Colonic nitrite and immunoglobulin G levels in canine inflammatory bowel disease

by

Subhadra C. Gunawardana

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INTRODUCTION

The term "Inflammatory Bowel Disease" (IBD) refers to a group of chronic disorders of the intestines, whose features are largely nonspecific. Of all chronic bowel inflammations, those that do not show characteristics of a particular etiological group such as bacterial, viral, parasitic and allergic disease, fall into this category. IBD has been known to affect many species. Being of unknown etiology, the only consistent feature of this disease is its chronic inflammatory nature, involving mucosal infiltration of inflammatory cells, whose type may vary between cases. The most common clinical feature is chronic recurrent diarrhea with mucoid and sometimes bloodstained stools. Other signs like vomition, weight loss and alteration of appetite are frequently present. IBD is common in canines, and presents a challenge to the veterinarian due to its nonspecific nature which makes both diagnosis and treatment quite difficult.

The standard diagnostic procedure begins with elimination of other possible causes which may bring about similar symptoms. The methods currently available for a relatively specific diagnosis of IBD are endoscopy of the colon and histopathological examination of biopsy specimens from affected areas. The treatments used in IBD include dietary management, anti-inflammatory therapy and symptomatic therapy which may or may not be successful in all cases. There are three types of dietary therapy used in IBD. One is withholding food whereby the bowel is placed

in a state of physiologic rest for 1-2 days. Another category is the feeding of a non-allergenic diet such as cottage cheese and rice, since some cases of IBD are caused by a sensitivity to a dietary protein. A non-allergenic diet preceded by bowel preparation by two days of fasting is recommended. Another helpful dietary approach is supplementation with fiber, which increases fecal bulk, stretches colonic smooth muscle and increases contractility. Anti-inflammatory therapy with drugs like sulfasalazine (antimicrobial and anti-inflammatory), prednisolone (corticosteroid) and azathioprine (immunosuppressant) is the basis of treatment in most cases of IBD, and helps to ameliorate the chronic inflammation. Symptomatic therapy involves decrease of fecal water and alteration of colonic motility by opiates such as loperamide (Sherding and Burrows, 1992).

Recent studies in humans (O'Mahoney et al., 1990, 1991; Choudari et al., 1993; Kubes, 1992; Roediger et al., 1986; Miller et al., 1992) have brought to attention two factors which may be useful in the diagnosis and treatment of IBD. These are: a) gut lavage fluid protein concentrations as a diagnostic marker of disease activity; and b) the role of nitric oxide synthase in the inflammatory process, which may be applicable in both diagnosis and therapy.

Several studies with whole gut lavage fluid (O'Mahoney et al., 1990, 1991; Choudari et al., 1993) have repeatedly shown elevated levels of Immunoglobulin G (IgG) and albumin in patients with active IBD as opposed to those of normal subjects or inactive IBD patients, indicating

this might be a suitable diagnostic parameter for identifying the active disease. Such studies have not been conducted in canines, and one objective of this investigation was to test whether gut lavage fluid IgG concentration is a reliable marker for active IBD in dogs.

Nitric oxide (NO), synthesized from L-arginine by the enzyme NO synthase, has been known to play a role in circulatory and neural physiology. Several recent studies have revealed that there may be an increase in NO production in IBD, which may be a contributory factor to the inflammatory process. According to Roediger et al. (1986), rectal dialysates of human patients with active ulcerative colitis contained elevated luminal nitrite levels, while nitrite was immeasurable in patients with quiescent colitis. Nitrite is the stable end product of nitric oxide. In a guinea pig model of ileitis, luminal nitrite levels were increased threefold and the inflammatory process could be prevented by oral administration of L-arginine antagonists which inhibit NO synthase (Miller et al., 1992). These studies suggest that enhanced NO synthesis may contribute to the mucosal injury in IBD, and this effect may be prevented by administration of L-arginine antagonists. Since the role of nitric oxide in canine IBD has not been investigated, a second objective of the present research was to evaluate the usefulness of colonic nitrite levels as a rapid diagnostic indication of active disease in the dog.

To determine the significance of these two parameters in IBD, it is necessary to assess the presence and degree of disease activity in

animals suspected of having IBD. In addition to histological examination of colonic biopsy specimens for inflammatory lesions, we measured the activity of the enzyme myeloperoxidase (MPO) in colonic lavage as an indicator of disease activity. MPO is an enzyme found in granulocytes, which are usually found in large numbers at sites of inflammation. The activity of this enzyme has been used as a measure of the degree of inflammation in several previous studies (Bradley et al., 1982; Miller et al., 1991).

