INTESTINAL LYMPHOMA AND INFLAMMATORY BOWEL DISEASE IN FERRETS

(*MUSTELA PUTORIUS FURO*)

by

PAOLA CAZZINI

(Under the Direction of Bridget Garner)

ABSTRACT

The objective of our first study was to identify correlations between enteric histological lesions and the severity of clinical signs in ferrets with inflammatory bowel disease (IBD). No significant correlation was identified using 2 grading schemes developed for cats and dogs. After dividing the histologic samples into groups based on clinical severity, other histologic characteristics that differed between groups were identified, and those correlated with clinical severity. The established retrospective grading scheme may have clinical utility in determining severity of IBD in ferrets. The objective of our second study was to determine the agreement in the diagnosis of IBD versus lymphoma reached solely using hematoxylin and eosin (HE)-stained sections versus using a combination of HE and immunohistochemistry (IHC). Results suggest that while IHC is not necessary to distinguish IBD from intestinal lymphoma in ferrets, it can be a useful tool in ambiguous cases.

INDEX WORDS: ferret, grading scheme, immunohistochemistry, inflammatory bowel disease, intestinal lymphoma

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DEDICATION

This work is dedicated to Alessandro.

Thank you for encouraging me and giving me the strength and stability I need to face any challenge.

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CHAPTER 1

INTRODUCTION

The domestic ferret (*Mustela putorius furo*, from the latin "mouse-eating, smelly thief") belongs to the Family Mustelidae, and the first recorded evidence of its domestication dates back to 350 BC.[1] Ferrets are becoming increasingly popular pets and are often used as laboratory animals.[2]

Inflammatory bowel disease (IBD) and lymphoma are common enteric diseases in ferrets.[3,4] Despite the common occurrence, there is very limited information in the literature regarding ferret IBD.[3] Clinical signs of IBD are nonspecific, and histopathologic examination of intestinal biopsies is necessary for a definitive diagnosis and accurate treatment. However, a universally adopted grading scheme for IBD has not yet been developed in ferrets, creating inconsistencies among pathologists. Furthermore, a histologic grading scheme would be more clinically significant if a correlation between histological severity and severity of clinical signs was established. In the first study (Chapter 3), we have tested two grading schemes used in other domestic carnivores[5,6] and correlated them with severity of clinical signs in ferrets. Additionally, we divided the samples based on severity of clinical signs to identify which histologic characteristics identified the subgroups. The universal adoption of a relevant grading scheme in ferret enteric disease is necessary for more consistent and accurate disease assessment, and therefore, a more efficient therapeutic plan. The treatment for IBD consists of removal of the

possible underlying cause of intestinal inflammation and suppression of the inflammatory response with azathioprine or corticosteroids.[4]

Accurate diagnosis is important, because if untreated, IBD can progress to lymphoma, which is the most common neoplasm of the gastrointestinal (GI) tract in ferrets.[4] The clinical signs associated with GI lymphoma are similar to those observed in IBD, and once again, a definitive diagnosis can only be made histologically. Given the known progression of IBD to gastrointestinal lymphoma, some cases of severe IBD might be difficult to differentiate from lymphoma, creating a quandary for clinicians with regards to appropriate treatment. Immunohistochemistry (IHC) is a powerful tool in immunophenotyping and distinguishing between a reactive, heterogeneous, inflammatory population of lymphocytes and a monomorphic, neoplastic population, and would therefore be useful in unclear cases. In our second study (Chapter 4), we have selected cases of IBD and intestinal lymphoma and compared the diagnosis of different pathologists based on the review of routine histology. Additionally, we applied common T and B lymphocytes markers to the samples to determine whether the use of immunohistochemistry is necessary to reach a definitive diagnosis. We also evaluated age and gender differences among ferrets diagnosed with IBD and enteric lymphoma, as well as T and B lymphocyte distribution in these two diseases.

CHAPTER 2

LITERATURE REVIEW

2.1. Inflammatory bowel disease

There are only few publications regarding IBD in ferrets. For this reason, in addition to a literature review on ferret IBD, a short review of feline and canine IBD are also provided.

2.1.1. Inflammatory bowel disease in ferrets

Presentation and possible causes

Inflammatory bowel disease is an idiopathic, chronic, inflammatory disease of the gastrointestinal tract, often involving both the stomach and intestine. Although it was virtually unheard of a decade ago, today IBD is considered the most common, yet most unrecognized disease in ferrets.[3,4] IBD is usually diagnosed in ferrets over 1 year of age, and many ferrets are asymptomatic or demonstrate only subtle signs of illness that are often overlooked by the owners.[4,7,8] Clinical signs of IBD are nausea (manifested by bruxism and salivation), anorexia, chronic weight loss, greenish to brown, mucoid to "birdseed" ("seeds" represent undigested globules of fat and proteins) diarrhea, melena, proctitis, and rectal prolapse.[4,7] No studies have pursued an association between the severity of the inflammatory infiltrate and clinical signs in ferrets. The underlying etiology of IBD in ferrets is unknown, but it is hypothesized that the

underlying cause is a prolonged or exaggerated immune response to infectious agents (such as *Helicobacter* or Coronavirus) or by exposure to dietary proteins that are new to this species.[4] Other known causes of gastritis and/or enteritis in ferrets include *Lawsonia intracellularis* (proliferative ileitis/ colitis), cryptosporidiosis, coccidiosis, and foreign body ingestion. These causes may be the trigger for an aberrant immune response; however, the inflammatory process is referred to as IBD if a known cause is not identified.[3]

Diagnosis and treatment

Microscopic examination of intestinal biopsies, ideally full-thickness, is necessary for a definitive diagnosis of IBD.[4,7,9] Histopathologically, IBD is characterized by shrinkage and blunting of the intestinal villi and by an inflammatory infiltrate of the mucosa and lamina propria. The inflammatory cells present in IBD are predominantly lymphocytes and plasma cells, but less commonly, an eosinophilic component is present.[4] Eosinophilic gastroenteritis (EG), also known as eosinophilic granulomatous disease, is a multi-organ disease characterized by the presence of elevated numbers of eosinophils.[7] The etiology of EG is not known, but allergic and parasitic etiology has been suspected.[10] Eosinophilic gastroenteritis should be distinguished from IBD, as EG involves multiple organs and is not gut oriented.[4,7]

Results of blood analysis and biochemistry may show increased concentrations of liver enzymes and serum globulins, and occasionally lymphocytosis.[7] In most confirmed cases of IBD, hyperglobulinemia ranging from 3.0 to 5.5 g/dL is present.[4] Eosinophilia and increased serum lipase may also be present with gastrointestinal inflammation.[3]

The treatment for IBD consists of removal of the possible underlying cause of intestinal inflammation and suppression of the inflammatory response with azathioprine or

corticosteroids.[4] It is known that if untreated, IBD can progress to lymphoma.[3,4] Ideally, protocols and dosages for long-term treatment should be based on intestinal histopathology,[4] but a universal grading scheme to analyze ferret IBD has not been established, creating confusion with regards to what should be considered mild, moderate, or severe IBD. An underestimation of IBD severity could lead to an inefficient treatment protocol and possible progression to intestinal lymphoma, while overestimation could lead to unnecessary and potentially life-threatening, drug side effects (such as immune or bone marrow suppression). A grading scheme is therefore necessary for more consistent and accurate disease assessment, and therefore, a more efficient therapeutic plan.

Diagnostic challenges in ferrets

Mesenteric lymph node biopsies are also helpful in differentiating IBD from lymphoma. Reactive lymph nodes in ferrets are challenging to interpret histologically, however, as they can exhibit considerable cellular and architectural atypia.[4] Lymphoid hyperplasia does not initially present a difficult challenge; however, over time, atypia can develop in the architectural features of the node and cytologic features of the lymphocytes, and the lesion may eventually transform into lymphoma.[3] Given the difficulty in interpreting atypical lymphoid populations in ferrets and the known progression of IBD to gastrointestinal lymphoma, some cases of severe IBD can be difficult to diagnose accurately.[9] IHC can be used to immunophenotype, and therefore distinguish between a reactive and heterogeneous, or neoplastic and monomorphic, lymphoid population. Although there are reports of lymphoma immunophenotyping in domestic ferrets,[11,12] there are no studies available on the efficacy of IHC for the distinction of IBD and lymphoma.

2.1.2. Inflammatory bowel disease in cats

Presentation and possible causes

As in other domestic animals, IBD in cats is believed to be caused by a complex interaction between environmental factors, such as diet and intestinal flora, and the mucosal immune system. In susceptible animals, this interaction results in chronic inflammation.[13,14] The importance of food in the pathogenesis of IBD in cats is suggested by the clinical benefit of dietary therapy in some cases.[13] In a study on 14 cases of IBD in cats, purebred cats seemed to be more predisposed to developing this disease than mixed-breed cats, supporting the idea of genetic predisposition.[15] In a 1992 study by Jergens, Siamese cats appeared to be prevalent (19%) than other cat breeds among the 26 cases examinated.[16] However, a causal genetic defect has not yet been identified.[13] Increased populations of mucosal-associated Enterobacteriaceae were strongly associated with inflammation in the intestine of cats with IBD. Furthermore, the number of some mucosal-associated bacteria were correlated with abnormalities in mucosal architecture, upregulation of cytokine mRNA, and the clinical signs exhibited by affected cats.[14] These findings support the possibility that abnormal mucosal flora may be involved in IBD etiopathogenesis in cats.

Cats with IBD are usually middle-aged, but animals younger than 2 years of age may also be affected.[16] Vomiting, diarrhea, anorexia, and weight loss are the most common clinical signs associated with IBD in cats.[13] These clinical signs are nonspecific and overlap with several other feline diseases. The clinical course of IBD is generally cyclical with spontaneous exacerbations and remissions.[13]

An index for the assessment of inflammatory activity in cats with chronic enteropathy has been recently designed and helps in measure the clinical disease activity.[17] The feline chronic enteropathy activity index (FCEAI) takes into consideration clinical signs, laboratory abnormalities, and histopathologic lesions. Each component is scored and the sum represents the FCEAI score, used to assess and monitor the progression of the inflammatory process and the response to treatment.[17]

Diagnosis and treatment

Other causes of chronic intestinal disorders have to be eliminated to reach a diagnosis of IBD. Infectious and parasitic agents, non-gastrointestinal disorders such as endocrinopathies, exocrine pancreatic insufficiency, structural abnormalities, food-responsive enteropathy, and alimentary lymphoma need to be ruled out for a final diagnosis of IBD.[13] Biochemistry, hematology, and other laboratory tests (such as feline pancreatic lipase, cobalamin, folate, and thyroxine), as well as abdominal radiographs and ultrasound, may also be useful in eliminating other disorders and reaching a final diagnosis.[13] Endoscopy can be used to directly assess mucosal abnormalities and to biopsy mucosal samples for histopathology.[13] Endoscopic abnormalities seen in cats with IBD correlate with both clinical activity and histopathologic lesions.[14,17]

Nutritional therapy, aimed to minimize the exposure to dietary antigens known to evoke sensitivity, pharmacological therapy such as corticosteroids, antibiotics, and immunosuppressive agents, and the use of pre- and pro-biotics, used to re-equilibrate the intestinal flora, are commonly used to treat IBD in cats.[13]

Diagnostic challenges in cats

Grading schemes for the evaluation of intestinal biopsies in cats have been proposed but they have not been correlated with the clinical severity of the disease. Moreover, although there have been attempts to standardize the interpretation of GI inflammation,[6] inter-pathologist variability persists.[13,18,19]

Differentiation between IBD and intestinal lymphoma may be problematic in cats and requires intestinal biopsies often combined with the use of immunohistochemistry or polymerase chain reaction for antigen receptor rearrangement (PARR).[13,18] In a study by Evans *et al.*, endoscopic biopsies were not useful in distinguishing between IBD and lymphoma in cats and full-thickness biopsies of the intestinal tract were preferred.[9] One of the reasons full-thickness biopsies are preferred is that intestinal lymphoma is significantly more likely to involve layers of the intestine deeper to the lamina propria when compared to IBD,[18] and evaluation of the deeper layers is an important aid in the differentiation of these two diseases.

