to two RBC; and large cell lymphoma consists of cells with a nuclear diameter equivalent to three RBC. A low mitotic index was defined as <3 mitoses/high power field (hpf); an intermediate mitotic index equated with 3-8 mitoses/hpf; a high mitotic index had >8 mitoses/hpf.

Antibodies:

Polyclonal rabbit anti-human CD3 (DAKO Corporation, Carpinteria, CA), monoclonal mouse anti-human CD79 α (DAKO), and polyclonal goat anti-ferret IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) were used on formalin fixed paraffin-embedded ferret lymphoid tissue. The CD3 antibody is directed against a conserved intracellular epitope of the CD3 epsilon chain and has been shown to cross-react with many species. The CD79 α antibody is directed against a conserved intracellular epitope of the B cell antigen receptor complex, and has also been shown to cross-react with many species. The IgG antibody is specific for ferret heavy and light chain immunoglobulin.

Antibody Specificity:

Lymph node, spleen, and thymus from 5 clinically normal ferrets were processed for immunohistochemistry to evaluate the affinity of the rabbit anti-human CD3 and the mouse anti-human CD79 α antibodies for ferret tissue. Non-immune rabbit serum and non-immune mouse serum were used as antibody controls. Human lymph node was used as a positive tissue control for both the rabbit anti-human CD3 antibody and the mouse anti-human CD79 α antibody.

Immunohistochemistry:

Slides were warmed to 56C for 30 minutes and subsequently deparaffinized and rehydrated in a series of xylene and ethanol baths at 25C. All subsequent manipulations

were carried out at room temperature. For the CD3 and IgG antibodies, endogenous peroxidases were inhibited by incubation in Methanol and 3% H₂O₂ for 20 minutes. Slides were washed with Tris-buffered saline, pH 7.4 (TBS), then incubated in pronase (200u/50ml TBS) for 10 minutes, washed extensively in H₂O, and then in TBS. For the CD79 α antibody, slides were processed by a heat-based antigen retrieval method. (42) Slides were immersed in Citrate buffer, pH 6.0, and boiled 2X for 5 minutes in a microwave oven. Endogenous peroxidases were inhibited by incubation in Methanol, 0.3% H₂O₂, and 0.1% sodium azide. Slides were subsequently washed in TBS. The primary antibody (CD3, CD79 α , IgG, non-immune rabbit serum, non-immune mouse serum, or non-immune goat serum) was applied at a 1:100 dilution for 60 minutes. The slides were washed in TBS and the secondary antibody (biotinylated goat anti-rabbit IgG, biotinylated rabbit anti-mouse IgG, or biotinylated rabbit anti-goat IgG) was applied at a 1:100 dilution for 30 minutes. Again, the slides were washed in TBS, and streptavidin-horseradish peroxidase applied for 30 minutes. Diaminobenzidine was used as the color substrate. Slides were allowed to air dry and were cover-slipped for subsequent microscopic evaluation.

Western Blot Analysis:

To confirm that the rabbit anti-human CD3 antibody recognizes ferret T cells and that the mouse anti-human CD79 α antibody recognizes ferret Bcells, western blot analysis was performed. Fresh lymphocytes (10⁸) were isolated from the thymus, spleen, and lymph nodes from healthy ferret donors. Cells were lysed in RIPA Lysis Buffer and run on a 12.5% acrylamide gel. The proteins were electrophoretically transferred to a nitrocellulose filter over 4 hours. The filter was blocked in 10% nonfat milk and 1% bovine serum albumin (Sigma) overnight at 4C, washed 3X in PBS/Tween and then incubated with the primary antibody (CD3 or CD79 α) for 1 hour at room temperature. The filter was washed 3X in PBS/Tween prior to incubation with the secondary antibody (peroxidase labeled anti-rabbit IgG or peroxidase labeled anti-mouse IgG) for 1 hour at room temperature. The filter was then washed 6X in PBS/Tween prior to detection via ECL system (Amersham Life Science

RESULTS (Table 3)

Clinical Presentation:

Ten young ferrets, with clinical, gross pathological, and microscopic evidence of lymphoma, were evaluated. The age of the ferrets at clinical presentation ranged from 6-18 months. The gender of the ferrets was equally split between males (5) and females (5). Six of the ten ferrets presented with an acute onset of dyspnea. Clinical signs were otherwise non-specific including lethargy, anorexia, and weight loss. Three exhibited a mild hindlimb ataxia. One presented peracutely and was found moribund.

Clinical Pathology & Serology:

Seven of the eight ferrets on which clinical pathological data was available had evidence of atypical circulating lymphocytes; five had an elevation in the absolute lymphocyte count. Five of the ferrets demonstrated a normocytic, normochromic anemia. Aleutian Disease Virus status based on serology and PCR was negative on all ferrets for which the data was available. Feline Leukemia Virus status based on serology and PCR was negative on the eight ferrets for which the data was available.

Gross Pathology:

A mediastinal mass was identified in nine of ten ferrets. Of these nine ferrets, seven had widespread disseminated lymphoma involving numerous organ systems. Two of the nine ferrets had lymphoma limited to the thymus and local lymph nodes. The one ferret which did not have a mediastinal mass had generalized abdominal lymphoma. The six ferrets which presented clinically with dyspnea all had large neoplasms in the mediastinum.

Histopathology:

All ten ferrets had a lymphoma which was characterized by an infiltrative, uniform population of round cells arranged in sheets with occasional fibrovascular trabeculae. In most cases, normal architecture was effaced by these round cells. Mitotic index was moderate in all ten cases. Half of the cases (5/10) were consistent with immunoblastic lymphoma (IB) as determined by the NCI classification system of lymphoid lesions. These lymphomas consisted of medium to large round cells with scant eosinophilic cytoplasm, single oval vesicular nuclei, and prominent basophilic nucleoli. Smaller lymphocytes, of normal morphology, were interspersed throughout the neoplasm as were larger cells resembling macrophages. The other 5/10 cases were consistent with small, non-cleaved lymphoma (SNC) according to the NCI classification system. These lymphomas were comprised of smaller round cells (approx - diameter) with a nucleus containing condensed irregular chromatin. A starry sky appearance was noted in these

cases consistent with a prominent macrophage infiltrative component. Three cases (2 immunoblastic and 1 small, non-cleaved lymphoma) were characterized by irregular, angular nuclei with numerous prominent nucleoli, and occasional multinucleated giant cells.

Immunohistochemistry:

The rabbit anti-human CD3 antibody and the mouse anti-human CD79 α antibody recognized ferret T and B lymphocytes, respectively. A similar staining pattern was observed in both the human lymph node and in the ferret lymph node. CD3 positive cells were identified in the cortical interfollicular region, the site of T lymphocytes. Few CD3 positive cells could be found in the follicles, the site of B lymphocytes. CD79 α positive cells were identified in the lymph node follicles but not in the cortical interfollicular region. In the ferret spleen, CD3 positive cells were identified in the peri-arteriolar lymphoid sheath (PALS). CD79 α positive cells were found in the splenic follicles. (Scattered IgG positive cells were seen along the periphery of the PALS region.) In the thymus, the majority of the cells were CD3 positive, and CD79 α negative.

Immunophenotypic characterization of the ten lymphoma cases revealed that nine were CD3+, CD79 α -, and IgG-, suggestive of a T-cell origin. (Figure 15) One case (ferret 5) was CD79 α +, and CD3-, suggestive of a B-cell lymphoma. (Figure 16) Antibody reactivity was evaluated in numerous tissues, including the mediastinal mass, lymph nodes, spleen, and liver from all ten cases.. The neoplastic lymphocytic infiltrate was extensive in all tissues with concurrent effacement of normal architecture. All tissues demonstrated strong antibody staining and were clearly positive for either CD3 or CD79 α reactivity. There were no detectable differences in the character, or intensity of the staining in any of the lymphomas, nor between the two classes of lymphoma, IB and SNC, that we observed in these young ferrets. All SNC lymphomas were CD3+ and CD79 α -, consistent with a T-cell phenotype. Of the five IB lymphomas, four were CD3+ and CD79 α -. One of the IB lymphomas was CD3- and CD79 α +, indicative of a B-cell lymphoma. All nine cases with gross evidence of a mediastinal mass were of a T cell phenotype. The one case

Table 3:CLINICAL, PATHOLOGICAL, & IMMUNOHISTOCHEMICAL FINDINGS IN 10 YOUNG FERRETS

Ferret	Age (months)	Gender	Clinical Presentation	Clinical Pathology	ADV Serology	Felv Serology	Gross Pathology	Histology	Immuno-histochemistry
1	12	F	Moribund	Anemia Lymphocytosis with Atypia	Neg	Neg	Mediastinal Mass Hepatomegaly Splenomegaly Lymphadenopathy	SNC	CD3+/CD79-
2	6	M	Malaise Dyspnea Posterior paresis	Lymphocytosis with Atypia	Neg	Neg	Mediastinal Mass Hepatomegaly Splenomegaly Lymphadenopathy	SNC	CD3+/CD79-
3	12	F	Malaise Dyspnea	ND	ND	ND	Mediastinal Mass Hepatomegaly Splenomegaly	IB	CD3+/CD79-
4	7	F	Dyspnea	Atypical lymphocytes	Neg	Neg	Mediastinal Mass	IB	CD3+/CD79-
5	8	F	Malaise Posterior paresis	Anemia Lymphocytosis with Atypia	Neg	Neg	Hepatomegaly Splenomegaly Lymphadenopathy	IB	CD3-/CD79+
6	18	M	Malaise	Atypical Lymphocytes	ND	Neg	Mediastinal Mass Hepatomegaly Splenomegaly	IB	CD3+/CD79-
7	9	M	Malaise Dyspnea Posterior Paresis	Anemia Atypical lymphocytes	Neg	Neg	Mediastinal Mass Splenomegaly Lymphadenopathy	IB	CD3+/CD79-
8	6	F	Malaise Dyspnea	Anemia Lymphocytosis	Neg	Neg	Mediastinal Mass Hepatomegaly Splenomegaly Lymphadenopathy	SNC	CD3+/CD79-
9	9	M	Malaise Dyspnea	Anemia Lymphocytosis with Atypia	ND	Neg	Mediastinal Mass Splenomegaly Lymphadenopathy	SNC	CD3+/CD79-
10	9	M	Malaise	ND	ND	ND	Mediastinal Mass	SNC	CD3+/CD79-