LITERATURE REVIEW

The Normal Large Intestine

Histology and physiology

The large intestine is the part of the gut that begins at the ileocecal junction and ends at the external orifice or anus. The parts of the canine large intestine are the cecum, the colon, and the rectum. In humans, the large intestine is more complex, having an initial blind pouch and ascending, transverse, descending and sigmoid regions in the colon. Its principal functions are reabsorption of water and electrolytes, and the elimination of undigested material. The histophysiological features of the large intestine are similar in canines and humans.

The histology of the large intestine is basically similar in general structure to all other parts of the gastro-intestinal tract. This general structure consists of four concentric layers, namely the mucosa, the submucosa, the muscularis and the serosa, respectively from the lumen outward. The mucosa consists of: a) a superficial epithelium in direct contact with the lumen b) the lamina propria, which is the underlying stroma containing loose connective tissue, blood vessels and immune cells, and c) the muscularis mucosa, which is a relatively thin layer of smooth muscle. The submucosa consists of collagenous connective tissue, containing few cells except for accumulations of lymphoid tissue. The submucosa also contains large blood and lymph vessels and nerve plexuses.

The muscularis consists of two layers of smooth muscle, the inner layer arranged in a circular fashion and the outer layer in a longitudinal fashion. The function of these muscle layers is to bring about movement of chyme along the lumen, the contraction of the circular muscles causing the constriction of the lumen and the contraction of the longitudinal muscles causing shortening of the tube. The serosa or adventitia, consisting of several layers of loose connective tissue, collagenous and elastic alternately, is the outermost layer of the gut (Neutra, 1988; Banks, 1993).

The structure of the large intestine is specifically arranged according to its functions. Since absorption of nutrients is very limited here, the mucosal surface is flat and without villi, in contrast to the small intestine. Straight tubular glands (crypts) from the surface extend to the muscularis mucosae. The epithelium of these glands contain large numbers of goblet cells, which secrete mucus to lubricate the surface and to facilitate the passage of solid and dehydrated luminal contents. This mucus also protects the mucosa from chemical and mechanical injury. Very large numbers of bacteria inhabit the lumen of the normal large intestine. Many of these bacteria help the host by digesting residual organic matter in the luminal contents and synthesizing trace amounts of nutrients, which may be absorbed through the surface epithelium. In protection against possible invasion by luminal bacteria, there is an abundance of lymphoid cells and nodules throughout the large intestine. The epithelium lining the surface and crypts of the large intestine is of

simple columnar type, and is constantly renewed. The crypt epithelium contains undifferentiated cells, immature absorptive cells, goblet cells and enteroendocrine cells, while mature absorptive cells and goblet cells predominate in the surface epithelium. The apical surfaces of the absorptive cells contain microvilli, which help in the absorption of water and small amounts of nutrients that occur in the large intestine. The bottoms of the crypts contain an abundance of undifferentiated cells, which gradually differentiate and become mature functional cells as they reach the surface. The colonic secretions have an alkaline pH, which helps prevent mucosal irritation by neutralizing acids produced by luminal bacteria (Neutra, 1988; Banks, 1993).

The colon has different types of muscular contractions to ensure proper function. There are slow wave contractions which cause peristaltic and antiperistaltic movements, segmental contractions which bring about mixing of the ingesta, and mass contractile movements to propel feces towards the anal orifice (Neutra, 1988; Banks, 1993).

Apart from water, the main substance absorbed in the large intestine is salt. For this purpose, the baso-lateral membrane of the surface absorptive cells is rich in Na-K-dependent ATPase. Sodium is actively absorbed and chloride is passively absorbed along with water, while potassium and bicarbonate are secreted into the lumen. Although dietary fat absorption is normally completed in the small intestine, sometimes lipids can enter the colon when there is incomplete absorption. In the surface epithelium of the ascending colon, absorption of fatty

acids, resynthesis of triglycerides, and formation and release of chylomicrons does occur, although less efficiently than in the small intestine. The passage of luminal contents is largely facilitated by the continuous secretion of mucus by the goblet cells, one of which occurs for about every four of the columnar cells. (Neutra, 1988; Banks, 1993).

Immunology

Defense mechanisms are very important to the gastrointestinal tract because of its continuous exposure to potentially harmful foreign substances which form the luminal contents. The surface epithelium is the only barrier between the mucosal blood vessels and this ever-changing mixture of antigens and microorganisms. Hence, to protect against possible invasion, the mucosa of all parts of the alimentary tract is heavily populated with immune cells. In fact, about one quarter of the mucosa is made up of lymphoid tissue, which includes lymphatic nodules (organized aggregates of lymphoid cells), dispersed lymphocytes and plasma cells in the lamina propria, and intraepithelial lymphocytes which are wandering lymphocytes insinuated between epithelial cells. Such lymphoid tissue is collectively referred to as "Gut Associated Lymphoid Tissue" (GALT). In some areas of the small intestine, many lymphoid nodules can occur together in closely packed clusters, with the epithelium flattened over it. Such areas appear macroscopically as oval shaped bodies referred to as "Peyer's patches". In the large intestine,

Peyer's patches are usually absent. In the human large intestine, Peyer's patches can be found in the appendix (Neutra, 1988; Banks, 1993).