2.1.3. Inflammatory bowel disease in dogs

Presentation and possible causes

Canine idiopathic IBD is a group of disorders affecting the small and large intestine that have a chronic and recurrent course. Conversely to humans, where the large intestine is the mostly affected by idiopathic diseases, the part of the GI tract that is most affected by IBD in dogs is the small intestine.[20,21]

Dogs affected by IBD may present with a variety of clinical signs, including vomiting, diarrhea, alterations in appetite, and weight loss.[16,21] Melana and hematemesis may also be present.[16]

As in other species, in canine IBD, the cause for the inflammation remains largely unknown, but a combination of environmental, genetic, and immunologic factors are believed to be key in the pathogenesis of this disease.[20] Supporting a fundamental role of genetics in the development of this disease, there are a number of forms of IBD that are unique to certain breeds, including histiocytic ulcerative colitis in the Boxer dog, immunoproliferative enteropathy of Basenjis, and protein-losing enteropathy in Soft-Coated Wheaten Terriers.[20,21] German shepherd dogs (GSD) have shown increased susceptibility for lymphocytic-plasmacytic IBD.[21] In support of the genetic basis for this predisposition, different patterns of Toll-like receptors (TLR) have been identified in GSD compared with other healthy breeds.[22] In another study, single-nucleotide polymorphisms (SNPs) in TLRs have been identified, providing even more solid evidence of a genetic predisposition of GSDs for IBD.[23] The expression of TLRs have been investigated in other studies on canine IBD, and a correlation between expression of TLR2 and clinical severity has been identified.[24] It is interesting to note that this study failed to find a correlation between histologic severity of IBD (evaluated according to Jergens 1992[16]) and TLR2 expression.

Several attempts to create a grading scheme for IBD have been made. A simplified canine IBD activity index (CIBDAI) was proposed by Jergens in 2003.[25] This index is based on six prominent gastrointestinal signs (attitude/activity, appetite, vomiting, stool consistency, stool frequency, and weight loss) that are individually scored based on severity. A total score is than calculated for each case, and overall IBD is classified as clinically insignificant, mild, moderate,

or severe. The CIBDAI had good correlation with histological subjective grading and the elevation in acute phase proteins, most notably C-reactive protein (CRP).[25]

Diagnosis and histological grading

As in other species, intestinal histopathology is essential to confirm a diagnosis of IBD.[21] Abdominal imaging, hematology, biochemistry, and cytology are helpful in supporting a diagnosis of IBD and ruling out other possible differential diagnoses (i.e. infectious agents).[16,26]

In dogs, IBD can be classified based on the inflammatory infiltrate present as lymphocytic-plasmacytic, eosinophilic, and granulomatous enterocolitis.[16] Classification can also involve the area of the intestine involved (i.e. idiopathic mucosal colitis).[27] The two predominant types of canine IBD are lymphocytic-plasmacytic and eosinophilic enteritis.[20] While in other species (i.e. cats, ferrets) these have been treated as separate entities, with eosinophilic enteritis being part of a more systemic disease, in dogs they are both considered variants of IBD.[27] Lymphocytic-plasmacytic enteritis is the most commonly reported form of IBD in dogs.[21]

Several grading schemes for evaluation of endoscopic specimens in dogs with IBD have been described. Some studies have suggested a quantification of inflammatory cells in the lamina propria.[28] Others have evaluated overall changes, such as mucosal epithelial injury or loss of architecture,[16] while in other studies, both cellularity and architectural changes were taken into consideration.[29] Despite many efforts to standardize histopathologic diagnosis of enteric diseases,[6] inter-pathologist variability still exists, and a universal grading scheme for canine IBD has not been officially adopted.

2.2. Lymphoma in ferrets

Neoplasia in ferrets, a historic perspective

Reports of neoplasia in ferrets have been rare until the second half of the 20th century. As a matter of fact, in the early 60s, it was speculated that ferrets were rather resistant to tumor development.[30] In a literature review on ferret neoplasia from 1965, 3 out of 6 tumors reported were of genital origin.[30] In 1993, another study on spontaneously occurring neoplasia in a colony of ferrets, reported lymphoma as the most common neoplasm, followed by genital tumors.[31] The life span of the animals in one of the breeding colonies examined was up to 5 years, with occasional animals that reached 7 years of age. A total of 130 animals had been part of this colony, and among those, only 7 tumors were recorded in 8 years, with an incidence of 5.4% for naturally-occurring tumors.[31]

As the ferret has become more and more popular as a pet, the incidence of tumors has seen an increase. The incidence of ferret neoplasia in the United States was reported to be 12% in a large–scale study published in 1998.[32] This may be due to more sophisticated diagnostic investigations, or to the increase in the lifespan of pet ferrets as compared to working, research, or breeding ferrets. Age was determined to be a risk factor for neoplasia in non-domestic ferrets.[33] Similarly, older ferrets seem to be more predisposed to neoplasia.[32] Other factors such as inbreeding, genetic predisposition, retroviruses, unnatural light cycles, and high-carbohydrate diets may also play a role in this increased incidence .[34] With the increase in spay and neuter in the ferret population, the number of genital neoplasms has been dramatically reduced, while endocrine neoplasms, such as adrenal tumors and insulinomas, have increased in incidence.[32,34]

There is convincing evidence that gonadectomy plays a key role in the development of adrenocortical tumors.[35]

Nowadays most ferrets are neutered at an early age and lymphoma is now the 3rd most common neoplasm in ferrets, preceded in frequency by adrenal cortical neoplasms and insulinomas.[32,36]

Diagnosis

While the suspicion of a neoplastic process is often achieved through clinical history, physical examination, and imaging, a diagnosis of lymphoma often involves visualization of the neoplastic cells by cytology and histopathology. Fine needle aspirates can be obtained from enlarged lymph nodes or abnormal organs. The expected finding in lymphoma is a monomorphic population of lymphocytes. When the neoplasm is composed of small mature lymphocytes rather than large, immature lymphocytes, often a final diagnosis is achieved by examination of tissue architecture through a biopsy sample. Immunocytochemistry can be supportive of a monoclonal population of lymphocytes; however, immunocytochemistry presents more challenges than immunohistochemistry as the cellularity of cytological samples may vary, and the retrieval of positive and negative controls may be cumbersome. Although histopathology offers the advantage of tissue architecture, severely reactive lymph nodes may be difficult to interpret, as there is overlap between the histologic appearance of lymphoma and other non-neoplastic causes of lymphoadenomegaly.[4,34] Enlarged peripheral lymph nodes should be preferred to intra-abdominal ones for histopathologic evaluation.[34] Immunohistochemistry can be used, in these cases, to help distinguish between reactive and neoplastic populations.

Classification of lymphoma in the ferret

Lymphoma in ferrets has been classified following several classification schemes.

Site of origin

Based on the site of origin, lymphoma can be classified as multicentric, mediastinal, gastrointestinal, cutaneous, and extranodal.[11,37]

- Multicentric lymphoma is the most common presentation of lymphoma in ferrets older than 3 years of age.[11,38-40] In an imaging study[40] and necropsy study[12] performed on ferrets with lymphoma, intrabdominal lymphadenopathy and splenomegaly were the most common findings. Multiple, enlarged, abdominal lymph nodes, especially the mesenteric ones, were a predominant finding in both studies. In a study on lymphoma in ferrets, mesenteric lymph nodes were suggested as the most frequent site of tumor development.[12] This suggestion was made because of their frequent involvement and because, occasionally, lymphoma was seen in the mesenteric lymph nodes as an accidental finding in ferrets euthanized for other reasons. In an imaging study, only in 1 of 14 cases was there peripheral lymphadenopathy,[40] while 3 in over 29 cases presented with enlarged peripheral lymph nodes in another study.[12] In a study by Onuma *et al*., in the majority of cases where multicentric lymphoma was present, there was generalized, symmetrical enlargement of superficial lymph nodes, mainly the cervical lymph nodes.[11]
- Mediastinal T cell lymphoma arising from the thymus usually has an aggressive disease course. Mediastinal involvement is more common in ferrets less than 3 years of age.[32,41] Ferrets with mediastinal lymphoma may present with tachypnea or dyspnea secondary to a space–occupying mass, and pleural effusions .[38] In a study by Coleman *et al*.,[41] atypical

lymphocytes were present in the peripheral blood in 7 of 8 ferrets with mediastinal lymphoma, and lymphocytosis was present in 5 of them.

- Gastrointestinal (GI) lymphoma: the GI tract was the second most common site for development of lymphoma in a 2008 study on 20 ferrets.[11] However, the GI tract was underrepresented as a site of lymphoma origin in other studies.[38,41]
    - Gastric lymphoma normally occurs in older ferrets, it is of B cell origin, and has been described in association with *Helicobacter mustelae* infection.[42] It is believed that, in ferrets, prolonged immune stimulation caused by gastric *H. mustelae* infection, can induce neoplastic transformation,[42] similarly to what happens in humans. As evidence for this theory, in a study on *Helicobacter*-induced gastric lymphomas in ferrets, the neoplasm developed in the antrum, where gastritis and *Helicobacter* infection were more severe. In all 4 cases described in this study, the ferrets were greater than 5 years of age. Three of the ferrets presented with a history of weakness and wasting, while in 1 case, there were no signs of gastrointestinal illness, and the animal died during a seizure. The tumors were classified according to the National Cancer Institute classification for non-Hodgkin's lymphoma: 2 low grade neoplasms were classified as diffuse small lymphocytic lymphoma (1 of which had plasmacytoid differentiation), while the remaining high-grade lymphomas were classified as immunoblastic. In all cases, the neoplastic cells were B cell in origin and were positive for IgG and immunoglobulin K light chain by immunostaining. Demonstration through immunohistochemistry of a predominance of κ or λ light chains, as opposed to a balanced mixture of the 2, helped in reaching a diagnosis of neoplasia.[42]

- Intestinal lymphoma has been described as epitheliotropic, causing severe chronic diarrhea and melena[12] or non-epitheliotropic. Diffuse inflammation or nodular lesions were observed grossly in the small intestine of ferrets with intestinal lymphoma in one study,[11] and this finding was often associated with enlargement of mesenteric lymph nodes.