Figure 15: Small, non-cleaved Lymphoma - T cell Phenotype

Top - H&E 100X

Left - CD3

Right - CD79 α

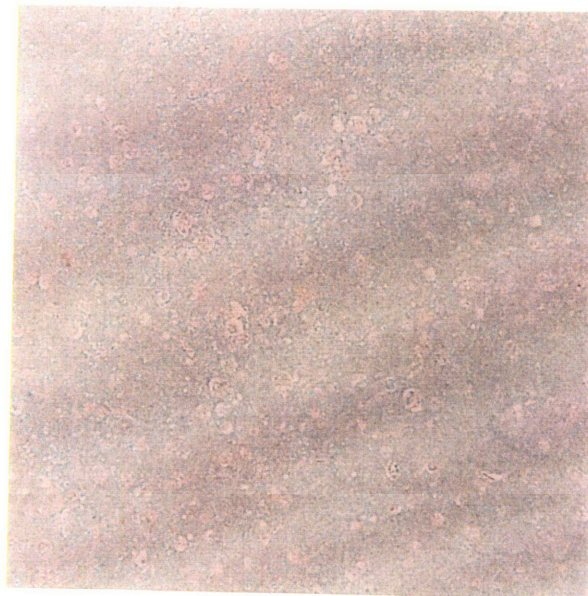
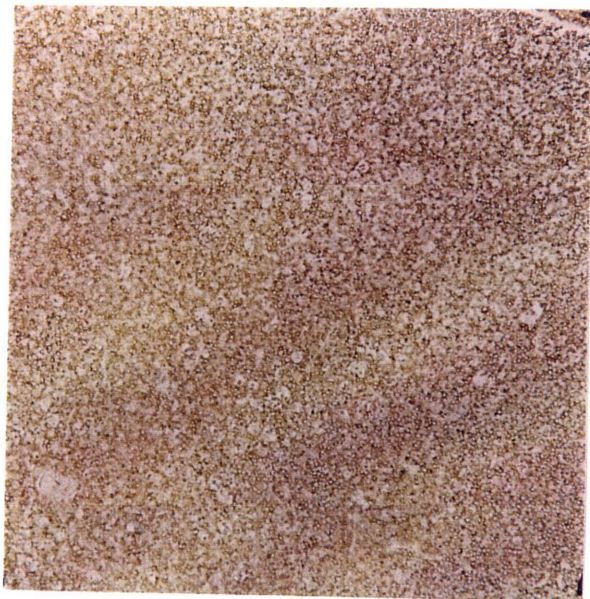
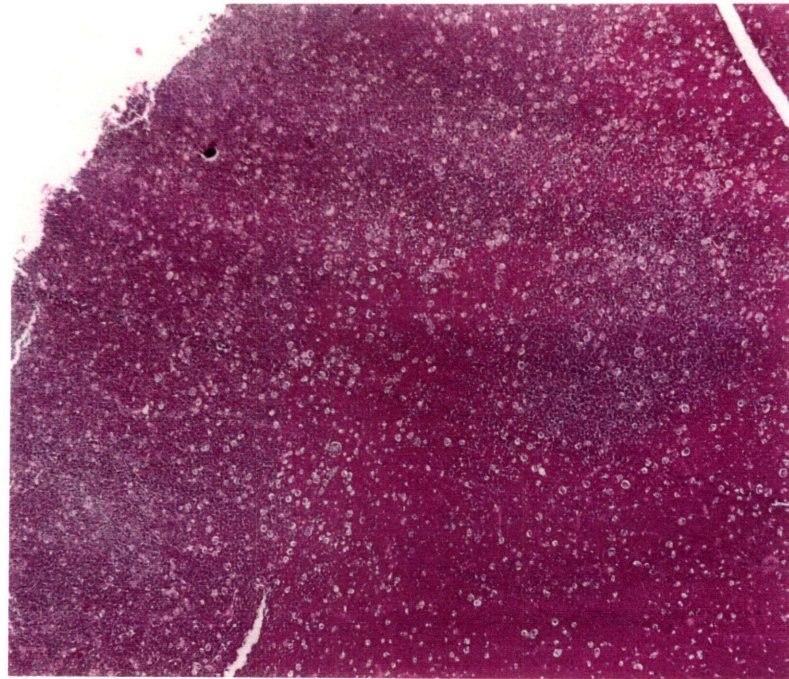
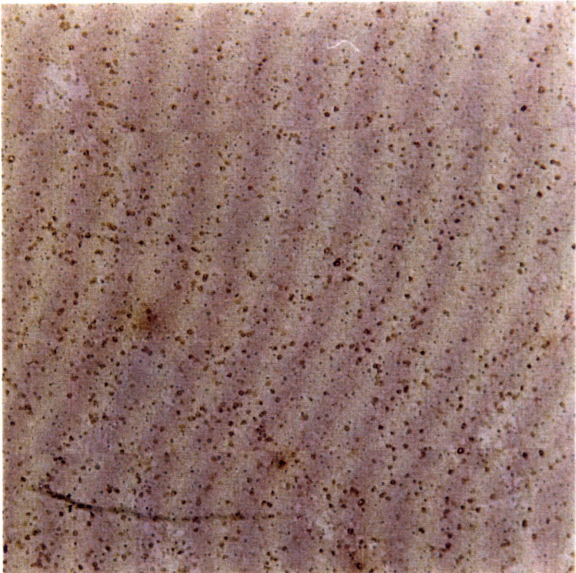
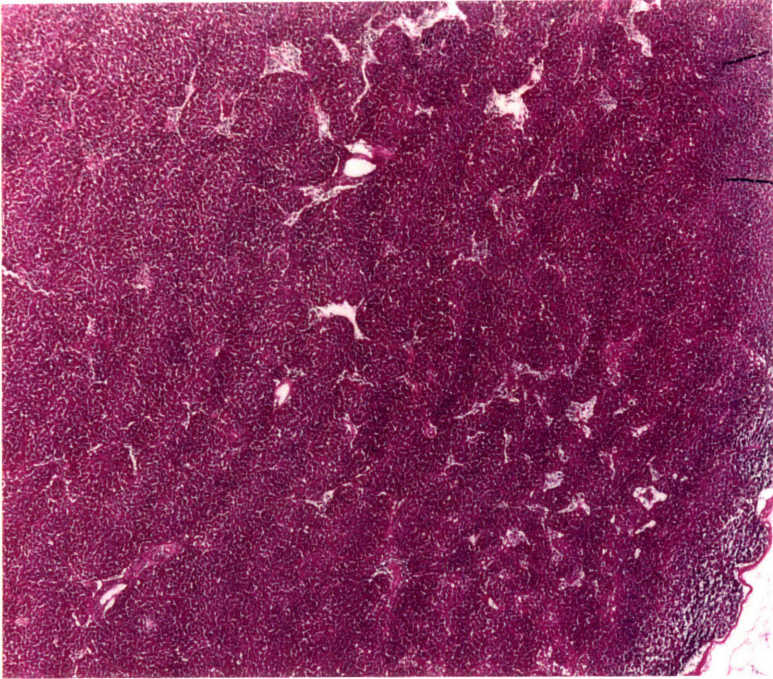


Figure 16: Immunoblastic Lymphoma - B cell Phenotype
Top - H&E 100X
Left - CD3
Right - CD79 α



which did not have a mediastinal mass but had widely disseminated lymphoma was phenotypically a B cell lymphoma.

Western Blot Analysis:

Western blot analysis confirmed that the rabbit anti-human CD3 antibody is recognizing an antigen, approximately 20kD, on ferret lymphocytes which corresponds with the known molecular weight of the CD3 molecule and that the mouse anti-human CD79 α is recognizing an antigen of 47kD corresponding with the known molecular weight of the mb-1 antigen of the B cell receptor.

DISCUSSION

Ten cases of high-grade lymphoma in ferrets less than eighteen months of age were evaluated and immunophenotyped. Nine of the ten ferrets had a T-cell lymphoma which appeared to originate in the mediastinum and was likely of thymic origin. One case was identified in which the lymphoma was of a B-cell phenotype. This ferret did not have any evidence of mediastinal or thymic involvement.

All ten cases had clinically aggressive tumors, irrespective of the phenotype. The majority of the cases presented peracutely with dyspnea and were moribund within 48 hours. The mediastinal involvement in the nine affected cases was extensive at necropsy. It appears that ferrets can compensate for an expanding thoracic mass and not show any clinical signs until there is very little space remaining in the thoracic cavity for the lungs to expand. Generally, by the time a clinical diagnosis is made in these young animals, it is too late to institute therapeutic intervention.

The microscopic evaluation of the lymphomas correlated with the rapid clinical demise. In all ten cases, the normal tissue architecture was destroyed and completely infiltrated with the neoplastic lymphocytic infiltrate. Cells were tightly packed in a honey-combed pattern consistent with a rapidly expanding tumor. Several of the tumors had areas of necrosis suggestive of rapid growth.

The clear delineation of lymphocyte phenotype seen amongst this group of lymphomas suggests that the T-cell lymphomas originated in the mediastinum, likely in the thymus, and then metastasized throughout the body. Our 9 cases of mediastinal lymphoma may represent a continuum of disease progression. Two cases were limited to

the mediastinum which may represent an early point in the continuum. Seven cases had evidence of extensive mediastinal involvement in addition to widespread neoplastic dissemination thereby representing a point later in the continuum. Interestingly, the only B-cell lymphoma in the group did not have evidence of mediastinal or thymic involvement.

The clinical, histologic, and phenotypic features correlate with that described in the young of other species. Unfortunately, the classification of lymphomas is not straight forward and numerous classification schemes have been proposed over the past 30 years. Ferret lymphomas have been described previously using the NCI working formulation (2-5) which differs from the terminology used to describe feline lymphomas and from the REAL (Revised European-American Lymphoma) classification scheme introduced in 1994 used to describe human lymphomas. (43) This disparity makes comparison of the lymphomas between species difficult. In general, we believe that the high-grade, intermediate to large cell lymphomas observed in these ferrets histologically resemble the lymphoblastic lymphomas described in cats and in children.

The association between an aggressive mediastinal mass and T-cell lymphoma is not uncommon in the young. Aggressive mediastinal T-cell lymphoma has been recognized in many species including humans, cattle, horses, dogs, cats, and mice. (41,76,77,80-82) The clinical course is generally very similar to that described in these ferrets. Affected animals succumb to an acute onset of dyspnea and respiratory distress due to a large mediastinal mass. The mass is typically an aggressive lymphoma often characterized as lymphoblastic in nature.

Juvenile-onset lymphoma in certain species is known to have an infectious etiology. This is best recognized in the domestic cat where aggressive T-cell mediastinal lymphoma has been linked with Feline Leukemia Virus infection. (76,77) In humans, virally-associated lymphoma has been described in both adults and in children. HTLV-I induces a T-cell leukemia/lymphoma in adults in certain geographical regions. (41) In children, Burkitt's lymphoma is the best described virally-associated lymphoma. (41) Burkitt's lymphoma, which is associated with EBV is characterized histologically as a small, non-cleaved lymphoma, and phenotypically as a B-cell tumor. (42) Histologically, some of our ferret lymphomas resemble Burkitt's lymphoma in that they are small, non-cleaved and have a characteristic "starry-sky" appearance. However, clinically and phenotypically the ferret lymphomas do not correlate with Burkitt's lymphoma. In the ferret cases that we describe, the SNC lymphomas were all of a T-cell phenotype. Burkitt's lymphoma

generally affects the abdomen, head and neck, and bone whereas our cases typically affected the thorax and abdomen. Burkitt's lymphoma is widespread, comprising about 35% of all non-Hodgkin's lymphoma cases. (42) Interestingly, in approximately 25-30 % of cases of children with non-Hodgkin's lymphoma, the presentation is a T-lymphoblastic mediastinal lymphoma, (42) which is similar to that described in the ferrets. No viral etiology has been definitively linked with this group of childhood mediastinal T-cell tumors, although it has been suggested that EBV may play a role in the etiology of certain peripheral T-cell neoplasms. (83)

There is evidence that a lymphotropic virus may exist in the ferret. Cluster outbreaks of lymphoma have been described in several large groups of co-habiting ferrets suggestive of a transmissible etiology. (4,5) In addition, horizontal transmission of lymphoma to naive ferrets has been described. Ferrets developed disseminated lymphoma following inoculation with either malignant lymphoid cells or a filtered cell-free supernatant from cultured malignant lymphocytes, suggestive of a viral etiology. (22) Feline Leukemia Virus has been suggested as a possible etiology underlying the high incidence of ferret lymphoma. Splenic lymphosarcoma was reported in a ferret that was FeLV positive based on serology. (7) However, we have tested our ferrets extensively for FeLV infection and have been unable to establish any correlation between FeLV infection and lymphoproliferative disease in ferrets. Aleutian Disease Virus has also been implicated as a possible etiology of lymphoma/leukemia in ferrets (14). Chronic ADV infection in ferrets has been shown to cause a hypertrophy of the thymus, however neoplastic changes have not been described. (12) Our ferrets consistently test negative for ADV despite the high incidence of lymphoproliferative disease observed.

Phenotypic analysis of these ferret lymphomas required the identification of lymphoid cell surface markers which would cross-react and recognize ferret lymphoid surface antigens. A ferret specific IgG antibody is commercially available, however this antibody has limited applications on formalin fixed tissue. Immunoglobulin antibodies tend to exhibit low specificity, resulting in a high degree of background. Immunoglobulin is secreted and can bind non-specifically to cell surfaces, as well as bind to a variety of cells via Fc receptors which can confound the interpretation of the staining. (41)

The commercially available polyclonal anti-human CD3 and monoclonal anti-human CD79 α antibodies were evaluated in ferrets since cross-reactivity with these antibodies has

been recognized in other species. (42,74,84,85) These antibodies proved to be very useful as they recognize antigens which are complementary and therefore allowed us to begin to broadly categorize ferret lymphomas. The CD3 antigen is expressed on all mature T lymphocytes. The CD79 α (mb-1) antigen is expressed on all B lymphocytes. CD3 is structurally and functionally associated with the T cell receptor. The CD3/TcR complex is important in the recognition of foreign antigen.(55) The B cell receptor is a heterodimer composed of the mb-1 and B29 polypeptides. The B cell receptor is structurally and functionally associated with surface immunoglobulins and is important in antigen recognition. The CD3 antibody recognizes a conserved intracytoplasmic epitope on the ϵ subunit, believed to be the most immunogenic, of the CD3 antigen. (57) Analogously, the CD79 α antibody recognizes the conserved intracytoplasmic C-terminus of the mb-1 polypeptide of the B cell receptor. (60)

These antibodies proved useful as they recognize the majority of T cells and B cells in normal and neoplastic tissue. Cross-reactivity of the CD3 and CD79 α antibodies with ferret lymphoid antigens was determined by observing characteristic anatomical labeling of ferret lymphoid tissue in situ. The antibodies recognized lymphocytes in anatomic locations specific for T & B lymphocytes, respectively, based on known distributions of T and B lymphocytes. Confirmation of cross-reactivity was achieved by Western blot analysis. Both the CD3 antibody and the CD79 α antibody labeled an appropriate weight antigen, 20 kD and 47 kD respectively, from a preparation of ferret lymphocytes. We believe that the Western blot in combination with in situ labeling of lymphocytes confirms that the antibodies are labeling the appropriate lymphoid surface antigen, and that lymphocyte distributions in ferrets are similar to that seen in other species. It is important to note, however that definitive determination of whether a neoplasm is of T-cell or B-cell phenotype is best determined by evaluation of DNA rearrangement of the T-cell Receptor gene or the Immunoglobulin gene.

Our results suggest that the majority of the lymphomas in young ferrets are of a T-cell phenotype. We are currently in the process of performing a genotypic analysis of these lymphomas to determine clonality and to definitively determine if they are of a T-cell or a B-cell origin. We believe that the ferret may make a good animal model of childhood mediastinal lymphoblastic T-cell lymphoma due to the similarity in disease presentation clinically, histologically, and phenotypically.