The epithelial barrier is sometimes inadequate to protect against all antigens (even in healthy subjects) so that antigens occasionally gain access to the intercellular spaces. Such antigens are then sampled and processed by intraepithelial lymphocytes, which are usually in contact with a nearby macrophage. These lymphocytes are mostly T cells while some B cells are also present. Antigen sampling is thought to occur most efficiently in the flattened epithelium overlying lymphatic nodules such as in Peyer's patches. In such areas there is a special type of epithelial cell called the "M cell", whose apical surface contains microfolds which can endocytose macromolecules. The endocytosed molecules are transported in vesicles to the intraepithelial lymphocytes which then migrate out of the epithelium to the lymphoid cell aggregates where B cells produce antibodies against the antigens.

The most common type of immunoglobulin produced by plasma cells in the lamina propria is secretory immunoglobulin A, which is dimeric. Other plasma cells produce polymeric secretory immunoglobulin M. There is a specific vesicular carrier system in the crypt epithelial cells which deliver both IgA and IgM to intestinal secretions. These antibodies then combine with luminal antigens (enterotoxins, microorganisms etc.) and protect the epithelial surface by "immune exclusion". In the lamina propria, there are also smaller populations of plasma cells which produce the monomeric antibodies IgG and IgE. A function for these antibodies is

not clearly elucidated yet, and they are believed to diffuse into the lumen where they are degraded. (Neutra, 1988; Banks, 1993).

Inflammatory Bowel Disease

The term "Inflammatory bowel disease" (IBD) refers to a group of disorders affecting the intestine, characterized by chronic inflammation with undefined etiology. In humans, IBD includes ulcerative colitis and Crohn's disease, which, although considered to be two separate disease entities, share numerous clinical and pathological features so as to be sometimes indistinguishable. In addition, several other forms of intestinal inflammation of unspecified etiology fall into this category, such as lymphocytic colitis, collagenous colitis, pouchitis and diversion colitis (Podolsky, 1991).

IBD is characterized by chronic diarrhea and microscopic mucosal inflammation with or without gross lesions. It has been difficult to produce a clear and specific picture of its pathogenesis due to the complex nature of the disease, its variable manifestations and the difficulty in reproducing the disease in animal models due to its unknown etiology (Podolsky, 1991). However, there has been some success in experimentally reproducing at least several features of IBD in some animal models by administration of exogenous agents, such as dextran sulfate which induces colitis in rodents (Okayasu et al., 1990). Other animal models which show some similarity to human IBD are the

spontaneously developing colitis in cotton top tamarin (Madara et al., 1985) and the endogenous bowel inflammation in transgenic rats expressing human HLA-B27 and beta macroglobulin (Hammer et al., 1990).

Etiology

Although the causative agents have not been specifically identified, a number of factors have been found to be associated with the incidence of IBD. For example, evidence of genetic predisposition is suggested by the increased prevalence of the disease among first degree relations of affected patients, increased incidence in certain populations (Bennet et al., 1991), and the high rate of disease occurrence in monozygotic twins of affected patients as opposed to dizygotic twins (Tysk et al., 1988). The nature of this genetic factor has not been identified, and is suspected to be an aberrant gene which encodes a factor causing structural alterations in the gastro-intestinal tract. Ulcerative colitis has been found to be associated with the appearance of a serum antineutrophil cytoplasmic antibody, which, while probably not directly involved in the pathogenesis of IBD, may be useful as a marker (Duerr et al., 1991).

Structural abnormalities in the gastro-intestinal tract are suspected to predispose to IBD by rendering the mucosa susceptible to damaging agents (Delpre et al., 1989). For example, abnormal structures have been found in colonic epithelial cells in IBD (Gibson et al., 1988) and the complex matrix of mucin glycoproteins showed alterations such as

selective reduction of one population of glycoproteins, which persists independently of disease activity in ulcerative colitis (Podolsky et al., 1983; Podolsky et al., 1988). Crohn's disease has been associated with increased intestinal permeability (Olaison et al., 1988). Some features of the disease have been attributed to a granulomatous angiitis causing ischemic infarcts (Wakefield et al., 1989; Wakefield et al., 1991).