- Cutaneous lymphoma may present with dermal or subcutaneous masses, or may diffusely involve the epidermis as epitheliotropic lymphoma.[43,44] In epitheliotropic lymphomas, neoplastic cells have a strong affinity for epithelial structures, such as epidermis and hair follicles. Lesions are commonly seen in the feet and extremities.[34] The survival time for this type of lymphoma is generally long, and can extend up to 4 years. Complete surgical excision of cutaneous masses may prolong disease-free intervals, while chemotherapy has been demonstrated to often be unsatisfactory in the treatment of this disease.[34]
- Extranodal lymphoma is commonly seen in the spleen.[38,40] After the spleen, the most common extranodal sites of neoplastic infiltration are liver, kidneys, and lung,[38] but radiographic or sonographic studies are less effective in detecting the presence of lymphoma in those organs.[40]

Cellular type and distribution

Ferret lymphoma has been classified in 3 main categories based on broad cell type and distribution.[34]

- Lymphocytic form is the most common form. This form occurs in ferrets older than 2 years of age (2 to 9 years), and it typically has a chronic course of disease.[34,39] Lymph nodes are often affected, and peripheral lymphadenopathy is common. Chronic lethargy,

inappetence, and weight loss may be nonspecific signs related to this disease. Neoplastic cells are well-differentiated, small lymphocytes.[34]

- Lymphoblastic form is usually seen in ferrets younger than 2 years of age. Thymus, spleen, and liver are the organs that are most commonly affected, and organomegaly is often seen.[34,39] Clinical signs are often associated with the organs involved. Neoplastic enlargement of the thymus can compress the lungs, resulting in dyspnea and pleural effusion. The incidence of bone marrow involvement is highest in this form of disease. Neoplastic cells in this case are large, immature lymphoblasts, and the disease has a more rapid course.[34]
- Immunoblastic polymorphous type is similar to the lymphoblastic form but can occur in ferrets of all ages and has varying degrees of peripheral lymphadenopathy and visceral organ involvement. As with the lymphoblastic form, this form has a short survival time after diagnosis. This category also includes Hodgkin's-like lymphoma, which is rarely seen in ferrets.[34] In a study by Erdman,[39] 3 cases of Hodgkin's-like lymphoma were associated with the presence of another type of lymphoma in the same animal.

National Cancer Institute Working Formulation

Variants of ferret lymphoma can be classified based on cytomorphology according to the National Cancer Institute Working Formulation (NCI-WF). This classification includes 3 grades of progression from low (indolent) to high (rapidly progressive). According to the simplification included in a study on cat lymphoma by Valli *et al.*,[45] each category comprises the following.

- Low-grade lymphoid neoplasms are formed by the small-cell lymphomas, i.e., small lymphocytic lymphoma (SLL), small lymphocytic lymphoma with plasmacytoid differentiation (SLLP), small-cleaved cell lymphoma (SCC), small lymphocytic lymphoma with intermediate differentiation (SLLI), mantle cell lymphoma (MCL), and chronic lymphocytic leukemia (CLL). Cleaved cell lymphoma was the most common type of lymphoma in a study on 60 ferrets with lymphoma.[38]
- The intermediate-grade grouping is formed by the follicular lymphomas, i.e., follicular small cleaved (FSC), follicular mixed (FM), and follicular large (FL) lymphomas, as well as the marginal zone (MZL) and mucosal-associated lymphoid tumor (MALT) lymphomas plus the large B-cell lymphomas. This category was the most prominent (12/20 cases) in a study by Onuma *et al.*[11]
- The high-grade lymphomas include the acute lymphocytic leukemias of both B- and T-cell type plus the lymphoblastic and small noncleaved-cell lymphomas. The lymphoblastic form and the immunoblastic variant type described by Williams and Weiss[34] are present in this group. In a study by Onuma *et al.*, [11] all 4 high-grade lymphomas were classified as diffuse immunoblastic type and had GI origin.

World Health Organization classification scheme

In 2002, the World Health Organization (WHO) published the Revised European- American Lymphoma (REAL) classification scheme for lymphoma in domestic animals.[46] This classification scheme is based on cellular morphology, genetic alterations, clinical features, and immunophenotype of neoplastic lymphocytes, therefore considering all aspects of this neoplasia. Lymphoma in ferrets has been classified by several authors using this classification scheme.[12,37]

- Peripheral T cell lymphoma (PTCL) was the most frequent (17/29 cases) lymphoma type in a study by Ammersbach *et al*.[12] Cells were described as small to medium, with only a few tumors having medium to large cells, and low mitotic activity. Cells were immunoreactive for CD3.
- Anaplastic large T cell lymphoma (ALTCL): tumors composed of highly pleomorphic cells with marked anisocytosis and anisokaryosis, high mitotic rate, and immunoreactivity for CD3 were classified in this category. The majority of ALTCL were confined to the abdominal or thoracic cavity, and extensively involved the central and peripheral nervous system.[12]
- Anaplastic large B cell lymphoma (ALCBL) is composed of large, highly pleomorphic cells with frequent nuclear "blebs", with high mitotic rate and immunoreactivity for CD79a.[12] Similar to ALTCL, ALCBL also mainly involves the abdominal and thoracic cavities.
- Diffuse Large B Cell Lymphoma, Centroblastic type (DLBCL-CB). Only one diffuse tumor in 29 cases belonged to this category[12] and was composed of medium to large cells with minimal anisokaryosis , high mitotic rate, prominent nucleoli, and immunoreactivity for CD79a.
- Hodgkin's-like lymphoma has also been described in ferrets. Hodgkin's-like lymphomas are characterized by the presence of large numbers of small T cells, admixed with smaller numbers of Reed-Sternberg-like cells, which are large, pleomorphic, often binucleated, round cells of B origin.[47] In one study, animals presented with a single enlarged lymph node, the biopsy of which led to a diagnosis. The neoplasm ultimately progressed to multicentric lymphoma.[12] Histologically, this type of neoplasia in ferrets has been

associated with large areas of necrosis and foci of fibrinolysis, and by the presence of very large, uni-, bi-, or multinucleated cells consistent with the Reed-Sternberg cells described in Hodgkin's lymphoma in humans.[12] This tumor type appears to rapidly progress and disseminate and to be poorly responsive to chemotherapeutic treatment.[12] A case of non-Hodgkin's lymphoma in a ferret was associated with hypereosinophilic syndrome, likely due to the overexpression of eosinophil mediators, such as IL5, from the neoplastic T cells.[47]

Immumohistochemical analysis of lymphoma

B versus T cells: CD3 and CD79a

Lymphoma in ferrets can be classified as either B or T cell origin, based on the use of immunohistochemical markers, such as CD79a, a marker of B cells, and CD3, a marker for T cells. A study by Hammer *et al*.[37] demonstrated that only specific clones of CD79a were immunoreactive with ferret B lymphocytes, and there was also variability in sensitivity and specificity among clones used for CD3 staining. Lymphomas are most commonly of T cell origin in ferrets.[11,32,37] In a study on ferret lymphoma,[37] 27/43 cases of lymphomas were classified as T cell, 14/43 cases were B cell in origin, and in 2/43 cases the neoplasia did not stain positive for either marker. In a 2008 study, immunohistochemistry was performed on 18 lymphoma cases, 16 of which were positive for CD3, while just 2 demonstrated CD79a immunoreactivity.[11] All gastrointestinal lymphomas in this study[11] were classified as T cell origin. The predominance of T cell lymphomas in the GI tract has also been reported in dogs and cats.[48,49] No statistically significant difference between age distributions and immunophenotype was seen in ferret lymphoma.[37]

Additional immunohistochemical biomarkers in ferret lymphoma

- Cellular line markers. Other immunohistochemical markers that have successfully been used in ferret lymphomas are CD45 and CD45RO (common leukocyte antigens), CD30 (an antigen expressed by activated B and T lymphocytes, anaplastic large-cell lymphoma cells, and by Hodgkin and Reed-Sternberg (H-RS) cells), MUM1 (a marker of late stage B cell differentiation), vimentin (a marker for cells of mesenchymal origin), and CD34 (a marker expressed by immature hematopoietic cells).[37] In a case report on ferret Hodgkin's-like lymphoma Reed-Sternberg like cells were positive for BLA.36, a B lymphocyte antigen.[47]
- Proliferation and anti-apoptotic markers. In a study by Hammer *et al*.,[37] Ki-67, a marker for cellular proliferation, was applied to 43 cases of lymphoma in ferrets. Based on Ki-67 staining, the majority (51.2%) of the neoplasms classified as T cell in origin had a high proliferative index, while 16.3% of T cell lymphomas had an intermediate proliferative index. This finding demonstrated that the majority of T cell lymphomas are composed of more malignant cell lines, as observed in other studies.[38,50]

  Other biomarkers that have been used in ferret lymphoma are Bcl2 (an oncogenic, anti-apoptotic protein), Bcl10 (an apoptotic protein), and p53 (a tumor suppressor protein).[37]

  In a case report on ferret Hodgkin's-like lymphoma, Reed-Sternberg like cells were positive for PCNA , a proliferative cell nuclear antigen.[47]

Proposed classification scheme

Given the lack of a standardized classification scheme for ferret lymphomas, a new classification scheme has been recently proposed.[51] This scheme is based on:

1) Clinical signs
2) Histologic evaluation (following WHO-REAL guidelines)[46]
3) Immunophenotyping (B vs T cell lymphoma)

The authors also included a proposed staging scheme that would take into consideration the anatomic location, the presence of single versus multiple tumors, invasion of local or distal lymph nodes, and the presence of neoplastic cells in circulation.[51]

Abnormalities associated with lymphoma in the ferret

Hematology and biochemistry

Lymphopenia has been observed commonly in older ferrets with lymphoma, whereas lymphocytosis and leukemia are most common in younger ferrets.[38] Non-regenerative anemia is often seen in association with lymphoma in ferrets and was detected in 22 of 27 cases.[12] Anemia was also the most common hematological finding in a study on 12 ferrets with lymphoma.[38] Neutropenia and thrombocytosis can be present and are often a result of myelophthisis.[12] Pancytopenia may also be induced by myelogenous suppression associated with the neoplasm.[39] Hypereosinophilic syndrome has been reported in a case of disseminated Hodgkin's-like lymphoma.[47] It is interesting to note that mesenteric lymph nodes from healthy ferrets often have an increased number of eosinophils in the absence of peripheral eosinophilia,[52] and this should not be confused with inflammation or a paraneoplastic syndrome. Hypercalcemia has been occasionally described in association with T cell lymphomas[12,38] and has been associated with generalized tissue mineralization in one case.[32] Polyclonal hyperglobulinemia has also been noted in a small number of cases of T cell lymphoma.[12] Hypoalbuminemia is often associated with intestinal lymphoma and appears more severe with the epitheliotropic variant.[12]

Osseous lesions

Osseous lesions may be present and are frequently located in the lumbar spine.[40,53] A myelo-osteolytic plasmablastic lymphoma was recently described in a 6-year-old ferret with severe femoral lysis.[54] Plasmablastic lymphoma is considered a variant of diffuse large B-cell lymphoma. The tumor was not classified as a multiple myeloma given the absence of hyperparaproteinemia and Bence-Jones proteinuria; however, the distinction between multiple myeloma and plasmablastic lymphoma is somewhat nebulous. Multiple myeloma has been rarely reported in ferrets, with only 3 cases reported.[54] A malignant B-cell lymphoma with Mott cell differentiation was described in a 3.5 year-old with spenomegaly.[55] No hyperglobulinemia or monoclonal peak in the globulin fraction were detected using protein electrophoresis.