Immunophenotypic Characterization of Ocular Lymphoma in Two Adult Ferrets

INTRODUCTION

Ocular lymphoproliferative lesions has been described in many species, including humans, dogs, cats, cows, and most recently, ferrets. (86) Ocular lymphoproliferative lesions can involve either intraocular or peri-ocular adnexal tissue. The lymphoproliferative lesions described range from reactive lymphoid hyperplasia to atypical lymphoid hyperplasia to lymphoma. (87) Ocular lymphomas are often very aggressive in nature. (88) Approximately 50% of ocular lymphomas become systemic in nature. (89) B-cell ocular lymphoma occurs with greater frequency, however T-cell ocular lymphoma secondary to retroviral infection is being increasingly recognized. (45-49) In humans, ocular involvement can be seen in Epstein-Barr virus associated T-cell lymphoma and in Human T-Lymphotropic Virus I (HTLV-1) associated Adult T-cell leukemia/lymphoma. In cats, ocular lesions have been recognized in association with Feline Leukemia Virus infection (FELV). (86)

Ocular lymphoma was described in two adult ferrets. The lymphoma was rapidly progressive and poorly responsive to therapy in both cases. The ocular lymphoma quickly became systemic in nature resulting in the death of both ferrets. Histologic examination of the affected tissues was consistent with the National Cancer Institute's definition of a small, non-cleaved lymphoma. Small non-cleaved lymphoma is one of the more common forms of lymphoma in ferrets of all ages. (3)

Immunophenotypic characterization of these two cases was performed in order to determine if they were of a B-cell or a T-cell phenotype. Lymphoma in young ferrets is typically T-cell in origin, whereas in adult ferrets both T-cell and B-cell lymphoma has been recognized. (Coleman, submitted for publication)

MATERIALS & METHODS

Ocular tissue from two adult ferrets with clinical and pathologic evidence of spontaneously occurring lymphoma was evaluated immunophenotypically. Both ferrets were pet ferrets which were referred to MIT's Division of Comparative Medicine for post-

mortem histopathologic evaluation. One ferret had an antemortem ocular biopsy which was sent to MIT for immunohistochemical staining.

Formalin-fixed paraffin-embedded tissue was thinly sectioned and placed on coated slides (Fisherbrand Colorfrost/Plus) for immunohistochemical staining. Polyclonal rabbit anti-human CD3 (Dako) and monoclonal mouse anti-human CD79 α (Dako) were used to detect ferret T and B cells respectively as previously described. (Coleman) Non-immune rabbit serum and non-immune mouse serum were used in parallel as negative controls. Briefly, slides were warmed to 56C, deparaffinized and rehydrated in a series of xylene and ethanol baths prior to incubation in methanol and hydrogen peroxide to inhibit endogenous peroxidases. For the CD3 antibody, slides were incubated in pronase to expose the antigen. For the CD79 α antibody, antigen retrieval was performed by a heat-based method. Slides were incubated in the presence of the primary antibody, washed, and incubated with a biotinylated secondary antibody. Slides were washed and incubated with streptavidin-horseradish peroxidase. Diaminobenzidine was used as the color substrate.

RESULTS

One ferret presented initially with unilateral exophthalmos and exposure keratitis. Biopsy of the ocular tissue revealed a lymphocytic dacryoadenitis. The ferret failed to respond to chemotherapy, and was euthanized due to a deteriorating condition. At necropsy, it was evident that the lymphoma had spread to involve both eyes. A general lymphadenopathy was noted, although all abdominal organs appeared grossly normal. Histopathologic examination of the ocular and peri-ocular tissue revealed a uniform population of immature lymphocytes consistent with a diagnosis of small, non-cleaved lymphoma.

The second ferret presented initially with unilateral exophthalmos and was treated with antibiotics for a periorbital abscess and cellulitis. The ferret failed to respond to chemotherapy. A generalized lymphadenopathy and peripheral lymphocytosis developed six weeks following the initial presentation. The ferret was euthanized due to a deteriorating condition. At necropsy, a mediastinal mass, splenomegaly with numerous white foci, hepatic white foci, and generalized lymphadenopathy was found. The ocular lesion remained confined to one eye although the mass had enlarged greatly since the initial presentation. The globe was intact and apparently not involved. The mass appeared to be entirely peri-ocular. Histologic examination of the affected tissue revealed a uniform

population of small to medium sized immature lymphocytes arranged in compact sheets. Scattered macrophages were present throughout. Histopathology was consistent with a diagnosis of small, non-cleaved lymphoma.

Immunohistochemical evaluation of all affected ocular tissue in both cases was consistent with a T-cell phenotype. (Figure 17) The ante-mortem biopsy from the first ferret contained many lymphocytes which did not stain with either antibody. Approximately 50% of the lymphocytes were CD3+ and <1% of the lymphocytes were CD79 α +. The post-mortem tissue from this ferret displayed uniform staining with the CD3 antibody. >90% of the lymphocytes were CD3+ and <1% were CD79 α +. Tissue from the second ferret demonstrated strong staining with CD3 (90%). Several lymphoid follicles within the affected ocular tissue were evident which were CD79 α +. Neoplastic infiltrates in the liver, spleen, and lymph nodes were also >90% CD3+.

DISCUSSION

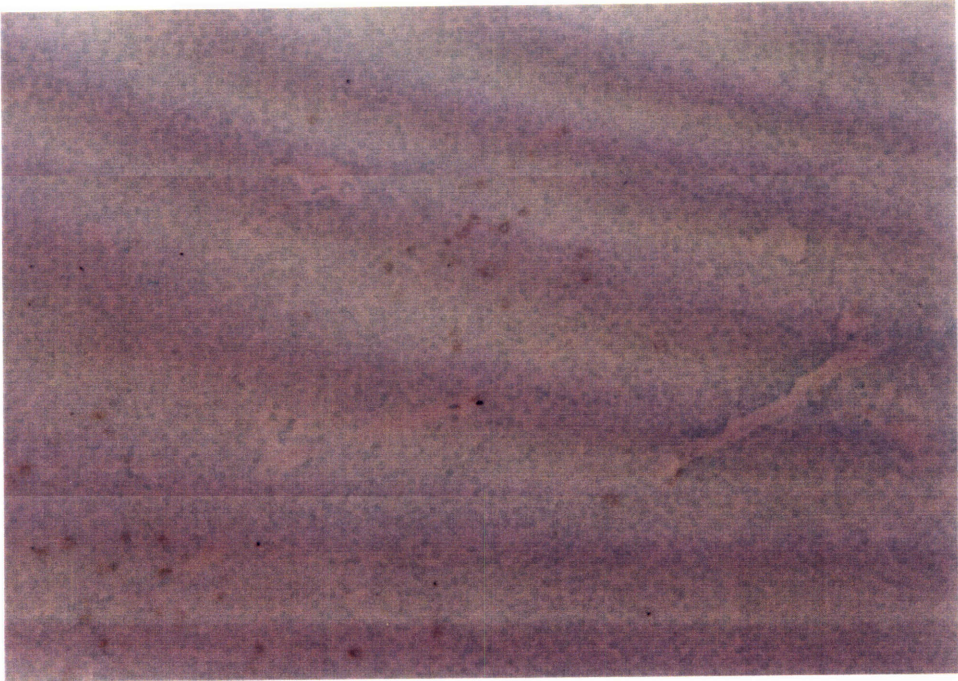
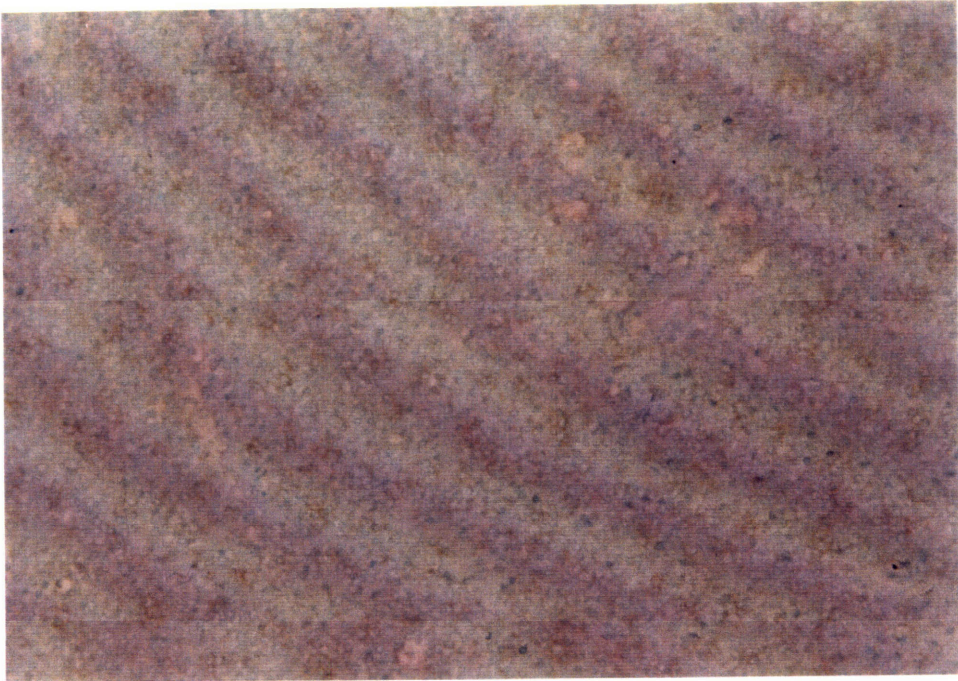
Lymphoma is one of the most common neoplasms of the ferret, however ocular lymphoma has been rarely described. (24) This report describes two adult ferrets that presented initially with clinical signs referable to ocular pathology. One ferret had an initial biopsy which was interpreted as an inflammatory response. The lesion eventually progressed to a neoplasm. One ferret developed a multi-centric lymphoma following an initial presentation of a peri-ocular mass. It appears that these two cases represent a continuum of disease progression from initial inflammation to a localized neoplasm and eventually to disseminated lymphoma.

Ocular involvement in association with systemic lymphoma is not uncommon. This phenomenon has been described in many species. (86) Ten percent of cows with lymphosarcoma develop ocular lesions. Clinically, cows present with exophthalmos and a secondary exposure keratitis due to peri-orbital lymphoma. Dogs, in contrast, develop endophthalmitis and uveitis in association with lymphosarcoma at an incidence of up to forty percent. The lesions in dogs are intra-orbital in nature. Cats can present with both intra-ocular and peri-ocular lesions in association with FELV infection or myeloproliferative disease of unknown etiology. In humans, it has been reported that 50% of patients diagnosed with ocular lymphoma will develop systemic lymphoma. (89)

Figure 17: Ocular Lymphoma - T cell Phenotype

top - CD3

bottom - CD79 α



The majority of human ocular lymphomas are non-Hodgkin's lymphomas, although ocular lymphoma can be a sequelae of infection with certain lymphotropic viruses. In general, intraocular lymphoma is associated with significant morbidity and mortality. (88)

The distinction between a chronic lymphoproliferative lesion and lymphoma is often a very fine line diagnostically, but extremely important prognostically. Generally, histopathologic assessment is used in conjunction with immunophenotypic and genotypic studies to determine if a lesion is proliferative or neoplastic in nature.(90,91) A neoplastic lesion should demonstrate phenotypic clonality or a genotypic rearrangement of the T-cell receptor or immunoglobulin genes. A retrospective Canadian study performed in 1996 compared the results of a histologic analysis of ocular lymphoproliferative lesions with a genotypic evaluation of the same cases. They reported that about 90% of patients diagnosed with ocular adnexal lymphoproliferative lesions actually had ocular lymphoma, which of course impacts therapy and prognosis. (89)

Phenotypic characterization of the ferret lymphomas is helpful in understanding disease etiology and progression. The two cases of ocular lymphoma in the ferret were determined to be a T-cell phenotype. We were unable to determine clonality of the lesion however we are developing a ferret specific T-cell receptor probe which will allow determination of gene rearrangement consistent with a T cell neoplasm.

The majority of human ocular lymphomas are small, lymphocytic non-Hodgkin's lymphoma of a B-cell phenotype. (87) T-cell ocular lymphoma is diagnosed far less frequently than B-cell ocular lymphomas. The most notable exception being a lymphoma associated with a viral infection, in which case these are most often T-cell lymphoma. Ocular involvement has been described in association with Epstein-Barr virus associated T-cell lymphoma (46). Human T-cell lymphotropic virus type I (HTLV-I) can induce a uveitis by a CD4+ T-cell immune mediated mechanism. (92,93). Intraocular lymphoma and retinal lymphoma have been identified in patients with HTLV-I infection. Histopathologic examination and electron microscopy of the affected eye is consistent with adult T-cell lymphoma/leukemia. (47,48) Ocular lymphoma has also been described in association with the Human Immunodeficiency Virus (HIV). (49)

It has been suggested that spontaneous lymphoma in ferrets may have an infectious etiology. Cluster outbreaks of lymphoma have been well documented amongst large

groups of ferrets in several different households. Horizontal transmission of lymphoma was demonstrated by inoculating naive ferrets with malignant cells or cell-free supernatant from ferrets diagnosed with multi-centric lymphoma. (22) Electron micrographs of neoplastic cells demonstrate budding cellular particles suggestive of a type C retrovirus. (unpublished data) Young ferrets develop a spontaneous T-cell mediastinal lymphoma consistent with the phenotype demonstrated in these ocular lymphomas. (Coleman) A T-cell lymphotropic virus would explain the high incidence of spontaneous lymphoma in ferrets, and the high incidence of a T-cell phenotype. The ferret may make a good model of virally-associated T-cell ocular lymphoma.