Although infectious agents have been suspected to contribute to the initiation of the disorder, evidence is inadequate to incriminate any particular organism. Mycobacterium paratuberculosis, the causative agent of Johne's disease, has been isolated from patients with Crohn's disease, but the rates of isolation were low and the same organism has been found in some control patients as well (Butcher et al., 1988). Some investigators have suggested indirect involvement of a biological agent, with damage produced by a product (such as formylated peptides) from a non pathogenic or normal luminal bacteria. A chronic granulomatous intestinal inflammation had resulted from injection of proteoglycans from bacterial cell walls (Sartor et al., 1989).

Pathogenesis

Regardless of the etiology, the pathogenesis of IBD involves immunologic mechanisms, as suggested by circumstantial evidence (Brandtzaeg et al.,1989). IBD may be initiated by an alteration of the physiological inflammatory response of the intestinal mucosa. The lamina propria of the intestine and colon possess a continuously present

"physiological" inflammatory response, which is presumed to result from the continuous influx of antigens through the lumen. There are regulatory mechanisms to prevent unrestrained activation of such immune and inflammatory responses. Failure or inadequacy of these regulatory mechanisms, resulting in exaggerated inflammatory response, is suspected to predispose to IBD (Podolsky, 1991). Numerous alterations in the general and mucosal immune response have been found in IBD patients. These include changes in immune cell populations, complement components and inflammatory mediators.

Increases in IgG bearing cells have been observed in IBD patients. This may result from altered homing mechanisms or an altered pattern of cytokines which control B cell differentiation (Hibi et al., 1990; MacDermot et al., 1981). The increase in cell numbers results in a general increase of the production of IgG as well as selective increases in specific subtypes in both blood and intestinal mucosa (MacDermot et al., 1989). These IgG antibodies may be directed against one or several antigens. One such antigen is a 40 Kd protein present in colonic tissue of both normal and diseased subjects (Takahashi et al., 1985).

The immune response could also be altered by changes in intestinal epithelial cells. This is suggested by a marked reduction in the induction of suppressor T cells by intestinal epithelial cells (Mayer et al., 1990). In Crohn's disease patients, elevated levels of cytotoxic Leu7+ cells (a subgroup of CD8+ T cells) in the peripheral blood as well as in the inflamed mucosa have been observed (Cantrell et al., 1990).

Increased numbers of intraepithelial lymphocytes (Entrican et al., 1987) and focal accumulations of leukocytes close to glandular crypts (Schmitz-Moorman et al., 1981) have been observed in ulcerative colitis and Crohn's disease. A preferential accumulation of antigen-primed memory cells is suggested by a significant reduction in the proportion of putative naive T cells in the intestinal mucosa and peripheral blood (Brandtzaeg et al., 1989). A significant increase in T4 lymphocytes and a decrease in T8 lymphocytes in the epithelium was observed in IBD when compared to the normal colon (Kobayashi et al., 1988).

In addition to changes in immune cell populations, there is deposition of complement components in the mucosa of patients with IBD (Ahrenstedt et al., 1990) and a marked induction of expression of major histocompatibility complex Class II antigens in intestinal epithelial cells, which suggests alteration in antigen presenting activity (Mayer et al., 1991).

Several features of IBD suggest that mucosal immune activation, such as alteration in the expression of several types of cytokines and lymphokines, may play a significant role in the disease. Increased expression of the cytokines interleukin-1 and interleukin-6 has been noted, in addition to altered expression of interleukin-2 and its receptor. Antigenic challenge leads to induction of interleukin-2 and its receptors in Crohn's disease, but the tissue levels were low as opposed to the circulating soluble interleukin-2 levels (Mahida et al., 1989; Mahida et al.,1990; Kusugami et al., 1989; Ligumsky et al., 1990;

Crabtree et al., 1990). In a rabbit model of colitis, the severity of inflammation was reduced by administration of interleukin-1 receptor antagonist (Comminelli et al. 1990). Other factors that regulate intestinal immune/inflammatory responses are likely to be peptide mediators of local and systemic neuroendocrine function (Shanahan et al., 1988).

Tissue injury may be produced by numerous noncytokine inflammatory mediators whose production is enhanced in IBD. These include eicosanoid products of arachidonic acid metabolism, such as prostaglandin and thromboxane products of cycloxygenase and thromboxane synthetase (Zirfoni et al., 1983). The inflammation may be further amplified by 5-lipoxygenase products, particularly leukotriene-B4 (Nielson et al., 1987; Sharon et al., 1984). That these nonspecific inflammatory mediators play a major role in sustaining the disease process is indicated by the fact that most available therapeutic approaches to IBD work by inhibiting these nonspecific pathways of inflammation.