Possible etiologic agents of lymphoma in ferrets:

Viral-induced lymphoma

Association between lymphomas in ferrets and retroviral infection has been long suspected but has not been convincingly confirmed.[56,57] Erdman investigated familial predisposition and viral origin for lymphoma in ferrets; however, his results were not conclusive for either.[56]

Intraperitoneal inoculation of fresh malignant cells or cell-free supernatant from cultured malignant cells resulted in new cases of lymphoma in 3 of the 6 ferret recipients within 3 years of inoculation.[57] Two of the recipient ferrets developing lymphoma had received fresh donor lymphoma cells, while the remaining ferret had received supernatant from donor cell cultures. Furthermore, increased reverse-transcriptase activity was observed in splenic cell cultures from

these affected ferrets, and electron microscopy (EM) of the recipients' tumor tissue revealed retroviral particles.

Electron microscopy performed on neoplastic cells from a spinal lymphoma in a ferret failed to reveal viral particles.[53] No viral particles were observed during EM analysis of a cell culture derived from the neoplastic lymphocytes. Furthermore, PCR-based analysis using conserved retrovirus primers did not detect retroviral nucleic acids in a sample collected from the tumor.[53]

Possible etiologic agents investigated in the ferrets included feline leukemia virus (FeLV) and Aleutian mink disease virus (AMDV). A cluster of outbreaks of mediastinal lymphoma has been observed in juvenile ferrets from a colony.[50] The ferrets were tested for both FeLV and Auletian disease virus (ADV), but were negative for both. Genetic predisposition of related ferrets or common exposure to a carcinogen were also considered in this case. A correlation with feline leukemia virus (FeLV) was suspected in ferrets living in the same environment as FeLV-positive cats,[58] but testing for FeLV virus in ferrets in this case and in others was negative.[39,58] However, a report of seropositivity for FeLV in a healthy ferret was published in 1983,[59] and a positive monoclonal antibody ELISA test for FeLV has been reported in a ferret with splenic lymphosarcoma.[60] Cross-reactivity with a ferret specific retrovirus could not be excluded in these cases.

Aleutian mink disease is a parvovirus-associated disease that presents with lymphoadenomegaly and splenomegaly and can cause a polyclonal gammopathy.[40] Cytologically, there is lymphoplasmacytic inflammation; persistent stimulation could theoretically lead to a neoplastic transformation. However, a convincing link between this virus and lymphoma has not been demonstrated,[61] and studies by Erdman found that there was no

association between infection with AMDV and lymphoma,[56] and that AMDV infection was rare in ferrets with lymphoma.[39]

Bacterial-induced lymphoma

Gastric lymphoma has been linked with infection with *Helicobacter mustelae*, a Gram-negative bacterium .[42] This association is similar to what is observed between human MALT lymphomas and *Helicobacter pylori*. As in humans and other domestic animal species, a link between IBD and lymphoma has been established in the ferret.[3,38] The common belief is that chronic antigenic stimulation can progress from lymphoid hyperplasia to eventually a neoplastic lymphoproliferative disorder.

Lymphoma prognosis

Responses to therapy are unpredictable and while some ferrets have been described as living up to 2 years without treatment after the diagnosis of lymphoma, others have died shortly despite chemotherapeutic treatment.[39] Similarly, in a study in which survival was available for 19 cases, the mean survival time for ferrets with T cell lymphoma was 5 months (4.3 months with chemotherapy and 5.7 months without), while for B cell lymphoma, mean survival time was 8.4 months (8.8 months if treated and 7 months without).[12] Based on the classification scheme by Williams and Weiss, the lymphocytic form, composed of mature lymphocytes, tends to have longer survival times, while the lymphoblastic and immunoblastic types tend to have a more grave prognosis and rapid disease course.[34] Similarly, in another study, neoplasia seen in older ferrets seems to respond better to therapy, while lymphomas in younger ferrets is poorly

responsive.[39] Cutaneous lymphoma also generally has a long survival time, especially if the cutaneous lesion is surgically resected.[34]

CHAPTER 3

PROPOSED GRADING SCHEME FOR INFLAMMATORY BOWEL DISEASE IN THE FERRET (*MUSTELA PUTORIUS FURO*) AND CORRELATION WITH CLINICAL SIGNS[1]

[1] Cazzini, P., Watson, M.K., Gottdenker, N., Mayer, J., Fox, J. G., Parry, N., Reavill, D., and K. Sakamoto. Submitted to *Veterinary Pathology*, 10/03/2014

Abstract

Inflammatory bowel disease (IBD) is a common, idiopathic, chronic, inflammatory disease of the gastrointestinal (GI) tract in ferrets. Clinical signs for IBD in ferrets are not always easily recognizable, and microscopic examination of intestinal biopsies is necessary for a definitive diagnosis. A universally-accepted grading scheme has not been established for ferrets but may reduce variability in histopathologic interpretation. Additionally, the association between the severity of histologic lesions and clinical signs in ferrets is unknown. The objective of this study was to evaluate enteric samples from ferrets diagnosed with IBD, compare histologic grading schemes for IBD, and to correlate the results with the severity of clinical signs. Enteric sections from 23 ferrets with IBD were analyzed using two grading schemes used in the assessment of IBD in cats and dogs, and a correlation between histologic lesions and clinical signs was evaluated. After dividing the histologic samples in groups based on severity of clinical signs, identification of the main histologic differences was performed. Age and sex were also assessed for correlation with clinical signs. No significant correlation was found between the 2 grading schemes and clinical signs (rho= -0.02, p= 0.90; rho= -0.03, p= 0.88). Degree of villous fusion, hemorrhage/fibrin, necrosis, inflammation density, and crypt abscess formation were additional histological characteristics that were compared with clinical severity. As expected, there was a statistically significant correlation between this third, retrospective grading scheme and severity of clinical signs (rho= 0.45 p= 0.02). Correlation between age (p=0.04) and females (p=0.007) with severity of clinical signs was also observed. The established retrospective grading scheme may have clinical utility in determining severity of IBD in ferrets.

Introduction

Inflammatory bowel disease (IBD) is an idiopathic, chronic, inflammatory disease of the gastrointestinal tract, often involving both the stomach and intestine. Although it was virtually unheard of a decade ago, today IBD is considered the most common, yet most unrecognized, disease in ferrets.[62] IBD is usually diagnosed in ferrets over 1 year of age, and many ferrets are asymptomatic or demonstrate only subtle signs of illness that are often overlooked by the owners.[7,62] Clinical signs of IBD are nausea (manifested by bruxism and salivation), anorexia, chronic weight loss, greenish to brown, mucoid to "birdseed" ("seeds" represent undigested globules of fat and proteins) diarrhea, melena, proctitis, and rectal prolapse.[7,62] It is known that if untreated, IBD can progress to lymphoma.[3,62]

The etiology of IBD in ferrets, as in other species, such as cats, dogs and humans, is unknown. It is hypothesized that the underlying cause involves a complex interaction between infectious agents and dietary proteins that are new to this species and the mucosal immune system.[62] This interaction in susceptible patients results in a chronic inflammatory response. Known causes of gastritis and/or enteritis in ferrets include *Helicobacter,* Coronavirus, *Lawsonia intracellularis* (proliferative ileitis/ colitis), cryptosporidiosis, coccidiosis, and foreign body ingestion. These pathogens or intestinal microbiotes may be the trigger for an aberrant immune response that may continue long after the primary cause has resolved.

In humans[63] and dogs,[23] defects in immune receptors for recognition of commensal bacteria resulting in an upregulation of inflammatory cytokines play a role in IBD susceptibility. Two separate studies in dogs with IBD demonstrated upregulation of Toll-like receptors (TLR), cell receptors that are mainly found in leukocytes and recognize invasive microorganisms, in intestinal samples.[24,64] Specifically, upregulation of TLR 2 was correlated with severity of

clinical disease in dogs.[24] However, it is unknown if genetic defects play a role also in the development of IBD in ferrets and cats.[21] Dietary factors may also play a role in susceptibility of domestic dogs, cats, and ferrets to IBD.[16,62] Ferrets are obligate carnivores and require a meat diet high in quality proteins and fat, and low in carbohydrates and fiber.[65] It is possible that domestic ferrets diets, including commercial formulations, may expose ferrets to 'novel' proteins and changes in dietary components (e.g. protein, fat, carbohydrates), potentially triggering IBD. Controlled or elimination diets often have a positive effect on feline and canine IBD.[21] Although the exact cause of IBD is unknown, treatment for IBD in dogs, cats, and ferrets consists of the removal of the possible underlying cause(s) of intestinal inflammation and suppression of the inflammatory response with azathioprine or corticosteroids.[62]

Although there is evidence of contributing causes, the exact etiology of IBD across species remains largely unknown, and diagnosis of IBD is achieved by the exclusion of infectious agents and environmental causes.[21] Microscopic examination of intestinal biopsies, ideally full-thickness, is necessary for a definitive diagnosis of IBD.[7,9,62] Histopathologically, IBD is characterized by shrinkage and blunting of the intestinal villi and by an inflammatory infiltrate of the mucosa and lamina propria. The inflammatory cells present are predominantly lymphocytes and plasma cells, but less commonly, an eosinophilic component is present.[62] IBD with eosinophilic infiltrates should be distinguished from the less common, eosinophilic granulomatous disease that involves multiple organs and is not limited to the gut.[7,62]

Ideally, protocols and dosages for long-term treatment should be based on clinical signs and intestinal histopathology,[62] but a universal grading scheme to analyze ferret IBD has not been established, creating confusion with regards to what should be considered mild, moderate, or severe IBD. Underestimation of IBD severity could lead to an inefficient treatment protocol

and possible progression to intestinal lymphoma, while overestimation could lead to unnecessary and potentially life-threatening, drug side effects (such as immune or bone marrow suppression). A grading scheme is therefore necessary for more consistent and accurate disease assessment, and therefore, a more efficient therapeutic plan.

Histopathologic interpretation is subjective, and it suffers from interobserver variability.[66] For this reason, several grading schemes have been proposed for the evaluation of intestinal specimens of dogs and cats with IBD.[5,6,16] A universal grading scheme will make diagnoses more uniform among pathologists, granting more consistency. In addition, a correlation between the degree of histopathologic lesions and clinical sign severity would be most useful in managing and assessing patients. The association between the severity of the inflammatory infiltrate and clinical signs in ferrets is not known. Accordingly, the aim of this study was to assess 2 grading schemes for intestinal IBD in ferrets and test their correlation with the clinical presentation. Additionally, we separated the intestinal samples based on the severity of clinical signs and designed a third grading scheme. Correlation between age and sex with severity of clinical signs was also tested.

Materials and methods

Study Population

We collected 63 specimens from ferrets. Specimens included full-thickness biopsy samples from small intestines. Often, multiple intestinal sections were available from the same ferret. Intestinal sections of 3 unaffected ferrets were used as controls. Inclusion criteria for this study included:

- Ferrets of more than 1 year of age; IBD is rarely present in ferrets younger than 1 year of age
- No other major lesions present; all cases where a secondary disease could have been the cause of all or part of the clinical signs described were excluded from this study
- Clinical history available
- Sample in adequate condition; if the histologic section was too small or contained large artifacts, it was excluded from this study

Among the initial 63 cases, 24 were excluded because of the presence of a severe secondary disease. Secondary diseases included lymphoma (10 cases), insulinoma (8 cases), intestinal carcinoma (2 cases), gastrointestinal foreign body (2 cases), adrenal carcinoma (1 case), and suppurative pyelonephritis (1 cases). Nine cases were excluded because the animals were younger than 1 year of age or the age was not specified. In 3 cases, no clinical history was provided, and for 1 case, the sample was inadequate.