Immunophenotypic Characterization of *Helicobacter* associated Chronic Gastritis and Gastric Lymphoma in Ferrets

INTRODUCTION

Chronic *Helicobacter pylori* infection has been linked with the development of gastric mucosa associated lymphoid tissue (MALT) lymphoma in people. (34-39,94) MALT is not a normal component of the gastric mucosa. (95) It is believed that MALT arises secondary to chronic antigenic stimulation associated with chronic *Helicobacter pylori* infection. (34,35) The presence of MALT in the stomach, in combination with other chronic environmental stimuli, as well as genetic factors may constitute the necessary setting for progression to a low grade gastric MALT lymphoma.(36)

The ferret has been widely used as an animal model of naturally occurring chronic *Helicobacter* infection. (17-21) Ferrets chronically infected with *Helicobacter mustelae* develop a chronic lymphocytic gastritis, primarily of the antrum, similar to that seen in people. (18) Primary gastric lymphoma has been described in aged ferrets (>5years) in association with chronic *Helicobacter mustelae* infection. (23) It is postulated that the chronic lymphocytic infiltrate in response to chronic *Helicobacter* infection serves as a precursor for the development of lymphoma in ferrets.

The histopathologic distinction between chronic gastritis and MALT lymphoma can be very subtle. Immunologic techniques have been used to distinguish between benign lymphoproliferative responses and malignant neoplastic lesions based on clonal expression of immunoglobulin light chains, in human B-cell gastric MALT lymphoma. (37, 96-102) Both kappa and lambda light chain restriction have been described in the human gastric lymphomas. In the ferret, the gastric lymphomas described also are of a B-cell origin.(23) Light chain restriction was demonstrated in the ferret neoplasms. The ferret syndrome appears to be a good model of *Helicobacter* associated gastric lymphoma.

To better define the ferret model of *Helicobacter* associated gastritis and gastric lymphoma, we were interested in evaluating cases of chronic gastritis in the ferret immunophenotypically to determine if we could identify cases of low grade MALT lymphoma analogous to that seen in people. Thirty-one cases of chronic gastritis in ferrets chronically infected with *Helicobacter mustelae* were evaluated histologically. The lymphocytic infiltrate was characterized with regards to T-cell and B-cell components. Evidence of monoclonality based on light chain restriction was assessed based on

distributions of kappa and lambda light chain immunoglobulins in these cases of gastritis. Morphologic features of chronic gastritis were correlated with immunophenotypic findings for lymphocytes to better understand the pathogenesis and progression of *Helicobacter*-associated gastritis and the development of MALT lymphoma.

MATERIALS & METHODS

Animals:

Stomachs were obtained from thirty-one ferrets chronically infected with *Helicobacter mustelae* and from nine ferrets specific pathogen free (SPF) for *Helicobacter mustelae*. The *Helicobacter* infected group consisted of thirteen pet ferrets which ranged in age from one to ten years (Ferrets 1-13), and eighteen research animals which ranged in age from one to two years. One of the pet ferrets (Ferret 13) was diagnosed with gastritis antemortem and monitored for one year. Gastric biopsy samples were obtained at 8 months and 3 months prior to her death, as well as at necropsy. (Figure 20) The remaining twelve pet ferrets were euthanized for reasons not related to gastric disease, and the stomachs were provided to us for histologic and phenotypic evaluation, post-mortem. Of the research animals, four were one year old ferrets used in ophthalmologic studies at an outside university. (Ferrets 14-17) The stomachs from these four ferrets were provided to us for histologic and immunohistochemical examination post-mortem. Fourteen were one to two year old ferrets used in research protocols at MIT. (Ferrets 18-31) These ferrets were euthanized for reasons unrelated to gastric disease, and the stomach was evaluated post-mortem. The nine SPF ferrets were research controls at MIT and were euthanized for reasons unrelated to gastric disease. The entire stomach was evaluated post-mortem.

Histopathology:

Stomachs were fixed in 10% neutral buffered formalin, paraffin embedded, and thinly sectioned (5µm) for haematoxylin and eosin staining and for immunohistochemical staining. Histopathologic evaluation was ascertained by 2 independent reviewers. The stomach was evaluated from the cardia to the pylorus. The fundus, proximal antrum, and distal antrum (the sites of inflammation) were graded independently. The lesions were graded on a scale of 0-5 based on a scale adapted by Wotherspoon et al. (34) (Table 4) *Helicobacter mustelae* infection was determined by Warthin-Starry stained sections. In nine cases, additional confirmation of *Helicobacter* infection was obtained by culture and urease results. (Ferrets 13, 24-31)

Table 4: HISTOLOGIC GRADING SCHEME OF GASTRIC MUCOSA OF FERRETS

<u>GRADE</u>	<u>DESCRIPTION</u>	<u>HISTOLOGIC FEATURES</u>
0	Normal	Rare lymphocyte or granulocyte in lamina propria
1	Chronic Mild Gastritis	Scattered lymphocytic infiltrate in lamina propria
2	Chronic Progressive Gastritis	Clusters of lymphocytes in lamina propria and submucosa
3	Chronic Gastritis with Lymphoid Follicles/Nodules	Lymphoid nodules/follicles in lamina propria and submucosa
4	Suspicious Lymphoid Infiltrate/Possible Lymphoma	Lymphoid nodules/follicles +/- atrophy +lymphoepithelial lesions +extension to lamina propria and submucosa
5	Low Grade MALT Lymphoma	Diffuse lymphocytes in lamina propria and submucosa + effacement of architecture

Adapted from Wotherspoon et al., Lancet 1993

Antibodies:

Polyclonal rabbit anti-human CD3 (DAKO) and monoclonal mouse anti-human CD79 α (DAKO) were used to detect ferret T and B cells respectively as previously described. Polyclonal rabbit anti-human kappa immunoglobulin light chain (Biomedica) and polyclonal rabbit anti-human lambda immunoglobulin light chain (Biomedica) were used to detect ferret B cell subsets. Specificity of the kappa and lambda antibodies for ferret B lymphocytes was determined by immunohistochemical staining of normal ferret lymphoid tissue and by flow cytometric analysis of ferret peripheral blood lymphocytes from healthy donors. In addition, the normal kappa:lambda ratio was established in the ferret before an assessment of clonal shifts could be made. (see antibody characterization)

Immunohistochemistry:

Slides were warmed to 56C for 30 minutes and subsequently deparafinized and rehydrated in a series of xylene and ethanol baths at 25C. All subsequent manipulations were carried out at room temperature. For the CD3, kappa, and lambda antibodies, endogenous peroxidases were inhibited by incubation in methanol and 3% hydrogen peroxide for 20 minutes. Slides were washed with Tris-buffered saline, pH 7.4 (TBS), then incubated in pronase (200 μ /50ml TBS) for 10 minutes, washed extensively in H₂O, and then in TBS. For the CD79 α antibody, slides were processed by a heat-based antigen retrieval method. Slides were immersed in Citrate buffer, pH 6.0, and boiled 2X for 5 minutes in a microwave oven. Endogenous peroxidases were inhibited by incubation in methanol, 0.3% hydrogen peroxide, and 0.1% sodium azide. Slides were subsequently washed in TBS. The primary antibody or non-immune control serum was applied to the slides for 60 minutes. CD3 and CD79 α were used at a dilution of 1:100. The kappa and lambda antibodies were pre-diluted by the manufacturer and were applied to the tissues neat. The slides were washed in TBS and the secondary antibody (biotinylated goat anti-rabbit IgG or biotinylated rabbit anti-mouse IgG) was applied at a 1:100 dilution for 30 minutes. Again, the slides were washed in TBS, and streptavidin-horseradish peroxidase applied for 30 minutes. Diaminobenzidine (DAKO) was used as the color substrate. Slides were counter-stained with 0.1% methyl green, allowed to air dry, and were cover-slipped for subsequent microscopic evaluation. Phenotypic analysis was graded on a scale of 1-5 based on a scale modified from Segal et al. (103) (Table 5) The fundus, proximal antrum, and distal antrum were evaluated independently for all four lymphocyte subsets.

Table 5:LYMPHOCYTE PHENOTYPE GRADING SCHEME

GRADE	% POSITIVE LYMPHOCYTES
1	<20
2	20-40
3	40-60
4	60-80
5	>80

*Each antibody (CD3, CD79 α ,kappa, lambda) evaluated individually

RESULTS

Histopathologic Assessment:

All forty ferrets exhibited some degree of lymphocytic gastritis. In general, there was a correlation between the age of the ferret, colonization with *Helicobacter mustelae*, and the degree of severity of gastritis. The SPF ferrets had evidence of a mild lymphocytic gastritis primarily of the antrum. (Grade 1-2) Scattered lymphocytes, eosinophils, and occasional small clusters of lymphocytes, were present in the deep lamina propria along the muscularis mucosa of the distal antrum. The ferrets colonized with *Helicobacter* generally had a more severe gastritis than the SPF ferrets. Lymphocytes were identified in the superficial layers of the mucosa, the site of *H. mustelae* colonization, as well as in the deep mucosa along the muscularis mucosa, as seen in the SPF ferrets. Many of these ferrets had involvement of the fundus as well as the antrum. The younger ferrets tended to have a mild to moderate gastritis, primarily of the antrum. (Grade 1-3) Older ferrets had a severe gastritis (Grade 4) and in some cases, there was histologic evidence of gastric lymphoma. (Grade 5) Mild gastritis was characterized by a diffuse lymphocytic infiltrate in the lamina propria, localized primarily in the antrum. As the gastritis increased in severity, clustering of lymphocytes in the lamina propria of the antrum was identified. These cluster of lymphocytes eventually form lymphoid follicles. With increasing gastritis, the infiltrate extended through the muscularis mucosa into the submucosa. The lymphoid follicles appear to continue to expand with progression to lesions suggestive of neoplasia. In the severe cases of gastritis, the lymphocytic infiltrate was extensive with involvement of the mucosa and submucosa. Mucosal gland atrophy, lymphoepithelial lesions, and general loss of normal architecture was evident in severe gastritis/low grade MALT lymphoma. The topography of the lesions was characterized by a marked involvement of the antrum, with a mild involvement of the fundus. (Table 6) (Figure 18) The gastritis correlated with the location of the *Helicobacter* organisms. *Helicobacter mustelae* colonized the gastric mucosa of the antrum primarily, and to a lesser extent the fundus. (Table 7) (Figure 19) Generally, colonization and thereby associated gastric pathology increased proximally to distally through the stomach.

Ferret 13 , a five year old pet male ferret with chronic gastrointestinal disease, was followed chronologically and biopsied three times over a course of eight months. The presenting complaint consisted of non-specific gastrointestinal symptoms, ie. anorexia, diarrhea, and vomiting. Initial gastric biopsy was consistent with a severe lymphocytic gastritis/low grade MALT lymphoma (Grade 4-5) Numerous *Helicobacter* organisms were

detected by a Warthin-starry stain. Additionally, gastric biopsy samples were urease positive and culture positive for *H.mustelae*. A short course of antibiotics (10 days) resulted in both clinical improvement and histologic improvement (Grade 2). Termination of antibiotic therapy, however resulted in a relapse clinically. At necropsy, 3 months later, he had a severe ulcerative lymphocytic gastritis (Grade 4). (Figure 20)

Immunophenotypic Analysis:

Mild gastritis (Grade 1-2) was characterized by a lymphocytic infiltrate comprised primarily of T cells diffusely scattered in the lamina propria of the antrum, with an occasional B cell. As the gastritis increased in severity, an accumulation of B cells was noted with subsequent B cell follicular development. (Grade 3-4) A mild diffuse T cell infiltrate in the lamina propria persisted throughout all stages of gastritis. The lesions which progressed to neoplasia were predominantly of a B cell phenotype with occasional T cells persisting in the lamina propria. In general, early gastritis was characterized by a T cell infiltrate and severe gastritis was predominantly characterized by B cells. (Table 8) (Figure 21)

Kappa and Lambda Antibody Specificity:

The anti-human kappa and lambda antibodies recognized ferret lymphocytes in situ and by flow cytometry. Semi-quantitative analysis of kappa+ and lambda+ B cells in normal ferret tissue correlated with the quantitative analysis of kappa+ and lambda+ B cells in the peripheral blood. A kappa:lambda ratio was determined to be 1.2:1 in the ferret.

Kappa/Lambda Light Chain Expression in Gastritis:

Kappa/lambda light chain expression was evaluated to elucidate the transition from B cell follicular proliferation to B-cell MALT lymphoma. Mild gastritis was primarily characterized by a T cell infiltrate. The B cells which were present were represented by both kappa+ and lambda+ cells equally and diffusely infiltrated in the lamina propria. In moderate gastritis, B cells accumulate, and there is a polyclonal distribution of kappa+ and lambda+ B cells consistent with benign hyperplasia. In cases of severe gastritis, clonal shifts of either predominantly kappa+ cells or predominantly lambda+ cells could be identified in the mucosa of the distal antrum. (Table 8) (Figure 22) This shifting may be indicative of an early transition to neoplasia. Gastric MALT lymphoma was characterized by large clonal populations of either kappa+ B cells or lambda+ B cells which is supportive of a diagnosis of neoplasia based on the concept of monoclonality.