A pathogenic mechanism for IBD has been proposed by Brandtzaeg et al., 1989. T cells are attracted to the intestinal epithelium by the initiating event or events. The stimulation of these T cells and macrophages lead to release of cytokines, which induce epithelial HLA class II expression. HLA class II renders the epithelium vulnerable to penetration by luminal antigens. The uptake and presentation of luminal antigens to mucosal T cells induces "help" rather than "suppression". This mechanism can be enhanced if luminal antigens get into the mucosa

through a break in the epithelium. Endothelial receptor mechanisms are altered by cytokines released from stimulated leukocytes. This results in attraction of B cells from the systemic immune system, which leads to overproduction of IgG1 and IgG2, and preferential production of monomeric IgA1. The immunologic homeostasis of the gut is changed by this imbalance between secretory and systemic immunity, which enhances further inflammation and tissue damage. As a result of transudation from vessels or local production, IgG and complement factors accumulate at the epithelial surface. Complement is activated due to immune complex formation, and this causes epithelial damage. Activated complement and immune complexes stimulate macrophages, which release biologically active substances like leukotrienes and complement factors. They also mobilize neutrophils which release lysosomal enzymes and oxygen radicals, which damage the epithelium. Substances released from macrophages can stimulate smooth muscles, leading to spastic contractions. They also bring about vasodilatation and increased vascular permeability.

Increased numbers of inflammatory cells such as neutrophils, lymphocytes, monocytes and mast cells are recruited to the intestinal mucosa in IBD. The mechanism whereby this occurs is not clear, but may be due to cell adhesion to endothelial surfaces with subsequent penetration into tissues. This is associated with the expression of a specific endothelial-cell-leukocyte-adhesion molecule (Podolsky: unpublished data, cited in Podolsky 1991, p.931). This molecule may be induced by cytokines present in high concentration as a result of tissue injury. The

attraction of neutrophils to the mucosa may also be mediated by chemotactic leukotrienes and formylated bacterial peptides. The inflammatory cells, once activated by these inflammatory mediators, go on to produce more of the same mediators so that the situation becomes autoamplifying, thereby generating the manifestations of inflammatory bowel disease.

The chronic colonic inflammation thus brought about, leads to disruption of the normal salt and water transport and alteration of the normal pattern of motility, which, in turn result in the chronic diarrhea that is the cardinal clinical sign of IBD (Sherding & Burrows, 1992).

In the normal colonic mucosa, there is a transmural potential difference related to active sodium transport, with the mucosal side negative to the serosa. In ulcerative colitis, there is a marked reduction in this potential difference, which results in the inability to absorb sodium against an electrochemical gradient, and an increase in the plasma to lumen sodium flux rate. This leads to an increased leakiness of the mucosa and increased levels of sodium in the lumen, which contribute to the diarrhea (Sherding and Burrows, 1992).

There is evidence that colonic inflammation disrupts normal colonic motor function. The spontaneous and urecholine-stimulated contractile activity of the colon was suppressed in periods of severe diarrhea in cats with diffuse experimental colitis. During healing and regeneration, this contractile activity was increased. Isolated colonic muscle from cats with "idiopathic summer diarrhea" showed a disruption of electrical

slow wave conduction, while there was significant derangement in the myoelectric characteristics of individual colonic muscle cells in dogs with experimental acute colitis. In human ulcerative colitis patients, an overall decrease in colonic motor activity has been observed including an abnormal response to food ingestion. In such patients, the major abnormality was found in smooth muscle contractility as opposed to relatively normal myoelectrical activity. This is evidence for a lack of coupling between the electrical and mechanical events. (Sherding & Burrows, 1992).

In summary, inflammatory bowel disease appears to stem from a failure in the colonic mucosal immune regulatory mechanisms which leads to an uncontrolled and exaggerated inflammatory response to the normal antigenic load. Chronic inflammation results in a disruption of the contractile activity as well as the salt and water transport mechanisms, which in turn leads to chronic diarrhea.

Clinical and laboratory findings

Ulcerative colitis and Crohn's disease, being the two major forms of human IBD, show a number of similar clinical and histopathological features. But there are significant differences that distinguish each disease. Although both diseases are characterized by chronic recurrent intestinal inflammation, in ulcerative colitis the inflammatory process usually starts from the rectum and extends proximally, being confined to the superficial layers of the bowel wall (mucosa and superficial