After applying the exclusion criteria, 26 ferrets (23 cases of IBD and 3 control samples) were included in this retrospective study. Twenty-one of the specimens had been submitted to the Zoo/Exotic Pathology Service (ZEPS, Sacramento, CA), and 5 had been submitted to Massachusetts Institute of Technology (MIT, Cambridge, MA). Clinical history was available for all of the cases evaluated and included a variety of clinical signs ranging from weight loss, anorexia, and diarrhea, to sudden death.

Slide preparation

Paraffin-embedded tissues were sectioned at 4 to 5 µm thickness, stained with routine hematoxylin and eosin (HE) stains, and examined microscopically.

Case Evaluation

For the first grading scheme, the following parameters were recorded for all HE sections, as in Kiupel *et al.* (2010):[5] location, distribution, and density of the lymphocytic population. The location was recorded as mucosa, submucosa, tunica muscularis, serosa, or intravascular, in each section corresponding to the deepest layer reached by infiltrates. Mucosal lymphoid infiltrates were classified as diffuse, and were further divided into low, medium or high-density, or multifocal. Lymphocyte infiltrates within the epithelium were classified as being present in the surface, in the crypts, or in both locations. Intraepithelial infiltrates were further categorized based on their distribution as single cells, nests, or plaques. In addition, the lymphocytic infiltrates were evaluated for monomorphism versus polymorphism, number of mitotic figures per 400X field, and cell size (large vs small).[5] Descriptive terms were converted into numerical

Table 3.1: Scoring system for grading scheme 1 (according to Kiupel *et al.*[5])

Score	**0**	**1**	**2**	**3**	**4**	**5**
Location	Absent	Mucosal	Submucosal	Tunica muscolaris	Serosal	intravascular
Density of mucosal infiltrates (if diffuse)	Absent	Low density (<2 cells)	Medium density (3-6 cells)	High density (>7 cells)		
Density of mucosal infiltrates (if multifocal)	Absent	Present				
T cells within epithelium	Absent	Surface	Crypts	Both		
Intraepithelial infiltrates	Absent	Single cells	Nests	Plaques		
Lymphocytes morphology	Monomorphic	Polymorphic				
Mitotic index	0	0-2	>2			
Cell size	small	large				

values attributing higher values to more severe manifestations of inflammation (Table 3.1).

For the second grading scheme, the following parameters were recorded for all HE sections, according to Day *et al*. (2008)[6] and following recommendations recently discussed by Willard and Mansen:[67] villous blunting, crypt abscesses, and lymphangectasia (Table 3.2).

Table 3.2: Scoring system for grading scheme 2 (according to Day *et al*.[6] and following recommendations by Willard and Mansen[67])

Score	0=absent	1= mild	2=moderate	3= severe
Villous blunting	No villi blunting	Villi reduced to 75% of normal length	Villi reduced to 50% of normal length	Villi reduced to 25% of normal length
Crypt abscesses	No crypt distention	10% of crypts distended	25% of crypts	50% of crypts
Lymphangectasia	No lacteal dilation	Lacteal up to 50% of width of the villous	Lacteal up to 75% of width of the villous	Lacteal up to 100% of width of the villous

A degree of clinical severity was assigned to each case based on the clinical signs reported (Table 3.3).

Table 3.3: Scoring system for severity of clinical sings

Degree	Absent	Mild	Moderate	Severe
Clinical signs described	• No GI - related clinical signs	• Mild Weight Loss • Inappetance • Mild Dehydration • "Not doing well" • Occasional vomiting	• Chronic weight loss • Moderate Diarrhea • Moderate Dehydration • Frequent vomiting	• Severe weight loss and diarrhea • Critically ill • Found dead • Emaciated

For the third, retrospective, grading scheme, samples were divided into groups based on the degree of clinical signs. The slides were examined as groups and new criteria were selected. Degree of villous fusion, hemorrhage/fibrin, and necrosis was considered to differ between the

various groups (Figure 3.1). In addition, among the criteria used in the other 2 grading schemes, inflammation density from the grading scheme by Kiupel,[5] and crypt abscesses, from the grading scheme by Willard and Mansen[67], seemed to correlate with the severity of clinical signs. The slides were re-scored once more in a blinded fashion using these paramenters, as illustrated in Table 3.4.

Table 3.4: Retrospective scoring system

Score	**0**	**1**	**2**	**3**	**4**
Villous fusion (crypt #:villous #):	absent	2:1	3:1	4:1	5+:1
Hemorrhage/fibrin:	absent	focal	multifocal	locally extensive	diffuse
Mucosal epithelial damage:	absent	vacuolation/ attenuation	focal necrosis	ulceration	
Inflammation density	absent	Low density	medium density	high density	
Crypt abscess	no crypt distention	mild crypt distention	moderate crypt distention	marked crypt distention or abscess	

Gender and age were also correlated with the severity of clinical signs.

Statistical Analysis

After converting the descriptive data into numerical values and summing scores so that higher severity corresponded to higher total scores (see tables 3.1 to 3.3), the first two grading schemes according to Kiupel *et al*. (2010)[5] and Willard and Mansen,[67] were correlated to the severity of clinical signs using Spearman's rank correlation.

The final scores on the retrospective grading scheme based on 3 new criteria (villous fusion, hemorrhage or fibrin, and mucosal epithelial damage), and on 2 criteria from the previously tested grading schemes (inflammation density, from Kiupel,[5] and crypt abscesses, from Willard

and Mansen[67]) were added (Table 3.4) then correlated to the severity of clinical signs. Spearman's rank correlation was used to determine the correlation between the score obtained with this third grading scheme and the severity of clinical signs.

The correlation between severity of clinical signs with age and sex of the ferrets was assessed using Spearman's rank correlation and Wilcoxon rank sum test with continuity correction, respectively.

Results

Neither of the first two grading schemes was a significant predictor of clinical severity. The correlation between the severity of clinical signs and the grading scheme for intestinal inflammation according to Kiupel *et al.* (2010)[5] was performed with Spearman's rank correlation (rho= -0.02, p= 0.90). The correlation between the severity of clinical signs and the grading scheme for intestinal inflammation according to Willard and Mansen[67] was also performed with Spearman's rank correlation (rho= -0.03, p= 0.88).

There was a moderate but significant, positive correlation between the third, retrospective grading scheme and clinical severity (Spearman's rank correlation; rho= 0.45 p= 0.02).

There was mild, positive correlation between ferret age and IBD severity of clinical signs (Spearman's rank correlation, p=0.04). There was a significant difference in IBD severity of clinical signs and sex (male vs female, not accounting for spayed or neutered), with females tending to have more severe clinical signs associated with IBD (Wilcoxon rank sum test with continuity correction, p: 0.007)

Discussion

No correlation was observed between either of the initial two grading schemes evaluated (both were developed for small animals) and the severity of clinical signs reported. This may be due to several reasons. In veterinary medicine, the registered clinical signs heavily rely on what is observed by the owners. There is great variability in the owner's attention for changes in pet behavior and for manifestations of pain, resulting in a potentially inaccurate and disparate collection of clinical signs in this study. Additionally, recognizing discomfort or pain in a ferret is often difficult[62] and requires careful observation of an undisturbed animal.[8]

Crypt distortion, villous blunting and fusion, and fibrosis were most commonly seen in cats with moderate or severe IBD in a study by Baez *et al.*,[68] and abnormalities in mucosal architecture, principally villous fusion and atrophy, were correlated with the number of clinical signs in a study on IBD in cats.[14] Correlation between clinical signs assessed using a canine IBD activity index (CIBDAI) and histopathologic changes has been successful in a study by Jergens and coworkers.[25] However, controversy still exists in the correlation between histology and clinical signs of IBD.[69] Part of the problem is due to interobserver variation among histopathologic evaluations of intestinal tissues.[66] Additionally, the absence of a unanimously recognized grading scheme for small animal enteric lesions has created disparity in diagnosis. Attempts have been made to promote universal criteria for histological evaluation of enteric samples;[6] however, even with this grading scheme, differences in tissue processing still creates disagreement among pathologists, especially in distinguishing mild and moderate processes.[19] Collecting surgical biopsy samples rather than endoscopic ones yield a more accurate diagnosis in enteric samples from cats and is preferable.[9] In this study, only full-thickness enteric biopsies were included. Another limitation in correlating clinical signs with histopathologic findings may

be due to the failure to biopsy the relevant area of the intestine that is causing the clinical signs.[70] In the present study, when multiple samples were available from the same animal, the one exhibiting the most severe histologic changes was considered. Unfortunately, in some cases, only one sample of intestine was available for evaluation, and the possibility of having missed a more severe lesion exists.

Given the retrospective nature of the third grading scheme, a positive correlation with clinical sings was expected, although the pathologist using the scheme was again blinded to the original diagnosis of each slide. Regardless, the identification of histologic characteristics that correlate with the severity of clinical sings is significant. A prospective study will be needed to test the efficacy of this grading scheme and its true correlation with clinical severity.

In this study, a mild correlation between severity of clinical signs for IBD and ferret age was observed. This may be due to the prolonged effects of this chronic disease accumulating over time or to a decrease in pain tolerance in older animals. Increase in age has been observed to decrease pain perception in a human study.[71] In a study by Jergens on 58 dogs and 26 cats with IBD, no age predisposition was observed.[16] However in Jergens' study, the severity of clinical signs was not correlated with the age of the animal. It is also possible that aging animals may be affected by concurrent diseases contributing to their clinical signs, although we tried to rule out this possibility when multiple tissues or diagnoses were provided.

Female ferrets had more severe clinical signs of enteric disease. Although the literature is often controversial regarding the effects of sex over sensitivity to pain in humans, females are generally considered more sensitive to noxious stimuli and are at higher risk for many common pain conditions.[72] In a 1992 study by Jergens, no sex predisposition for IBD was observed in

either dogs or cats,[16] and sex was not reported to affect the density of immune cells in the lamina propria or epithelium in dogs with enteropathies.[73]

Limitations of this study consist of a small sample size available for analysis. Unfortunately, the frequent concomitant occurrence of other diseases in ferrets with IBD, lead to exclusion of a high number of cases because of a possible confounding effect on clinical signs. Furthermore, because this was a retrospective study, the clinical history was often limited and was not always centered on the GI signs. The use of a questionnaire with guided questions may be advised in prospective studies. Additionally, standardization of sampling sites and the examination of multiple biopsy samples from the intestine of the same patient may avoid missing the most severe lesion causing the clinical signs.

In conclusion, although the use of previously established grading schemes for small animal IBD did not correlate with severity of clinical signs in ferrets, the histologic characteristics identified in the third, retrospective grading scheme in this study revealed a possible correlation between intestinal lesions and clinical signs. Additionally, female and older ferrets may be more predisposed to show more severe clinical signs of IBD.