Table 6: Histologic Grade of 31 cases of Chronic Gastritis in the Ferret

CASE	Fundus	Proximal Antrum	Distal Antrum
1	0	2	1
2	2	2	2
3	2	2	4
4	1	1	3
5	3	4	5
6	1	2	4
7	1	1	2
8	3	2	3
9	1	2	3
10	1	3	NE
11	1	5	5
12	4	NE	NE
13	3	4	5
14	1	4	5
15	2	3	3
16	2	4	5
17	2	5	5
18	NE	2	4
19	1	1	1
20	1	2	2
21	1	2	3
22	1	2	3
23	NE	1	4
24	2	3	4
25	NE	2	NE
26	NE	3	4
27	1	2	3
28	0	1	1
29	1	2	2
30	NE	3	4
31	1	2	2

NE=not evaluated

Figure 18:Chronic Lymphocytic Gastritis
top - Grade 2
bottom - left:Grade 3/right:Grade 4

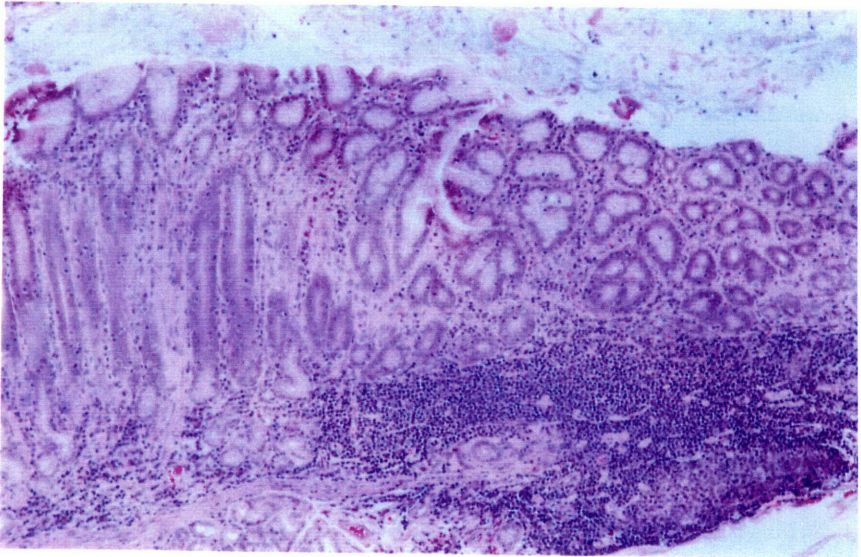
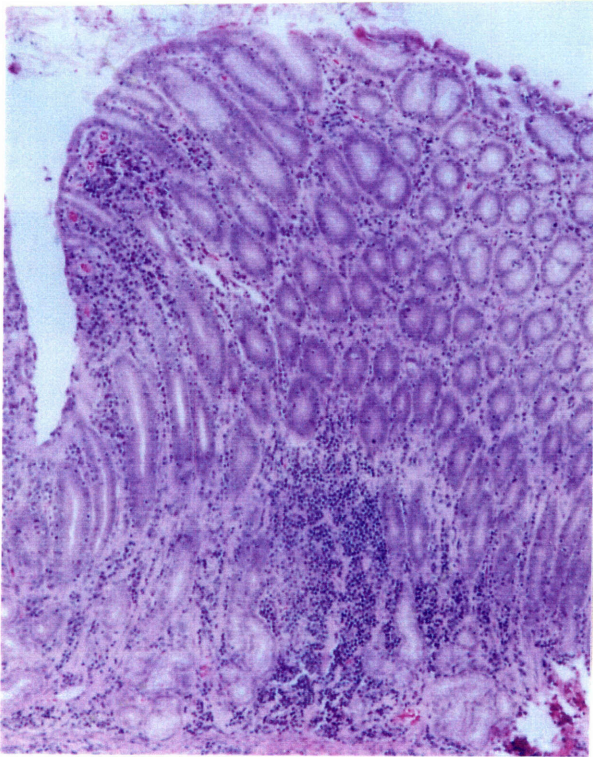
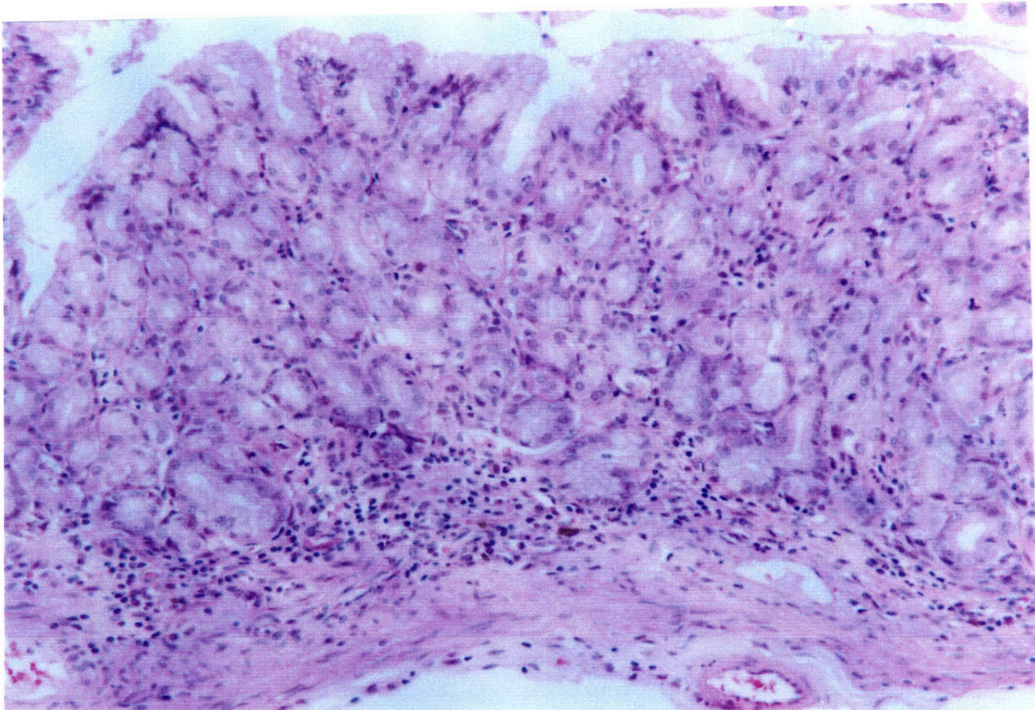


Table 7: *Helicobacter mustelae* Colonization - Chronic Gastritis

CASE	Fundus	Proximal Antrum	Distal Antrum
1	-	-	+
2	-	-	-
3	-	-	+
4	+	+	+
5	+	+	+
6	-	-	+
7	+	+	+
8	-	+	-
9	-	-	+
10	-	-	+
11	+	+	+
12	-	-	+
13	-	+	+
14	+	+	+
15	-	+	-
16	+	+	+
17	+	+	+
18	-	+	+
19	-	-	+
20	-	+	-
21	-	+	+
22	+	+	+
23	-	-	+
24	-	-	+
25	-	-	-
26	-	+	+
27	-	+	+
28	-	-	+
29	+	+	NE
30	NE	+	+
31	-	+	+

Figure 19: *Helicobacter mustelae* Localized to the Gastric Pits of the Antral Mucosa (Warthin-Starry)