submucosa), whereas in Crohn's disease the inflammation involves the deeper layers and may even spread transmurally. Both diseases are characterized by dense infiltration of inflammatory cells, where the predominant cell types are neutrophils and lymphocytes in ulcerative colitis, while macrophages and lymphocytes predominate in Crohn's disease. In addition to the cellular infiltration of the lamina propria, the main histopathological features in ulcerative colitis are formation of microabscesses within the crypts, depletion of mucin from goblet cells, and absence of inflammation in the deeper layers of the bowel wall. Extensive superficial ulceration is characteristic, as opposed to the deep linear ulcers seen in Crohn's disease. In the early stages of Crohn's disease, dense areas of lymphoid tissue contain overlying aphthoid-like ulcers. As the disease progresses, these ulcers spread deep into the underlying tissue in a fissure-like or serpiginous manner while the intervening tissue may be relatively preserved, which gives the mucosa a segmented or "cobblestone" appearance. This contrasts with the extensive and continuous superficial ulcers occurring in ulcerative colitis. These are macroscopically seen to be covered with a mucopurulent exudate. In about 50% of Crohn's disease patients, non-caseating granulomata develop due to aggregation of macrophages. Another common feature in Crohn's disease is collagen deposition, which may lead to stricture formation. As in ulcerative colitis, the most common areas involved in the inflammation are the terminal ileum and colon, but unlike ulcerative colitis, Crohn's disease can affect any site within the

alimentary tract and even extend beyond it by fistula formation. Another distinguishing feature is that the rectum is rarely affected in Crohn's disease. Enterocutaneous fistulas often lead to perineal disease or "blind" sinus tract formation (Podolsky, 1991).

In both diseases the prominent clinical feature is recurrent diarrhea, which is bloodstained in ulcerative colitis owing to the extensive mucosal ulceration. This is accompanied by abdominal pain in Crohn's disease due to inflammatory masses. Obstruction may occur in Crohn's disease due to severe inflammatory edema or stricture formation due to fibrosis. Bleeding is much less common in Crohn's disease than in ulcerative colitis, while systemic signs like fever and weight loss are more common in Crohn's disease. Although the cardinal clinical symptom is diarrhea, constipation does sometimes occur in ulcerative colitis, probably due to disruption of peristaltic patterns (Podolsky, 1991).

Canine Inflammatory Bowel Disease

As in humans, a number of poorly defined colonic diseases in dogs is collectively referred to as canine IBD. These diseases, whose principle feature is colonic inflammation causing chronic diarrhea, include plasmacytic lymphocytic colitis, eosinophilic colitis, granulomatous colitis and canine histiocytic ulcerative colitis. As the names imply, these forms are classified according to the nature of the

inflammatory infiltrate, there being no significant differences in the history or clinical picture (Sherding & Burrows, 1992).

Like human IBD, canine IBD is believed to stem from a defect in the mucosal immunoregulation, where a breach in the mucosa may lead to a self perpetuating, exaggerated immune response to bacterial or dietary proteins. Such inflammation may persist long after the inciting cause is removed. The original cause might be anything that brings about mucosal injury, such as hookworm or whipworm infections (Sherding & Burrows, 1992).

Cases of canine IBD are usually presented with a history of chronic mucoid diarrhea, with poor response to conventional antidiarrheal therapy. Clinically, semiformed or liquid feces, often bloodstained and mucoid, are observed. The most frequently reported clinical symptoms are chronic diarrhea, vomition, weight loss and alteration of appetite. The region of the gastrointestinal tract affected and the extent of cellular infiltration determine the severity of these symptoms, which show a cyclic rather than progressive clinical course. Hematemesis and/or melena may occasionally occur due to severe ulceration of the intestinal mucosa. Symptoms of abdominal pain may occur but are less common (Jergens et al., 1992).

The etiopathogenesis is unknown. One suspected cause is a hypersensitivity reaction to antigens in the bowel lumen or mucosa. IBD is diagnosed by methodical elimination of other possible causes which produce similar symptoms, such as parasitic infections. Endoscopy and

histopathological evaluation of biopsy specimens are the main criteria for differential diagnosis of various forms of IBD (Jergens et al., 1992).

Upon colonoscopy, the normal mucosa appears as a smooth glistening surface which reflects the endoscopic light uniformly. The mucosa is translucent so that the superficial submucosal blood vessels are visible. The normal pink color of the mucosa may or may not be changed by hyperaemia depending on the type of solution used for enema. The normal mucosa is somewhat flexible, distending readily upon air insufflation and not being damaged by rubbing with the tip of the endoscope (Sherding and Burrows, 1992).