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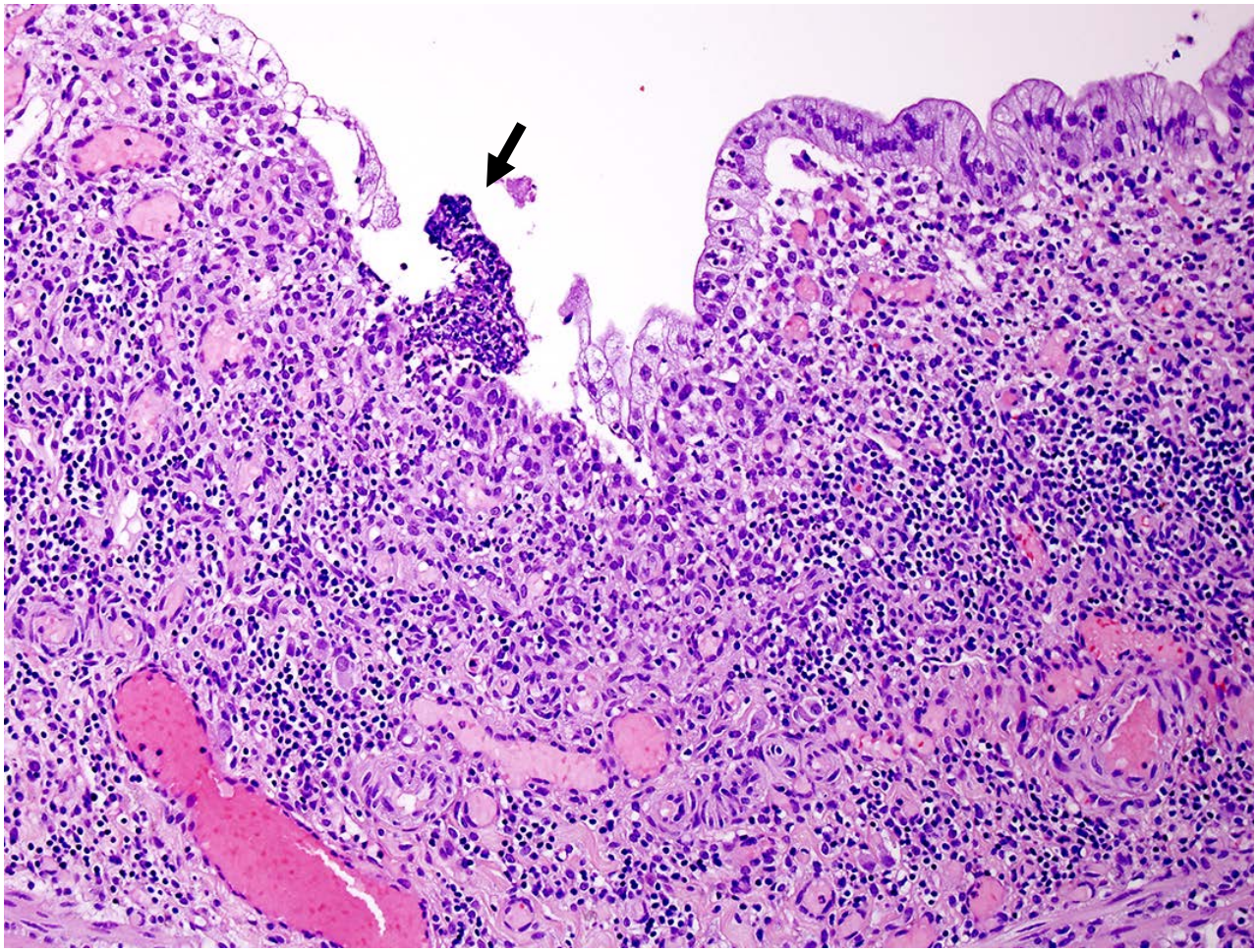

Figure 3.1. Severe inflammatory bowel disease, small intestine, ferret. This image shows severe villous blunting and necrosis (arrow). HE.

CHAPTER 4

HISTOPATHOLOGY AND IMMUNOHISTOCHEMISTRY OF SEVERE INFLAMMATORY BOWEL DISEASE VERSUS LYMPHOMA IN THE FERRET (*MUSTELA PUTORIUS FURO*)[2]

[2] Cazzini, P., Watson, M.K., Mayer, J., Gottdenker, N., Reavill, D., Parry, N., Fox, J.G., and K. Sakamoto. To be submitted to *Veterinary Pathology*

Abstract

Inflammatory bowel disease (IBD) is a common chronic disorder of the gastrointestinal tract in ferrets that may progress to lymphoma. Although routine histopathology is commonly used for distinguishing between IBD and lymphoma, misclassifications may occur. Immunohistochemistry (IHC) is commonly used to distinguish between IBD and lymphoma in small animals. The objective of this study was to determine the agreement in the diagnosis reached solely using hematoxylin and eosin (HE)-stained sections versus using a combination of HE and IHC. Enteric sections from 47 ferrets previously diagnosed with IBD or intestinal lymphoma by pathologists with expertise in ferret pathology were analyzed. A pathologist blinded to the original diagnosis assessed the same HE-stained sections. Analysis was then repeated using HE sections in parallel with sections stained using antibodies against CD3 and CD79a. No significant difference was found between pathologists ($p=0.91$) or between HE versus HE and IHC ($p= 0.16$). Diagnostic agreement between HE and IHC was 93.8% for cases ultimately diagnosed as lymphoma and 96.4% for IBD cases. There was no significant age ($p= 0.29$) or sex ($p= 0.29$) difference between diagnoses. The histologic location of CD3+ T cells did not differ between inflammatory and neoplastic diseases. In lymphoma cases, microanatomic location was not predictive of neoplastic cell immunophenotype. Results suggest that while IHC is not necessary to distinguish IBD from intestinal lymphoma in ferrets, it can be a useful tool to reach a definitive diagnosis in cases of severe IBD.

Introduction

Inflammatory bowel disease is a common chronic disorder of the gastrointestinal tract in ferrets over 1 year of age.[4] There are two common types of IBD in the ferret: multiple

eosinophilic syndrome and lymphoplasmacytic enteritis. Clinically, lymphoplasmacytic enteritis is a subtle disease that may not be noticed by the trained eye of the clinician or owner until it is severe. Although the exact etiology of IBD is unknown, many hypotheses exist, including genetic background, hypersensitivity reaction, or an atypical immune response.[7] There is speculation that severe gastrointestinal (GI) tract inflammation could be caused by commercial ferret diets, as seen in small animals with cases of diet-responsive, chronic enteropathy.[74] Ferrets are strict carnivores. Their extremely short GI tract and lack of a cecum result in a highly motile and excitable GI tract with a transit time of approximately three to four hours.[75] Because they are true carnivores, ferrets require a diet of high fat and protein, with low carbohydrate and fiber content. Many commercial pelleted ferret diets contain greater amounts of carbohydrates and fiber,[76] which with chronic exposure could lead to irritation and/or inflammatory effects on the GI mucosa.

The GI tract of domestic animals is rich in lymphoid tissue. Lymphoid cells in the mucosa are predominantly T lymphocytes,[5] while submucosal lymphoid follicles are predominantly composed of B cells.[77] Overstimulation or lack of regulation of intestinal lymphocytes may lead to two of the most common diseases seen in the intestinal tract of ferrets: inflammatory bowel disease (IBD) and lymphoma. Multiple, full-thickness GI biopsies are used ante mortem to distinguish between these two lymphoproliferative diseases and to guide optimal therapeutic management.

In 2008, the World Small Animal Veterinary Association (WSAVA) published a histologic grading system for gastrointestinal inflammation in the dog and cat.[6] Currently, there is no grading system utilized when analyzing ferret intestinal samples to distinguish between severe IBD and lymphoma, leading to possible disparity between pathologists. Due to the lack of

clear standards or classification of the histopathology, it is difficult to clearly separate the stages of IBD from each other, and misclassification between severe IBD and lymphoma may occur. The current theory is that chronic prolonged exposure to GI irritants, such as an abnormally high amount of carbohydrates, could induce a spectrum of disease severity ranging from mild, moderate, to severe IBD, eventually leading to lymphoma.[4,78] A similar correlation has been established in ferrets between chronic *Helicobacter mustelae*-associated gastritis and gastric lymphoma.[42] The basis of this correlation is that during chronic infection, lymphoid follicles enlarge and may eventually progress to low-grade lymphomas, or mucosa associated lymphoid tissue (MALT)-omas, which exhibit lymphoepithelial changes, germinal centers, and B cell phenotype.[42,79]

Lymphoma is the most common malignant neoplasm in the ferret.[39,61,80] In order to classify the specific type of GI lymphoma in ferrets, as in other species, further immunophenotyping diagnostics, such as immunohistochemistry (IHC), are often performed. Immunophenotyping studies have determined that in ferrets, B cell lymphomas have a better prognosis than T cell lymphomas.[12] With increased understanding of ferret neoplasia and advanced diagnostic capabilities, treatment options can be optimized to the individual disease and patient.

Immunophenotyping of lymphoma in ferrets shows a predominance of T cell lymphomas in the GI tract .[11,12,37] In addition to predicting prognosis, lymphocyte immunophenotyping can be used for making a definitive diagnosis when a histopathologic diagnosis of neoplasia is difficult to make based solely on HE-stained sections. IHC can be used to distinguish between a reactive and heterogeneous, or neoplastic and monomorphic lymphoid population. Despite the importance of IHC when examining intestinal sections in ferrets, no studies have characterized

the immunophenotype of severe IBD or determined whether IHC is necessary in distinguishing IBD from lymphoma.

It is commonly believed that T cell lymphomas of the GI tract tend to exhibit epitheliotropism, and neoplastic cells are often located in the lamina propria, while B cell lymphomas arise from the submucosal germinal center.[81] In a recent study on feline gastrointestinal lymphoma, the normal intraepithelial distribution pattern of T lymphocytes was echoed in T cell lymphomas, the majority of which were classified as mucosal lymphomas.[82] In the same study, all of the B cell lymphomas had a diffuse distribution.[82]

The purpose of this study was to investigate the diagnostic accuracy of tissue analysis using HE staining alone or in combination with IHC to reach a final diagnosis of IBD or lymphoma. We hypothesized that there would be a difference in diagnoses between intestinal samples using HE alone or those in conjunction with IHC, and that HE staining alone would significantly underdiagnose lymphoma. We also hypothesized that IBD would be more prevalent in younger animals, while lymphoma would be more common in older animals, with no difference in the sex of animals with regard to either diagnosis. We expected that the percentage and distribution of T and B lymphocytes in the various intestinal layers would differ between IBD and lymphoma, and in T versus B cell lymphoma cases. Furthermore, we hypothesized that microanatomical location would be a good predictor for neoplasia and immunophenotype, because typically T lymphocytes are more prevalent in the mucosa and B lymphocytes are more prevalent in the submucosa and tunica muscularis.[5,77]

Materials and methods

Study Population

Forty-seven ferrets were included in this retrospective study. Forty-one of the specimens had been submitted to the Zoo/Exotic Pathology Service (ZEPS, Sacramento, CA), and 6 had been submitted to the Massachusetts Institute of Technology (MIT, Cambridge, MA). Specimens included full-thickness biopsy samples from the intestine. Often multiple intestinal sections were available from the same ferret; however, since small intestine was more consistently available, we elected to limit our study to this part of the intestinal tract. Based on histopathology only, all ferrets had been diagnosed as either IBD (29 cases) or intestinal lymphoma (15 cases) at the institutions of origin by board-certified, veterinary pathologists with expertise in ferret pathology. For the IBD cases, only cases affected by the lymphoplasmacytic variant were considered in this study. Intestinal sections of 3 healthy ferrets were used as a control. In 4 ferrets, some intestinal sections were diagnosed as lymphoma, while other sections from the same ferret were diagnosed as inflammatory. Clinical history was available in 44 of the 47 cases evaluated and included a variety of clinical signs ranging from weight loss, anorexia, and diarrhea, to sudden death. For all cases, the age and sex of the animal were also available. Age and sex distribution for included cases are shown in Table 4.1. Ferrets less than 1 year of age were excluded from the study.