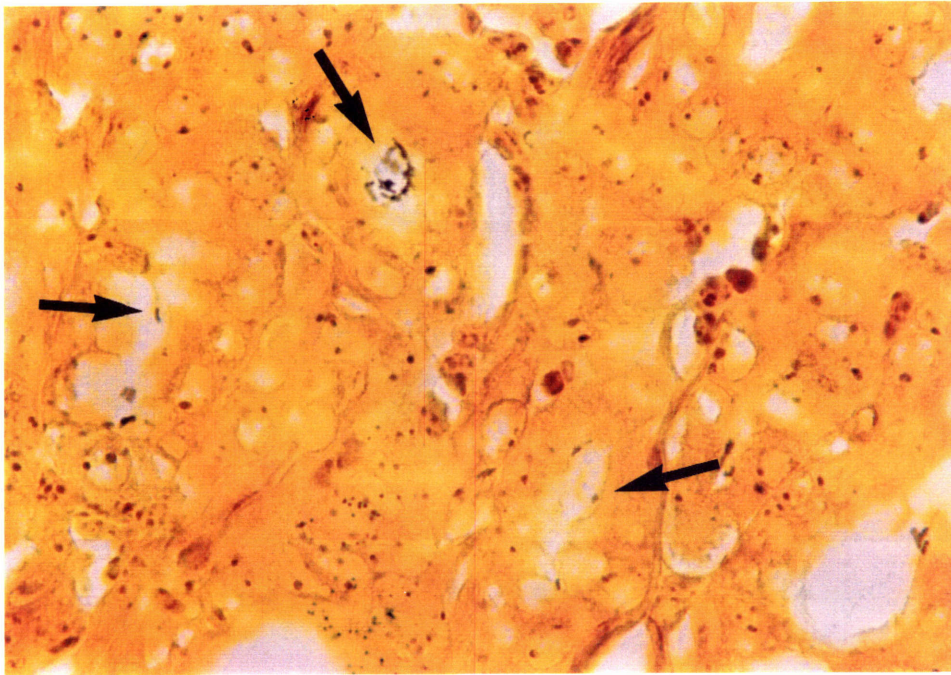


Figure 20: Progression of Ferret 13

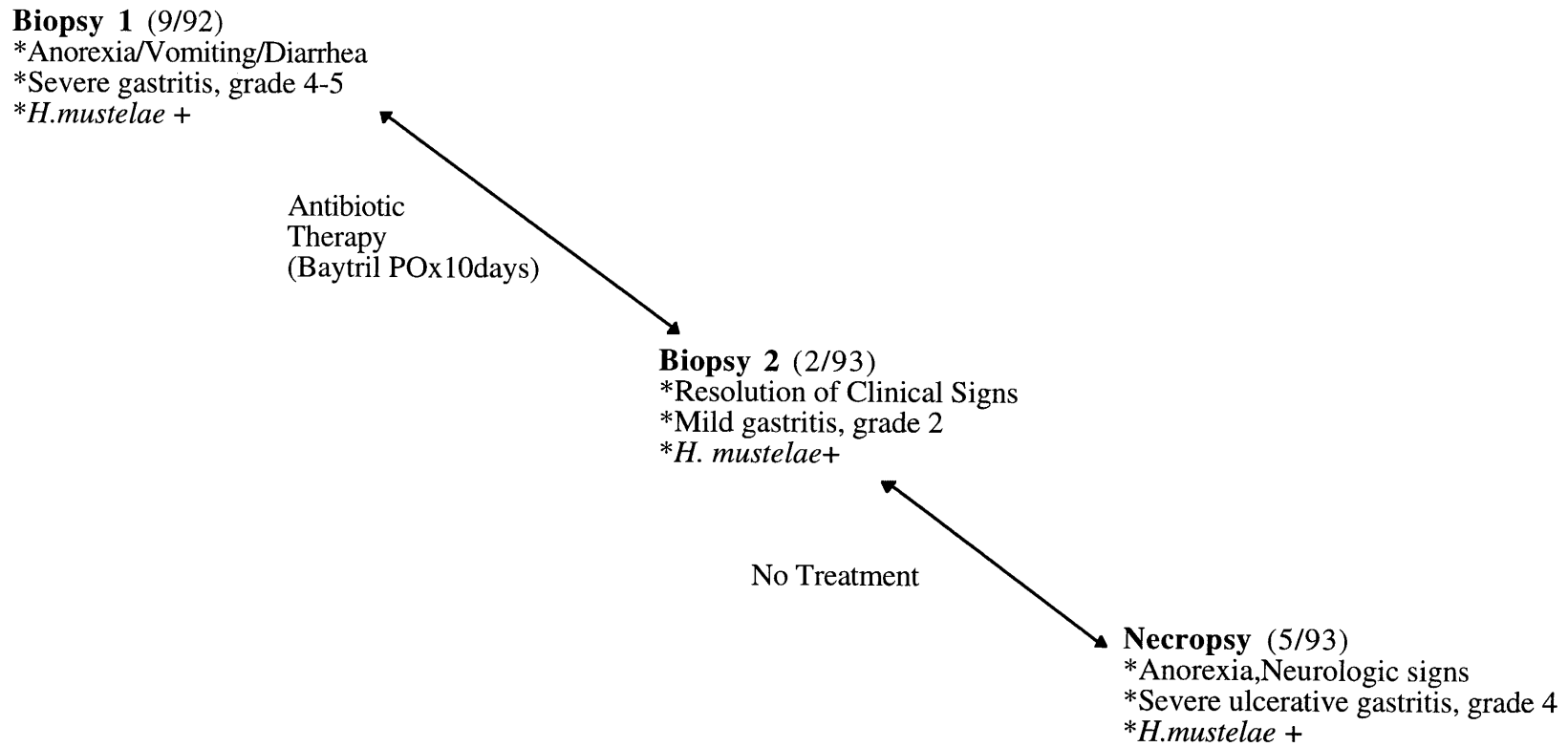
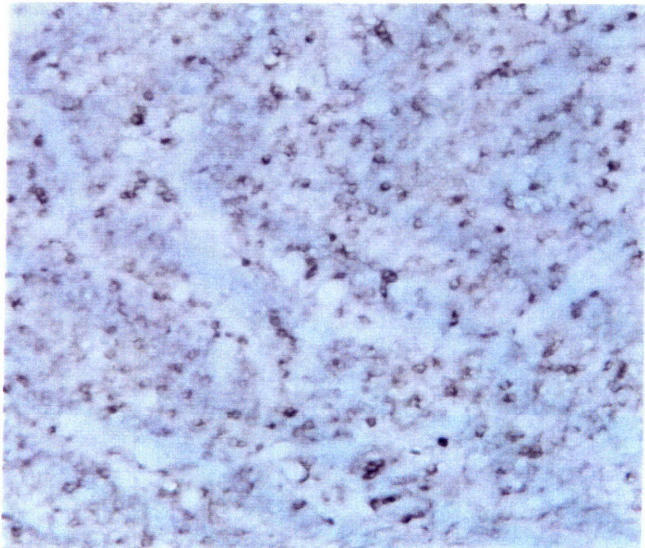
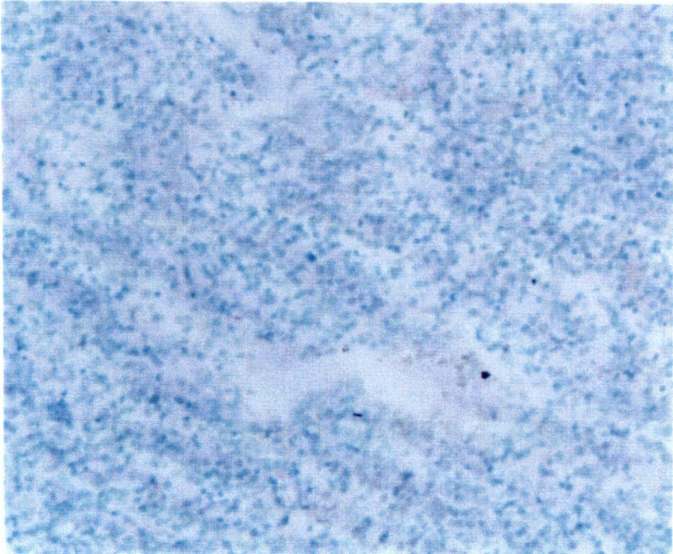
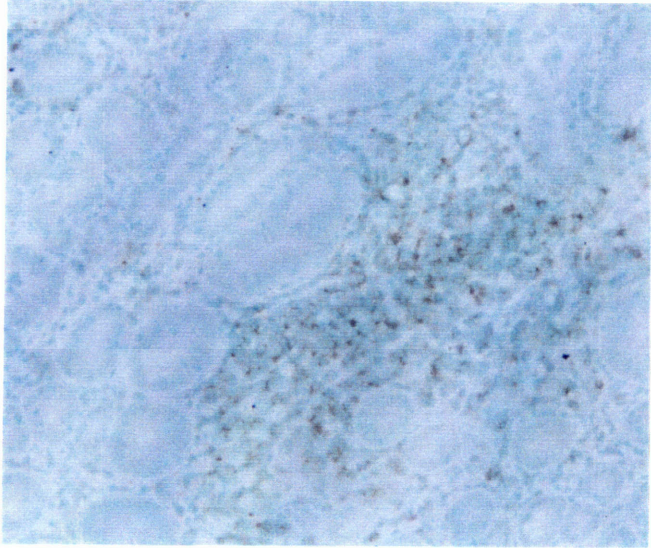
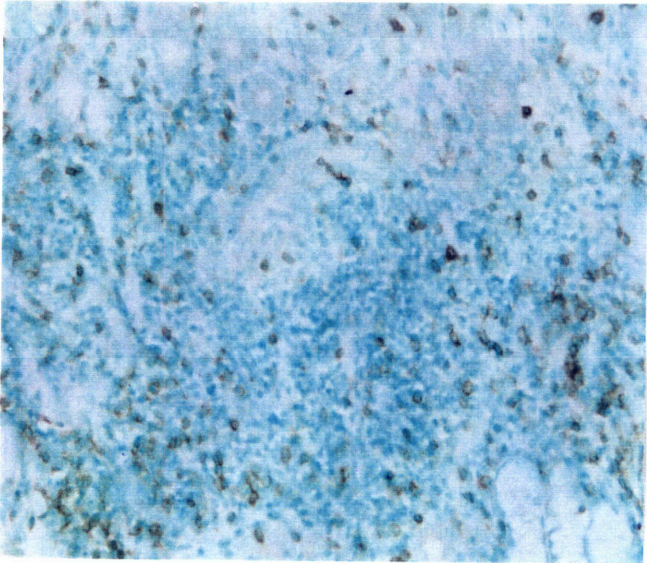
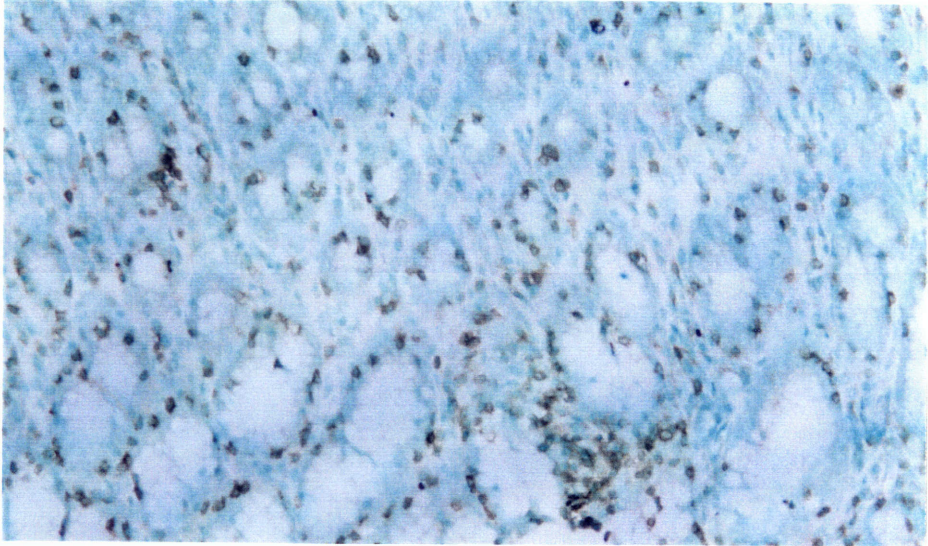


Table 8: Immunophenotypic Analysis of the Antrum in Chronic Gastritis

CASE	CD3	CD79 α	Kappa Ig	Lambda Ig
1	4	2	1	2
2	3	2	2	2
3	4	3	1	2
4	3	3	3	2
5	4	2	2	2
6	3	3	1	1
7	3	2	2	1
8	3	3	2	3
9	3	3	1	2
10	2	2	1	1
11	2	4	1	3
12	3	3	2	2
13	2	2	0	1
14	3	2	2	1
15	3	2	2	2
16	3	3	2	1
17	3	3	2	3
18	2	2	1	2
19	3	1	1	1
20	3	3	1	1
21	3	3	1	2
22	2	3	1	1
23	2	3	1	1
24	3	3	2	3
25	3	1	1	2
26	2	4	1	3
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28	5	1	1	1
29	5	1	1	2
30	2	4	1	2
31	5	1	2	2

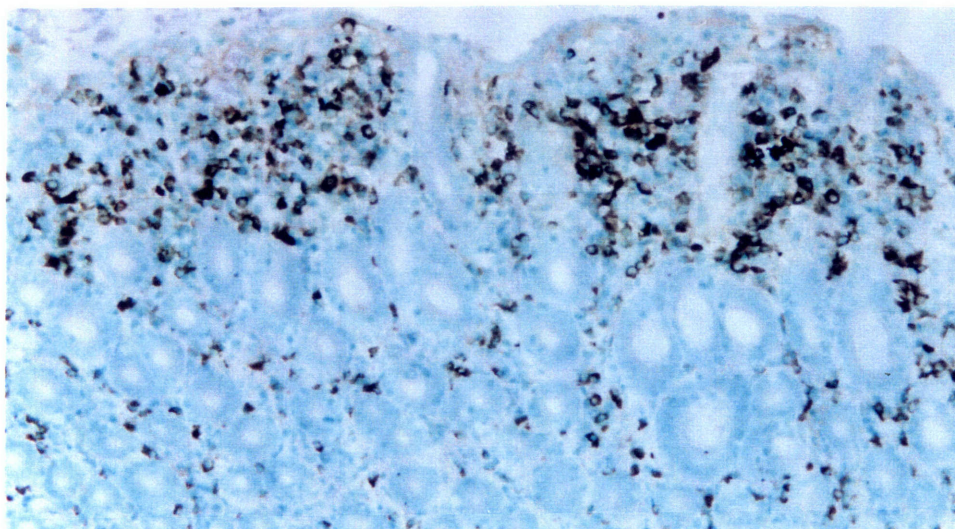
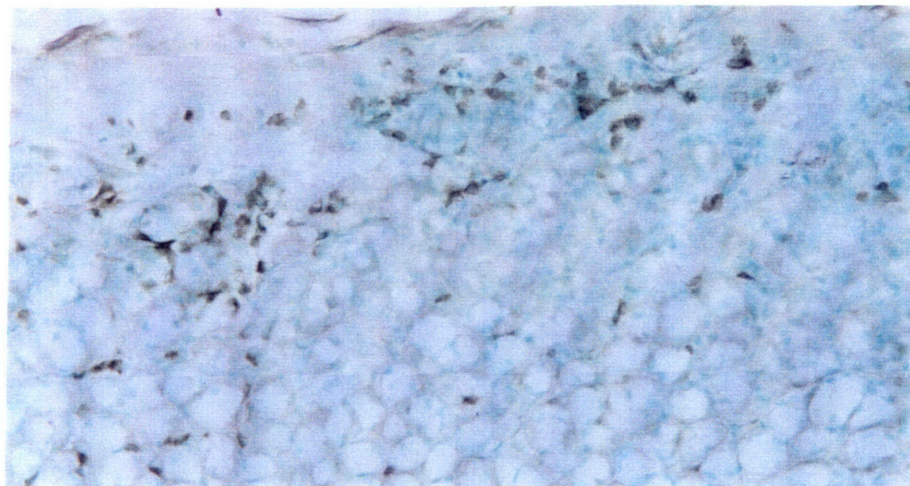
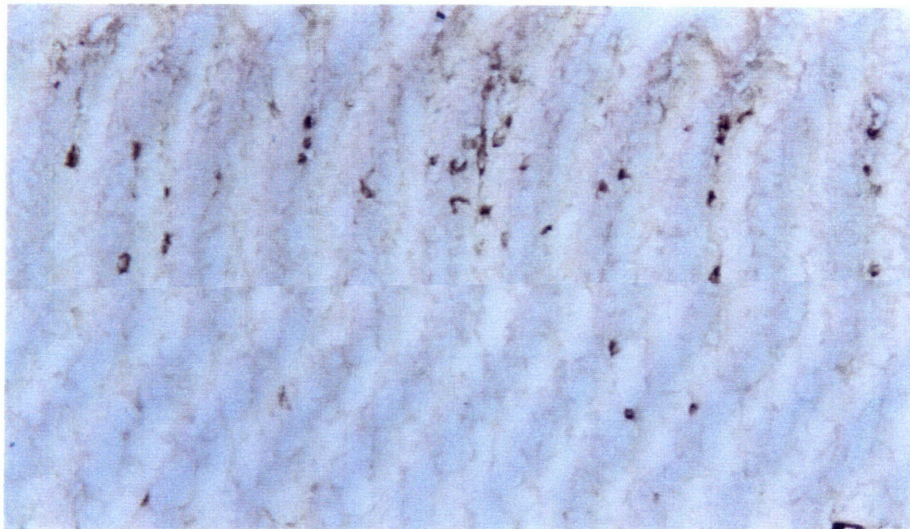
Figure 21: Phenotypic Analysis of Chronic Lymphocytic Gastritis

top: Grade 2 - diffuse CD3+ lymphocytes in lamina propria
middle: Grade 3 - CD3+ lymphocytes (left) - neg lymphoid cluster
CD79 α + lymphocytes (right) - pos lymphoid cluster
bottom: Grade 5 - CD3+ lymphocytes (left) - <10% positive
CD79 α lymphocytes (right) - >90% positive



**Figure 22: Phenotypic Analysis of Chronic Lymphocytic Gastritis
Demonstrating a kappa Ig Shift**

top: Grade 2 - scattered κ Ig lymphocytes
middle: Grade 3 - scattered κ Ig lymphocytes
bottom: Grade 4 - κ Ig Clonal shift present



DISCUSSION

The histologic and phenotypic features of *Helicobacter* associated lymphocytic gastritis in ferrets correlates with that described in humans. (18,23,34,35,38,94,104) A continuum from mild lymphocytic gastritis to gastric MALT lymphoma was demonstrated both histologically and phenotypically. The gastritis increases in severity over time and the phenotypic character of the gastritis changes markedly as the histopathologic features progress. A mild T lymphocytic gastritis progresses to a severe B cell lymphocytic gastritis and eventually to a B cell gastric MALT lymphoma.