In IBD, the mucosa appears inflamed and edematous so that the submucosal blood vessels are invisible. Irregular thickening of the mucosa causes a granular appearance in place of the smooth and glistening surface in the normal bowel. Inflammation causes the mucosa to be friable so that it will easily bleed when touched. There may be ulceration in variable degrees and a thick tenacious mucus adhering to the wall or free in the lumen. In granulomatous colitis, severe mucosal proliferation with granulomatous lesions is characteristic. This results in narrowing of the lumen and failure to dilate on air insufflation. Ulceration may occur, and the lesions are of a segmental nature with relatively normal areas interspersing with inflamed areas, and thus, this disease is analogous to Crohn's disease in humans. Multiple strictures can also occur in

histiocytic ulcerative colitis, along with deep bleeding ulcers (Sherding and Burrows, 1992).

Histological examination of mucosal biopsy specimens is the most reliable method of diagnosis of IBD. The histopathological picture is similar in all forms of canine IBD, and is characterized by mucosal thickening and edema, infiltration of inflammatory cells, depletion of goblet cells, and occasionally fibrosis of the lamina propria causing the glands to be separated from each other. The inflammatory reaction may be acute or chronic, and focal or diffuse, depending on the stage and severity of the disease. Both acute and chronic reactions may appear in a single biopsy, indicating the progressive nature of the disease (Sherding and Burrows, 1992).

The different forms of canine IBD are identified by a few distinguishing histopathological features. For example, in granulomatous colitis the inflammation is of a segmental nature, with severe granulomatous proliferation of the mucosa. Eosinophilic colitis is characterized by circulating eosinophilia as well as heavy mucosal infiltration of eosinophils. Infiltration of PAS positive macrophages and progressive superficial ulceration are typical in histiocytic ulcerative colitis, whereas in plasmacytic lymphocytic colitis the predominant cell types in the infiltrate are plasma cells, lymphocytes, neutrophils and sometimes eosinophils (Sherding and Burrows, 1992). In addition to colonoscopy and histopathology, results of a hemogram may aid in the diagnosis. Frequent hematologic findings in IBD are hypoalbuminemia,

hypergammaglobulinemia, and mild microcytic hypochromic anaemia caused by persistent chronic bleeding. Mild neutrophilia with a left shift may be seen in lymphocytic plasmacytic colitis, while eosinophilic colitis is characterized by eosinophilia and increased serum IgE levels (Sherding & Burrows, 1992).

A recent study on clinical cases suggests there is no age, sex or breed predisposition for IBD, although previous studies have reported a higher incidence in older animals. The same study has indicated a higher incidence among purebred dogs (Jergens et al., 1992).

Association of Immunoglobulin G with Inflammatory Bowel Disease

The primary form of humoral immunity in mammals is the class of plasma protein referred to as immunoglobulins or antibodies. These are glycoprotein molecules with a specific structure, synthesized and secreted into the plasma and tissue fluids by plasma cells (B lymphocytes). While some immunoglobulins are produced at all times, most are synthesized only in response to antigenic stimulation, have a structure specifically complementary to the said antigen, and have the ability to inactivate the antigen by binding to it.

Immunoglobulins are part of the gamma-globulin fraction of plasma proteins. Although they share a common basic structure, there is a considerable degree of structural heterogenicity between different subclasses of immunoglobulins. The basic structural unit of an

immunoglobulin molecule is composed of two pairs of polypeptide chains, namely the heavy chains and light chains, linked together by interchain disulfide bonds. Each unit contains a fragment for antigen binding and another fragment which usually binds to a cell surface. (Sell, 1987).

The main classes of immunoglobulins are immunoglobulin A (IgA), immunoglobulin G (IgG), immunoglobulin M (IgM), immunoglobulin D (IgD) and immunoglobulin E (IgE). Each class, in turn, is divided into several subclasses.

IgG, on which attention has been focused in this investigation, is the most abundant of the immunoglobulins. Its concentration in the serum, which is around 1240 mg/dl, is significantly higher than the concentrations of other Ig classes which range from 0.03 mg/dl (IgE) to 270 mg/dl (IgA). IgG is a monomer with a molecular weight of 150,000, a sedimentation coefficient of 7, and a relatively long half life of 23 days. IgG is found in high concentrations in both vascular and extravascular spaces, and is able to cross the placental barrier. A specific property of IgG is the ability to fix complement, which is part of its protective mechanism. The function of IgG is to provide immunity against blood-borne infectious agents such as bacteria, viruses, parasites and fungi. It is also active in tissues, and receptors for IgG are found in human monocytes and lymphocytes. Hence, IgG can be considered to play a major part in providing protection within the body, as opposed to IgA which is the major immunoglobulin found in secretions and mucosal surfaces which come into contact with the external

environment. Unlike IgA, IgG is not known to play a significant role in the intestinal lumen, and is found only in trace amounts in normal subjects (Sell, 1987; Evans, 1984).