Histopathology

Full-thickness biopsies from the small intestine were available for all ferrets included in the study. For all cases, formalin-fixed, paraffin-embedded samples were available for HE and

IHC. Tissue samples embedded in paraffin were routinely processed and sectioned at approximately 5 μm thickness. All tissue sections were stained with HE.

Table 4.1: Age and sex distribution of the population of ferrets with samples included in this study. All ferrets under one year of age were excluded from analysis.

Variable	IBD	Lymphoma
Total cases	29	15
Mean age	4.1 years old	6.8 years old
Median age	4 years old	5 years old
Age Range	1.5 – 8 years	2 – 7 years
Sex	Female: 6 Male: 23	Female: 6 Male: 9

Immunohistochemistry

Serial sections were deparaffinized and rehydrated for routine IHC to detect surface expression of CD3 (1:800 dilution; Polyclonal Rabbit Anti-Human CD3 Antibody [A0452], Dako, Carpinteria, CA) for T cells and cytoplasmic expression of CD79a (1:50 dilution; Monoclonal Mouse Anti-Human CD79αcy Clone HM57 [M7051], Dako, Carpinteria, CA) for B cells. Antigen retrieval methods consisted of heat-induced epitope retrieval (HIER) using citrate buffer at a pH of 6.0 (HK086-9K, Biogenex, San Ramon, CA). Endogenous peroxidase was blocked using 3% hydrogen peroxide (H312-500, Fisher Scientific, Fair Lawn, NJ). Protein blocking was performed using Power Block (HK085-5K, Biogenex, San Ramon, CA). Positive immunohistochemical controls consisted of formalin-fixed, paraffin-embedded, canine tonsil and lymph node. As a negative control, the primary antibody was eliminated and substituted with

purified rabbit immunoglobulin (NC495H, Biocare Medical, LLC, Concord, CA) or purified mouse immunoglobulin (NC494H, Biocare Medical, LLC, Concord, CA) for CD3 and CD79a, respectively.

Case Evaluation

Histologic sections of small intestine stained with HE were evaluated by a board-certified veterinary pathologist blinded to the original diagnosis, and based on the appearance of the most severely affected section, a diagnosis of IBD or lymphoma was made subjectively. The specimens were categorized according to the World Health Organization Histological Classification of Hematopoietic Tumors of Domestic Animals.[46] The same pathologist analyzed the HE-stained sections a second time, in parallel with the serial IHC preparations for CD3 and CD79a, and without knowledge of the previous results. These markers were chosen based on previous studies using IHC in intestinal lymphoma in ferrets.[11,12,37] A diagnosis of IBD or lymphoma was made again. The diagnoses based solely on HE were compared with those made after immunophenotyping.

For each case, the age and the sex of the animal were compared with the final diagnosis (obtained with the aid of IHC) to see if a correlation was present. Subjective percentages of B and T lymphocytes, identified by IHC, that were present in the lamina propria, submucosa, and tunica muscularis were also recorded for the cases ultimately diagnosed as lymphoma. In the lymphoma cases, the location of the majority of the lymphocytes was recorded. If multiple layers were affected, the one closest to the serosal surface was recorded.

Statistical analysis

Statistical analyses were performed using the computing environment R, version 3.0 (http://CRAN.R-project.org/doc/FAQ/R-FAQ.html). An unweighted Cohen's kappa test was used to assess the agreement between the diagnosis obtained using HE alone versus the diagnosis achieved using a combination of HE and IHC, as well as agreement between diagnosticians. The percentage of agreement between diagnoses (IBD, lymphoma) using the two methods (IHC, HE) was also calculated. Wilcoxon rank sum test was used to compare ferret age with diagnosis (IBD vs. lymphoma). Fisher's exact Chi squared test was used to compare sex and diagnosis (IBD vs. lymphoma). Wilcoxon rank sum test was used to identify a difference in the percentage of T or B cells amongst lymphocytes present in different layers of the intestine (lamina propria, submucosa) in the lymphoma cases. A Fisher exact test was used to determine if an association was present within lymphoma cases between the location of the neoplastic cells and the immunophenotype determined by IHC. For all statistical tests, the p value considered statistically significant was $p<0.05$.

Results

Sixteen cases were ultimately diagnosed with lymphoma. Based on IHC, 11 were T cell lymphomas (69%), and one of those cases also had a prominent B cell component. Four of the remaining cases were B cell lymphomas (25%), and in one case, the neoplastic cells were negative for both B and T cell markers. Original diagnoses by pathologists with experience in ferret pathology were compared to the blinded pathologist in our study. There was no significant difference between pathologist diagnoses ($p = 0.91$). No significant difference was present between the diagnosis made by using only HE and the diagnosis made with the aid of IHC

(Cohen's Kappa, z= 1.14, p=0.16). In only one case was the diagnosis changed and was from lymphoma to IBD (Fig. 4.1). An example of a case that was diagnosed as lymphoma, with agreement after IHC is shown in Figure 4.2. The diagnosis agreement between HE and the combination of HE and IHC was 93.8% for the cases ultimately diagnosed as lymphoma, and 96.4% for the cases ultimately diagnosed as IBD. There was no significant age (Wilcoxon rank sum test, W = 133.5, p = 0.29) or sex (Fisher exact test, p = 0.29) difference between cases ultimately diagnosed as IBD or lymphoma.

Table 4.2: Median number of CD 79a$^+$ B cells based on intestinal layer and neoplastic immunophenotype. The median percentage of CD79a$^+$ B cells was significantly different between B and T cell lymphomas in every intestinal layer, with B cell percentages greater in the B cell lymphomas.

Intestinal layer	**B cell Lymphoma**	**T cell lymphoma**	**P value**
Lamina propria	60	10	0.02
Submucosa	50	10	0.001
Tunica muscularis	70	1	0.004

The median percentage of CD3$^+$ T cells was not different between B and T cell lymphomas in any location (Wilcoxon rank sum test; lamina propria, p = 0.11, 0.94; submucosa, p = 0.24; tunica muscularis, p = 0.94), indicating that reactive T lymphocytes were always present in cases of B cell lymphoma. However, the median percentage of CD79a$^+$ B cells was significantly different in every intestinal layer between B and T cell lymphomas, with B cell percentages greater in the B cell lymphomas (see Table 4.2 for p values and Fig. 4.3), indicating

that T cell lymphomas were not accompanied by inflammatory B cells. No statistical difference (Fisher exact test, p=0.55) was seen between microanatomical locations of the neoplastic mass in T and B cell lymphomas.

Discussion

No statistical difference was present between the diagnosis obtained via HE alone and the diagnosis obtained with the addition of IHC. Although not statistically significant, there was a 6.2% disagreement between cases originally diagnosed as IBD that were reclassified as lymphoma after IHC testing, and conversely, a lower percentage of cases (3.6%) that were originally diagnosed as lymphoma were reclassified as IBD after IHC. These results are consistent with another study in cats in which lesions previously diagnosed as lymphomas were reclassified as severe inflammation based on IHC findings.[83] Another study found that often a third pathologist opinion was necessary in distinguishing lymphoplasmacytic enteritis and low grade alimentary lymphoma in cats.[18] One limitation of the present study was the small sample size, and a statistical significance may be seen in a larger study. These results indicate that although IHC is not routinely required to make a diagnosis of IBD or lymphoma from full-thickness intestinal biopsies in ferrets, its use may be crucial for a small percentage of animals for which a definitive diagnosis is difficult to reach based on HE staining only. For this reason, the use of IHC should be considered in cases in which an HE diagnosis is uncertain; otherwise the lesion may be misdiagnosed.

For many institutions, IHC is considered the gold standard in determining the immunophenotype of a lymphoma after initial diagnosis and prior to instituting treatment.[84] Our findings are consistent with a recent study in cats that recommended a diagnostic algorithm that

first uses histologic assessment, followed by immunophenotyping, and then PCR to determine clonality of the lymphocytes to more accurately differentiate between intestinal lymphoma and IBD.[5] Follow-up IHC after histologic diagnosis was recently recommended in ferrets.[51] Clonality studies have not yet been performed or validated on lymphoma in ferrets.

The predominance of T cell lymphomas in the ferrets in this study is consistent with the results of other studies.[11,37,41,53] Gastrointestinal lymphoma in dogs is commonly of T cell origin.[49] Although findings are variable in cats, when analyzing lymphomas affecting only the intestine, T cells are also the predominant cell type.[5,48]

In this study, age and gender were not a determining factor for IBD versus lymphoma. Only animals older than 1 year of age were used in this study, since both IBD and lymphoma are not commonly diagnosed in animals of less than one year.[32,85] The range of ages of the animals included in this study was 1.5 to 8 years of age. With a reported lifespan of 5-11 years,[86] our samples cover at least part of the typical life expectancy. We expected a positive correlation between mean group age and neoplasia, as in general, older animals are more likely to have a neoplastic transformation in the lymphocyte population. Additionally, studies on domestic and non-domestic ferrets indicated age as a risk factor for neoplasia;[32,33] however, no statistically significant correlation between age and diagnosis was observed. This could be related to bias introduced by sample population and size. Conversely, the lack of statistical difference between gender and diagnosis was expected, as no sex predominance has been identified for lymphoma in ferrets[32,37] or other domestic animals.[87]

There was no significant difference between the percentages of CD3+ T lymphocytes present in the various layers of the intestine in B or T cell lymphomas. Even when the final diagnosis was B cell lymphoma, a T lymphocyte component was always present. This finding is

in agreement with what was seen in another study on ferret lymphoma,[37] as well as a study in cats,[18] and is most likely due to the normal predominance of T lymphocytes in the intestinal mucosa.[5] On the contrary, B lymphocytes in the intestine are usually confined to the submucosa as opposed to the mucosa,[77] and their presence in the different intestinal layers was only significantly correlated with B cell lymphomas (ie. B cells were rare in T cell lymphomas).

Despite the location of normal intestinal B and T lymphocytes, no statistically significant difference was seen between locations of T and B cell lymphomas. This result differed from a recent study on feline gastrointestinal lymphoma, where the majority of the T cell lymphomas had a mucosal distribution.[82] The present study indicates that the location of the neoplasm in the intestine cannot be used as a predictor for immunophenotype in ferrets.

Diet has been investigated as a possible contributing factor in IBD in humans. Dietary products are the source of luminal antigens in the bowel, and possible mechanisms for inflammation include: direct antigenic effects, alteration of gene expression, modulation of inflammatory mediators (e.g., eicosanoids), changes in the composition of the enteric flora, and effects on gut permeability.[88] In human medicine, the relation to chronic IBD, such as Crohn's disease, and intestinal lymphoma is being debated, and some evidence exists that there is an increased risk of developing GI lymphoma among patients with Crohn's disease.[89] In addition, there is conflicting evidence that shows underlying IBD may be a significant causal factor in the development of intestinal non-Hodgkin lymphoma (NHL) of the GI tract.[90] One of the conflicting points is that the data suggest that immunosuppressive drugs used to manage the different forms of IBD can significantly increase the risk of NHL in these patients.[90]

Along with the speculation that IBD may lead to lymphoma, it is also hypothesized that if diagnosed and treated early, the disease progression from IBD to lymphoma may be prevented.