Lymphoid tissue is not a normal component of the gastric mucosa. (95) It is believed that the chronic presence of *Helicobacter* organisms in the gastric mucosa induces the development of MALT in the gastric mucosa. Chronic antigenic stimulation is believed to result in lymphoproliferation which may eventually develop into a MALT lymphoma in a continuum of pathology. (Figure 23) Humans chronically infected with *Helicobacter pylori* develop a gastric MALT lymphoma which has been shown to regress following eradication of *H. pylori* with antibiotic therapy. (39) This observation adds further support to the theory that chronic antigenic stimulation may be a key contributor to the development of MALT lymphoma. This observation has not been studied extensively in ferrets. Ferret 13, however, was treated with a short course of antibiotics. Antibiotic therapy did seem to have an effect on the progression of gastritis. Microscopically, the gastritis regressed from a low grade MALT lymphoma to a mild lymphocytic gastritis following antibiotic therapy. Resolution was short term as termination of antibiotic therapy resulted in the recurrence of a severe gastritis. One could infer from Ferret 13 that ferrets with a low grade MALT lymphoma may demonstrate a similar regression of lymphoma following eradication of *H. mustelae* as demonstrated in humans with *H. pylori*.

The distinction between severe benign lymphoproliferation and neoplasia is often a fine line. In human medicine, light chain restriction based on phenotypic analysis is an important criteria in the diagnosis of B cell malignancy. (96,98,105) The underlying premise is that neoplasia arises from a single cell (clone). Since B cells express either kappa or lambda light chains, malignancy can be inferred if the majority of the cells in a lesion express either kappa or lambda B cells. Light chain restriction can not be used as the sole criteria for diagnosis of neoplasia. Recently, genotypic analysis of either B cell

Lymphocytic Gastritis <<<<>>> Lymphoma

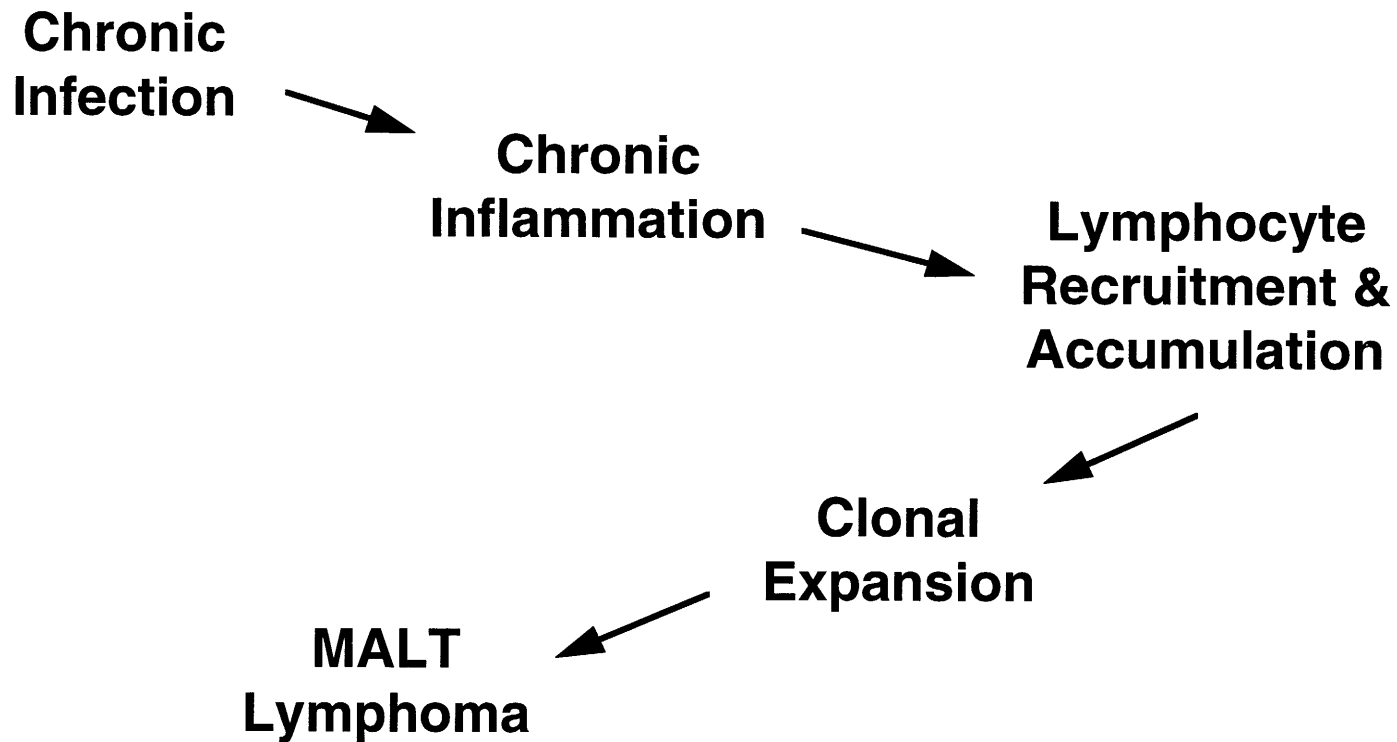


Figure 23. Progression of lymphocytic gastritis to lymphoma.

immunoglobulins or the T cell receptor has been used to further substantiate a diagnosis of B cell or T cell malignancies, respectively. (37, 97,101,102,106-109)

Light chain restriction analysis was used in the ferret to distinguish between benign and malignant lesions in a manner similar to that used in humans so that we could make correlations between the ferret model and the human disease. Kappa and lambda light chain immunoglobulin antibodies were found to be useful in the ferret both on formalin-fixed tissue and on fresh cell suspensions. Analogous to humans, mild chronic gastritis in ferrets is characterized by a polyclonal distribution of kappa and lambda expressing B cells. As the gastritis increases in severity, a clonal shift was identified in several cases. In the most severe cases of gastritis/low grade MALT lymphoma, monoclonal expression of either kappa or lambda expressing B cells was identified which we used as confirmation of neoplasia. (Figure 24) To date, we do not have the genotypic tools to identify immunoglobulin gene rearrangements in ferret B lymphocytes to confirm a diagnosis of neoplasia based on light chain analysis.

Raptopoulou-Gigi et al. phenotyped the lymphocytic infiltrate of the gastric mucosa of people with chronic gastritis. (104) They found that the majority of the infiltrating lymphocytes were CD4+ T cells in mild gastritis. As the gastritis increased in severity, the B cell population increased proportionally in a direct correlation with gastritis severity. Our findings in ferrets with chronic gastritis correlate with this phenotypic analysis of human chronic gastritis. In addition, Raptopoulou-Gigi et al. reported an increase in IL-2 receptor positive cells in the mucosa of chronic gastritis which they attributed to a T cell role in the pathogenesis of chronic gastritis. It has been speculated that T-cell dependent B-cell proliferation is the mechanism underlying B-cell MALT lymphoma development. In vitro studies performed by Hussell et al. demonstrated that neoplastic B cells obtained from patients with *H.pylori* associated B-cell gastric MALT lymphoma would proliferate only in the presence of T cells. They hypothesize that the response of low-grade B-cell MALT lymphoma is dependent on *H.pylori* specific T cells, and not a direct response to the *Helicobacter* organisms. (110)

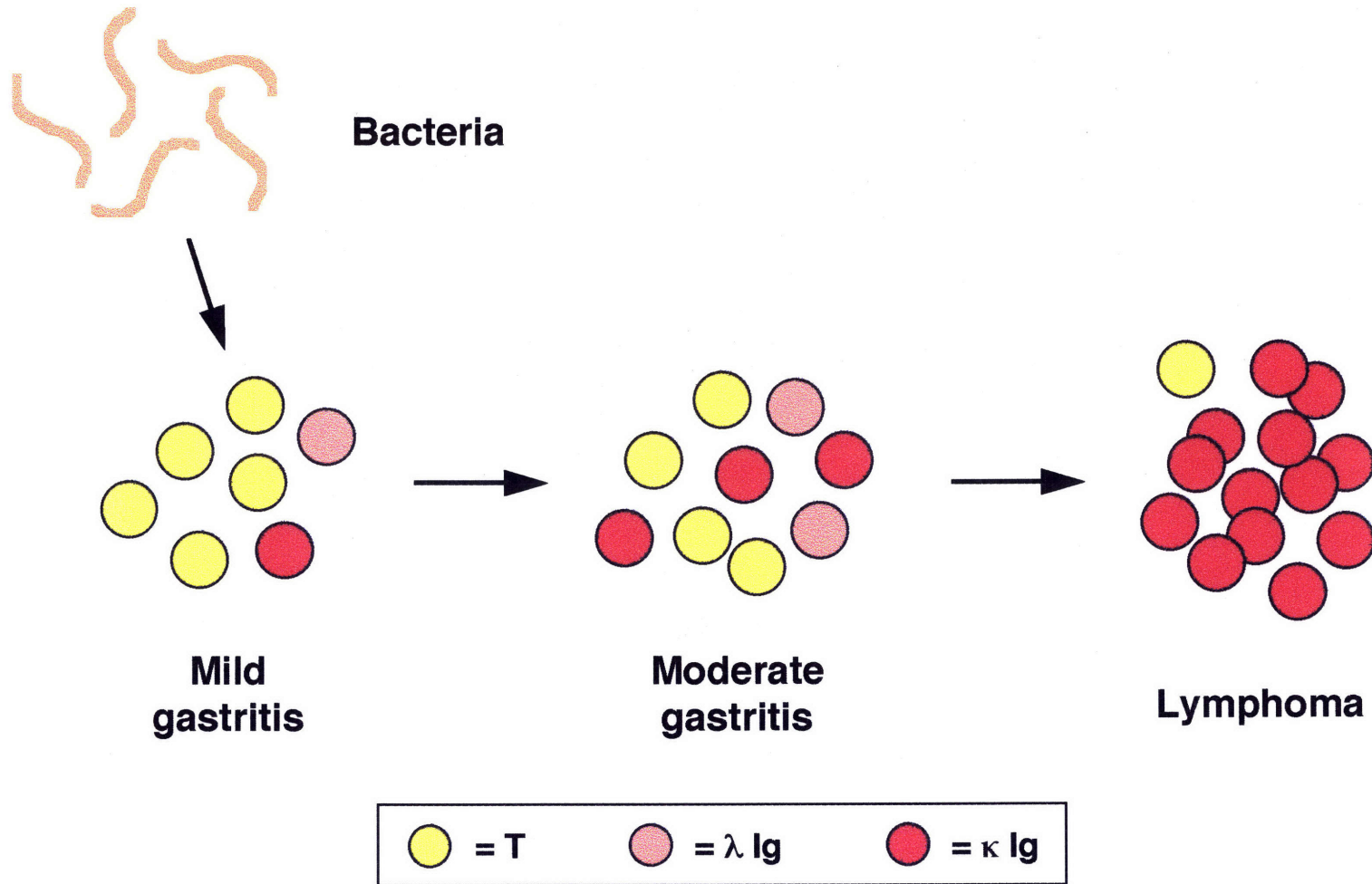


Figure 24. Schematic of light chain restriction in progression to neoplasia.

The ferret is a good model of *Helicobacter* associated gastric pathology. The ferret demonstrates the continuum from chronic antral gastritis to B-cell MALT lymphoma analogous to humans in response to chronic *Helicobacter* infection. As further tools are developed to study the ferret lymphocytic response to *Helicobacter*, we may be able to further elucidate the pathogenesis of the development of lymphoma in response to chronic bacterial stimulation.

DISCUSSION

The ferret (*Mustela putorius furo*) has a high incidence of lymphoproliferative disease, and in particular, of naturally occurring lymphoma. (1-7) Phenotypic characterization of the lymphocytic infiltrate in these syndromes is helpful in the elucidation of the etiology and pathogenesis of lymphoproliferative disease in ferrets. Determination of the lymphocyte cell type involved is instrumental in dissecting out the cellular pathways and underlying mechanism which contribute to these diseases. Elucidation of the lymphocyte phenotype can provide information about the factors (chemokines and interleukins) which initially attract the cells to a particular tissue, as well as information about the subsequent environment created by the resident lymphocyte population, ie interleukins & other chemoattractants, or free radicals, which may be produced by the lymphocytes. The entire cellular environment must be studied in order to truly understand the pathogenesis of disease.

As there are few immunologic tools commercially available to characterize ferret lymphoid tissue, it was necessary to identify and assess potential tools which could be used to study ferret lymphoma, and hopefully begin to elucidate the complex mechanism underlying this common neoplasm of ferrets. The first goal was to find a means to identify the two major subclasses of lymphocytes - B and T lymphocytes. To further study lymphocyte biology, it was necessary to identify subclasses of the B and T lymphocytes. Finally, it was necessary to apply these tools in a manner which allowed the assessment, or hypothesis, of function. Eventually, once the character of the cellular infiltrate is established, then the cellular environment at the molecular level can be examined to fully elucidate the disease pathway.

Ideally, one would create monoclonal antibodies specific for ferret lymphocytes. An attempt was made to develop a ferret specific T cell marker, however this was unsuccessful due to a lack of large quantities of antigen. It became apparent that there were commercially available antibodies which would potentially cross-react with ferret lymphocyte surface antigens. A variety of antibodies were evaluated for cross-reactivity with ferret peripheral blood lymphocytes. Dog, cat, and mink lymphocyte subset markers were assessed. The only marker which proved to be useful was a human CD8 marker, OKT8, which had already been shown to cross-react with and recognize mink CD8 T lymphocytes. (58) In turn, it was decided to evaluate other human specific lymphocyte antibodies for cross-reactivity with ferret lymphocytes. Several human specific lymphocyte antibodies were identified which recognize highly conserved epitopes and therefore proved

to cross-react and demonstrate specificity for ferret lymphocyte surface antigens. These markers proved to be extremely valuable in the study of ferret lymphoid tissue and lymphoproliferative disease.