Unfortunately, clinical signs are often non-specific or undetected. Ferrets are frequently diagnosed with IBD by histopathology with no clinical signs noted by the owner. This leads to the clinical conundrum of whether to treat non-clinically affected animals, as well as whether to empirically treat clinically affected animals without a definitive diagnosis. Treatment with prednisone alone before instituting chemotherapy in lymphoma cases can reduce the efficacy and median survival times.[61] Other therapies for treatment of IBD, such as azathioprine or metronidazole as a first line, may be indicated. However, as previously mentioned, in human medicine, there appears to be an increased incidence of non-Hodgkin lymphoma in IBD patients on immunosuppressive therapy.[90] One of the recommended drugs to treat IBD in ferrets is azathioprine.[51] This drug has been evaluated in humans for its potential to cause GI lymphoma, and it was determined that the benefits of the drug outweigh the risk of lymphoma.[91]

Clinically, IBD is often a diagnosis of exclusion. A definitive diagnosis is only obtained by histopathology; however, in ferrets, these findings often may not correlate with clinical signs. Full-thickness gastrointestinal biopsies are recommended during any abdominal exploratory procedure in a ferret. However, in many mildly affected ferrets, owners are less likely to proceed with aggressive diagnostics, such as exploratory surgery, and a definitive diagnosis may not be obtained. In gastric B cell lymphomas, demonstration through immunohistochemistry of a predominance of κ light chain, as opposed to a balanced mixture of both κ and λ light chains, helped in reaching a diagnosis of neoplasia.[42] Other diagnostic techniques that do not require surgery or full-thickness biopsies have been used to determine immunophenotype in small animals, such as flow cytometry and PCR for antigen receptor rearrangements (PARR). These diagnostic tests only require blood samples or fine needle aspirates of lymph node or organ affected in suspension, which can often be obtained via ultrasound guidance without anesthesia.

Stained cytologic specimens can also be used to extract DNA material for PARR analysis.[92] Other future studies may consider investigation of these diagnostic tests in non-surgical cases.[84]

Limitations to our study include a limited sample size and select populations of ferrets. In some of the ferrets, different tissue samples were involved in the original diagnosis, contributing to a diagnosis of lymphoma; however, our study only analyzed samples from the small intestine, as this was the tissue that was most consistently available. The future direction of this study will involve developing a scoring system to be specifically utilized in ferret tissue samples that will standardize histopathology in IBD cases. In conclusion, although IHC is not necessary to distinguish IBD from lymphoma in ferret intestinal samples, it is useful when a definitive diagnosis is difficult to reach by routine histopathology. In addition, IHC is also indicated for prognostic purposes after intestinal lymphoma is diagnosed, as IHC is the only diagnostic tool currently available to distinguish T and B cell lymphomas in ferrets.

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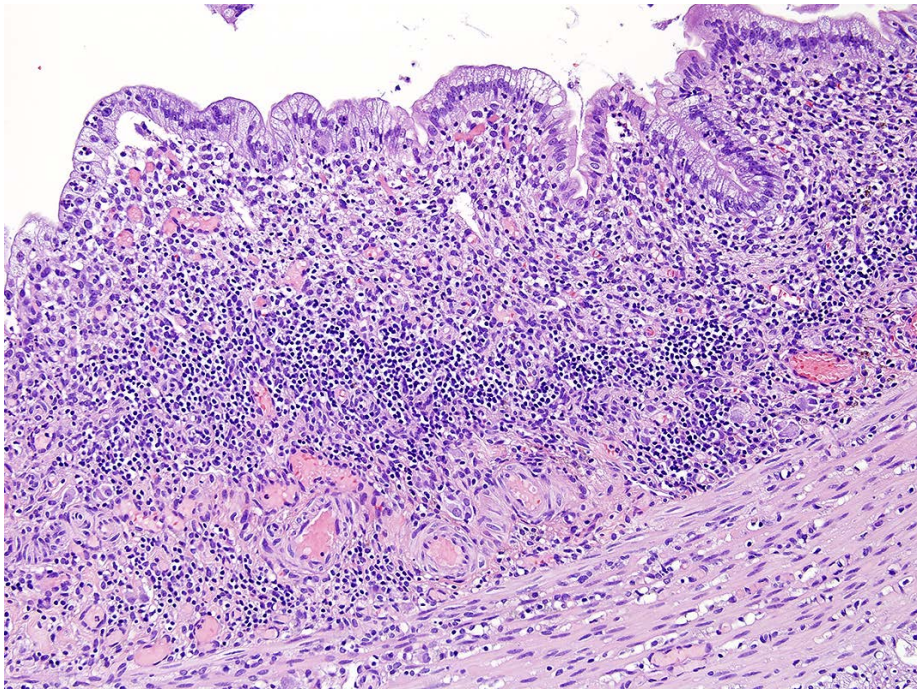

Figure 4.1. Severe inflammatory bowel disease, small intestine, ferret. In this case, IHC was useful in correcting a previous diagnosis of intestinal lymphoma. HE.

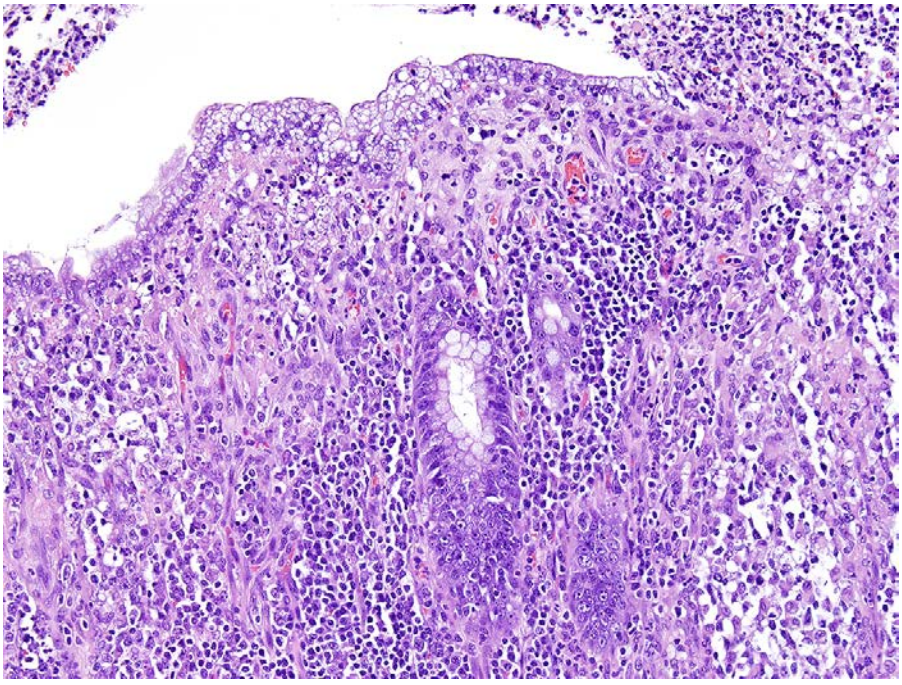

Figure 4.2. Intestinal lymphoma, small intestine, ferret. An example of a case that did not require IHC for correct diagnosis. HE.

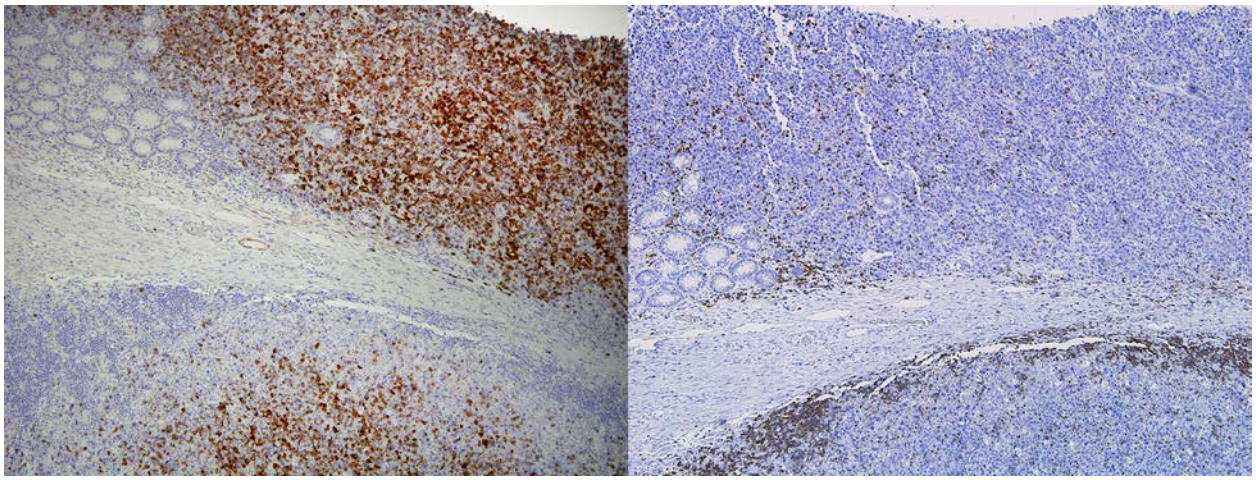

Figure 4.3. Intestinal lymphoma, small intestine, ferret (IHC). In intestinal B cell lymphomas, most of the cells throughout the transmural neoplasm stain positively for plasmalemmal CD79a (a), whereas some cells are CD3-positive (b). IHC for CD79a or CD3 with hematoxylin counterstain.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Although a lot remains to be unraveled regarding IBD and lymphoma in ferrets, our studies provided useful information that may guide pathologists and clinicians in the future. Our first study (Chapter 3) was focused on testing grading schemes developed for IBD in small animals in the ferret and on finding a correlation between those results and the severity of clinical signs. There was no significant correlation between the grading schemes used and severity of clinical signs. After dividing our samples into groups based on severity of clinical signs, several characteristics appeared to differ in severity among groups. These characteristics were used to create a third grading scheme that showed good correlation with severity of clinical signs. It would be interesting to conduct a prospective study on IBD in ferrets collecting full-thickness biopsies and a detailed and guided clinical history. The third grading scheme could be applied to the histologic sections, and its correlation with clinical signs could be tested once more. If clinical significance of this grading scheme is demonstrated in the prospective study, this grading scheme could be universally adopted to identify the severity of IBD in ferrets and to monitor therapy. Our study showed a positive correlation between female and older ferrets with severity of clinical signs for IBD. It would be interesting to see if this correlation persists in a study on larger numbers of ferrets.

The use of more standardized and meaningful parameters to histologically grade enteric samples in ferrets with IBD will hopefully help in shedding more light into this emerging and yet

poorly understood disease. Ferrets are often used as animal models for human diseases[2,93] and a better understanding of disease patterns in this species might also reveal important information for humans.

Our second study (Chapter 4) concluded that HE can be used alone to correctly diagnose IBD versus lymphoma in ferrets, but that IHC may be useful in cases of severe IBD. As in other domestic animals, IHC can be used in the ferret for prognostic purposes after intestinal lymphoma is diagnosed. Polymerase chain reaction for antigen receptor rearrangement (PARR) is another diagnostic tool used in small animals to distinguish between a polyclonal (reactive) and monoclonal (neoplastic) population of lymphocytes.[92] This diagnostic technique has never been published for ferrets. Future directions for the diagnosis of lymphoma in ferrets will be to validate the use of this technique in ferrets.

Similarities have been found between lymphoma of the gastroenteric tract in ferrets and humans. Notably, in ferrets as in humans, B cell gastric lymphoma can be caused by excessive antigenic stimulation from *Helicobacter* infection, and ferrets have been extensively used as animal models for this disease.[42,79,94-96]

Better understanding of lymphoid disorders of the GI tract in ferrets will certainly be useful for both pet and laboratory ferrets.

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