Dako's polyclonal rabbit anti-human CD3 and monoclonal anti-human CD79 α antibodies demonstrated specificity for ferret T and B lymphocytes, respectively *in situ*. Several research groups have demonstrated that these human specific antibodies will cross-react and recognize T and B lymphocytes, respectively, in numerous species including mice, rats, cats, dogs, horses, cows, pigs, sheep and goats. (51,84,85) This high degree of cross-reactivity can be attributed to the antigen which the antibodies recognize. Both antibodies recognize an intracellular epitope on a conserved region of either the T cell receptor or the B cell receptor complex, a highly expressed lymphocyte antigen. (55-57, 59-62) The CD3 and CD79 α antibodies can be considered analogs as both recognize epitopes which are expressed on the majority of T and B lymphocytes. T cells express the CD3-T cell receptor complex early on in T cell development and maturation. Expression persists through the mature T cell stage. (52-57) Likewise, B cells express the Ig- B cell receptor complex early on in B cell development. Expression persists through the plasma cell stage. (59-62). The CD3 and CD79 α antibodies have proven to be very useful tools in dissecting out ferret T and B lymphocyte populations *in situ*. These antibodies can be used on formalin-fixed paraffin-embedded tissues, thereby allowing the dissection and study of archival cases of naturally occurring ferret lymphoma.

Human specific lymphocyte surface antibodies were also employed to identify ferret T lymphocyte and B lymphocyte subsets. Ideally, one would like to initially divide T lymphocytes into CD4+ and CD8+ lymphocytes as their functions are quite different. An antibody which recognizes fresh, viable ferret CD8 lymphocytes was identified (OKT8, Ortho). This antibody is not useful on formalin-fixed paraffin-embedded tissues which limits its usefulness on studying archival cases of naturally occurring lymphoma. To date, we have been unable to identify a lymphocyte marker which will recognize ferret CD4 lymphocytes. With regards to B lymphocytes, markers were identified which can distinguish between mature B cells on the basis of their immunoglobulin light chains. These antibodies, human specific kappa light chain immunoglobulin (Biomed) and lambda light chain immunoglobulin (Biomed), have proven to be valuable in the elucidation of certain B cell lymphomas and lymphoproliferation. The kappa and lambda antibodies will recognize both fresh viable ferret lymphocytes as well as formalin-fixed, paraffin-

embedded lymphoid tissue allowing us to study current, as well as archival cases of lymphoma and lymphoproliferative disease.

In summary, five antibodies were determined to be useful in the characterization of ferret lymphocytes. A pan T-cell marker, CD3, and a pan B-cell marker, CD79 α , recognize ferret lymphocytes *in situ*. Two B-cell subset markers, polyclonal kappa and lambda light chain immunoglobulin antibodies, recognize ferret lymphocytes *in situ* and in suspension. One T-cell subset marker was identified which will recognize ferret CD8 lymphocytes in suspension. It is important to note that there is one commercially available ferret specific antibody. Goat anti-ferret immunoglobulin (heavy and light chain) is produced by Kirkegaard & Perry, Laboratories. This antibody is useful in studying suspensions of fresh, viable lymphocytes and is therefore a good complement to the studies performed with the OKT8 antibody and the kappa and lambda light chain immunoglobulin antibodies. The ferret specific Ig antibody has limited application with respect to the study of archival tissues. As a result, the antibody was not used extensively in these studies of naturally occurring lymphoma.

These antibodies were used to dissect out the cellular phenotype of three different syndromes of naturally occurring lymphoma. Lymphoma has been identified in most organ systems of the ferret. The neoplasm has been described in all ages of ferrets as well. Young ferrets typically develop mediastinal lymphoma. This lymphoma is often very aggressive, with multicentric metastasis. The clinical course is characterized by a peracute onset of dyspnea and a high mortality rate. (1-3,5) Many features of the mediastinal lymphoma resemble that seen in young cats infected with Feline Leukemia Virus (FeLV). FeLV associated mediastinal lymphoma is typically a T-cell phenotype, likely originating in the thymus. (76,77) Similarly, children which develop a mediastinal lymphoma present with respiratory difficulty secondary to a large space occupying mass which is often a T-cell lymphoblastic lymphoma, and which is likely thymic in origin. (42,44) Elucidation of the ferret syndrome enabled us to make comparisons between young ferrets afflicted with lymphoma and the young of other species. Such comparisons contribute to the study of comparative medicine as a whole, both in regards to enriching the health of ferrets, and for contributing to the study of lymphomagenesis in general.

Adult ferrets develop lymphoma which is typically more varied in its origin, aggressive and metastatic nature, pathology, and phenotype. (1-4) To begin to elucidate the

adult lymphomas, two very different syndromes were chosen for phenotypic evaluation. Ocular lymphoma has been described rarely, although both cases exhibited very similar clinical case histories and progression. Ocular lymphoma was recognized in two aged adult ferrets, both of whom presented initially for exophthalmos which was attributed to inflammation secondary to an infectious etiology. Neither case responded to chemotherapy. Both cases progressed to develop lymphoma, which in one case metastasized throughout the body.(24) Ocular lymphoma has been recognized in numerous species, and in many cases, can be linked to a viral etiology. Cats infected with FeLV may develop an ocular or peri-ocular lymphoma. (86) Cows have been reported to develop ocular lymphoma with relative frequency which may be related to Bovine Leukemia Virus. (86) In humans, the majority of ocular lymphomas are idiopathic in nature, and are typically a B cell phenotype. (45) A proportion of human ocular lymphomas have been linked with viral agents, including Human T-cell Lymphotropic Virus I (HTLV-I), Human Immunodeficiency Virus (HIV), and Epstein Barr Virus (EBV). The virally associated ocular lymphomas of people are typically T-cell in origin. (46-49)

The third syndrome of adult lymphoma in ferrets which was evaluated phenotypically was *Helicobacter* associated gastric B-cell mucosa associated lymphoid tissue (MALT) lymphoma. This syndrome has great importance with respect to ferret biology and with respect to comparative medicine as it is a very important model of human *Helicobacter* associated gastric B-cell MALT lymphoma. Ferrets are naturally infected at weaning with *Helicobacter mustelae*. The incidence is close to 100%. The majority of adult ferrets develop some degree of lymphocytic gastritis by twelve months of age which tends to worsen as the ferrets age. (18) Aged ferrets are at risk for developing a MALT lymphoma analogous to the syndrome of people. (23) To adequately compare the ferret syndrome with that seen in humans, it was critical that we phenotype the ferret syndrome. In humans, chronic gastritis has been described as a T-cell mediated B-cell proliferative process which may ultimately progress to a B-cell MALT lymphoma. (110) Elucidation of the cells involved in ferret chronic gastritis was paramount to further development of the ferret as a model of *Helicobacter* associated gastric disease.

The immunophenotypic characterization of these three syndromes provided important information with regards to ferret biology and to ferret model development. The CD3 and CD79 α antibodies were used initially to characterize each lymphoma as T-cell or B-cell in origin. Mediastinal lymphoma of young ferrets is a T-cell lymphoma with many

shared clinical, histologic, and phenotypic features with childhood T-lymphoblastic mediastinal lymphoma of people. The syndrome is also similar to FeLV associated T-lymphoblastic mediastinal lymphoma of kittens. In both humans, and in ferrets, it has been speculated that there may be a lymphotropic virus associated with mediastinal lymphoma analogous to that seen in FeLV positive cats. A retroviral etiology could explain many of the features of ferret mediastinal lymphoma. The incidence is relatively high for a young onset. Clustering of mediastinal lymphoma has been described suggestive of an infectious etiology. (5) In vitro studies have demonstrated elevated reverse transcriptase activity in cultured peripheral blood lymphocytes as well as budding retroviral type C-like particles in adult ferrets lymphocytes. (S Erdman)

The two cases of ocular lymphoma described were phenotypically T-cell in nature, which correlates with the theory that there may be a ferret lymphotropic virus contributing to the relatively high incidence of naturally occurring multi-centric lymphoma in ferrets. T-cell ocular lymphoma in other species has been uniformly linked with virus etiologies. T-cell ocular lymphoma of humans is relatively uncommon except in association with a T-lymphotropic virus. Ferret ocular lymphoma has not been widely studied. It is too preliminary to determine the potential pathogenesis underlying the development of this neoplasm. One can speculate, however, that the neoplasm may be virally induced based on current studies of adult ferret lymphomagenesis ongoing in the laboratory and by making comparisons with other species that demonstrate similar clinical, histopathologic, and phenotypic features.

Helicobacter mustelae associated chronic gastritis and gastric MALT lymphoma correlate with that described in humans in association with chronic *Helicobacter* infection. (104) Early mild chronic lymphocytic gastritis is characterized by a diffuse T-cell infiltrate in the gastric antral mucosa. As the gastritis progresses in severity, clusters of B-cell accumulate in the mucosa. Eventually, B-cell follicles form. As the follicles expand, mucosal glandular atrophy is evident with lymphocyte extension through the muscularis mucosa into the submucosa. Eventually, B cell proliferation is the primary feature of the gastritis. A transformation occurs at some point, and the hyperplasia becomes neoplastic and a B-cell MALT lymphoma is detected histologically and phenotypically. (36) Differentiation of a B cell neoplasm and B cell hyperplasia was obtained by the use of the B cell subset antibody markers, kappa and lambda light chain immunoglobulin antibodies. Light chain analysis was used to differentiate between a benign polyclonal expansion of B cells and a neoplastic monoclonal expansion of B cells by a similar technique as that used in

human medicine. (67-71) Several severe cases of gastritis showed evidence of clonal expansion of either a kappa or lambda expressing B cell suggestive of an early shift to neoplasia. B cell gastric MALT lymphoma was diagnosed in several ferrets based on a phenotypic clonal shift in addition to certain histopathologic features consistent with neoplasia. Phenotypic analysis of the ferret syndrome is suggestive of a T-cell mediated process similar to that described in humans. It has been speculated that there may be a synergistic interaction between a T-lymphotropic ferret virus and *Helicobacter mustelae* in the pathogenesis of gastric lymphoma.

Immunophenotypic characterization of certain lymphoproliferative diseases in ferrets has enabled us to begin to elucidate the cellular components involved in these syndromes. The pathogenesis is likely to be complex, and will involve the interactions of both T and B lymphocytes. There may be an infectious etiology with a predilection for T lymphocytes, however an agent has not been isolated to date. As we continue to phenotype more ferret syndromes, trends will become apparent which could aid in deciphering the pathogenesis of ferret lymphoma. Additional tools will need to be developed which can further dissect out ferret lymphocyte populations in normal and diseased tissues. Mechanisms of disease still need to be elucidated to truly understand lymphomagenesis, however, we are one step closer to unraveling the many complex components of ferret lymphoma.

Table 9: Proposed Models of Ferret Lymphoma

Ferret Lymphoma	Phenotype	Proposed Model of Human Disease
Mediastinal Lymphoma	T-cell	Childhood T-Lymphoblastic Mediastinal Lymphoma
Ocular Lymphoma	T-cell	Virally Associated Ocular T-cell Lymphoma
Gastric MALT Lymphoma	B-cell	<i>Helicobacter pylori</i> Associated B-cell Gastric MALT Lymphoma

CONCLUSION

The ferret has been used extensively as an important model in comparative medicine of viral diseases and more recently, of lymphoproliferative diseases. (18-30) The high incidence of naturally occurring lymphoma in ferrets of all ages is an important disease to study both to benefit ferret medicine as well as to increase our understanding of these syndromes in general. Lymphoproliferative diseases have been described in most species, yet are often poorly understood with respect to etiology and pathogenesis. The ferret is an easy model with which to work, and is a model which shares many clinical and histopathologic features of other species with similar disease syndromes.

To study lymphoproliferative disease in ferret, immunologic tools were identified and characterized which could be used to study lymphocyte populations in normal and diseased fresh and formalin-fixed tissues. A pan T-cell marker, CD3, and a pan B-cell marker, CD79 α , were paramount in the elucidation of lymphocyte phenotype in archival cases of lymphoma. To further dissect out B cell proliferation's, it was determined that kappa and lambda light chain immunoglobulin antibodies were useful in the recognition of ferret B cell subsets both in formalin-fixed tissue as well as on freshly isolated lymphocyte suspensions. These antibodies proved to be essential in the characterization of B cell proliferations.

These immunologic tools were used to characterize mediastinal lymphoma in young ferrets, ocular lymphoma of adult ferrets, and *Helicobacter* associated chronic gastritis and gastric MALT lymphoma in adult ferrets. Phenotypic analysis of these syndromes allowed us to draw parallels with syndromes of other species. Mediastinal lymphoma in ferrets is typically T-cell in origin, and has many shared features with other species which develop mediastinal lymphoma. (42,44,76-78) Ocular lymphoma, also a T-cell phenotype, may be similar to virally induced T-cell ocular lymphomas of other species. (45-49) *Helicobacter* associated chronic gastritis and gastric B-cell MALT lymphoma of ferrets correlates with the human syndrome, and is a very useful model of *Helicobacter* associated gastric disease. (18, 23,34,35,38, 94)

Immunophenotypic characterization of these syndromes is important in further development of the ferret as a model of human disease, and for its further use a significant contributor to the field of comparative medicine.

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