The protein levels in the intestinal lumen can be quantified by testing whole gut lavage fluid (WGLF), which is the clear fluid passed following initial colonic cleansing prior to procedures like endoscopy or surgery. This fluid is a gut perfusate containing secreted proteins such as immunoglobulins. Although different types of proteins can normally be found in gut lavage fluid, it has been reported that IgG, albumin and alpha-1 antitrypsin are found only in trace amounts in normal subjects (Choudari et al., 1993). In this study, gut lavage fluid was tested for the levels of these proteins in 45 human IBD patients. The results revealed that the levels of these three proteins are markedly elevated in IBD, in proportion with the disease activity. IgG showed the highest correlation with the disease activity.

Previous studies by the same authors, using two separate groups of IBD patients, have indicated that gut lavage IgG concentrations in IBD patients are significantly higher than those of controls (O'Mahoney et al., 1990; O'Mahoney et al., 1991), and that there is a positive correlation between secreted IgG levels and albumin levels, suggesting a leakage of proteins across the mucosa (O'Mahoney et al., 1991). This study (Choudari et al., 1993) has revealed that elevated levels of IgG, albumin and alpha-1 antitrypsin (AlAT) are relatively specific markers of active IBD, even though high levels are occasionally found in other forms

of colitis. However, rather than use as screening tests for IBD in general, these protein assays are more suitable for differentiation between active and inactive IBD, since the levels of these proteins were found to be normal in the inactive disease.

The source of IgG in whole gut lavage fluid was initially considered to be the IgG bearing cells, as evidenced by an increased number of these cells in IBD. An excess of IgG containing cells located deep in the mucosa of IBD patients were revealed by immunohistochemistry (Baklien et al., 1976; Saverymuttu et al., 1986) and high amounts of IgG were found to be secreted by isolated mononuclear cells from IBD patients (MacDermott et al., 1981; Verspaget et al., 1988). Plasma cell IgG subclass distribution was found to be different in IBD patients from that of normal subjects (Kett et al., 1987). However, recent studies have suggested that at least some of this IgG is derived from plasma rather than cells in the mucosa (O'Mahoney et al., 1990; O'Mahoney et al., 1991; Choudari et al., 1993) evidenced by the positive correlation between IgG and albumin in the gut lavage from patients with active IBD. In addition, luminal concentrations of all three types of proteins were low in inactive Crohn's disease patients, even with extensive chronic ulcers known to be rich in IgG bearing cells (Choudari et al., 1993).

The appearance of these proteins in the lumen may not be the mere result of bleeding, since in active IBD the relative concentrations of each protein in WGLF significantly differed from those in plasma. Furthermore, a selective increase in the mucosa to lumen permeability for

these three proteins is suggested by the fact that the molecular weights of the three are not similar (IgG: 150,000 daltons, albumin: 69,000 daltons and AlAT: 65,000 daltons). One reason for the albumin levels not being as high as the IgG levels may be that albumin is more susceptible to proteolysis by intestinal enzymes prior to processing of the lavage fluid (Choudari et al., 1993).

There are several reasons to consider gut lavage fluid protein quantification to be a better approach for measurement of disease activity than other existing scientific approaches. Tests like erythrocyte sedimentation rate (ESR), platelet count, acute phase proteins etc., while useful, are not always reliable, since they may appear normal in active IBD, and positive in inflammatory states without gut involvement. Techniques like labeled leukocyte studies involve exposure to radioactivity and require complete fecal collection, in addition to being expensive. The results of these tests are influenced by concurrent dietary and pharmacological treatments, while WGLF is not (Choudari et al., 1993). Gut lavage is a procedure widely used for bowel preparation for endoscopy, radiology and surgery, and the lavage fluid can be easily obtained in the course of the diagnostic and therapeutic procedure. Also this fluid can be stored for several months at -70°C without deterioration, and is aesthetically acceptable and easy to handle. The most important factor is that IgG concentrations in gut

lavage fluid may distinguish between active and inactive IBD and accurately grade the degree of disease activity.

Nitric Oxide: General Overview

Nitric oxide (NO) is a highly volatile gaseous compound synthesized from L-arginine by the enzyme nitric oxide synthase (NOS). NO has been found in tissues of the nervous, immune and cardiovascular systems, and is believed to play a major regulatory role as a physiologic messenger molecule. NO is also suspected to be involved in the pathophysiology of various conditions such as neurodegenerative diseases, septic shock, hypertension and stroke (Bredt & Snyder, 1994).

Nitric oxide synthase (NOS) has been isolated and purified from a number of tissues including the brain, macrophages and vascular endothelium, and its structure and function have been elucidated by molecular cloning of the cDNA. Each isoform of NOS has been named according to the tissue from which it was isolated, namely bNOS (brain NOS), macNOS (macrophage NOS), eNOS (endothelial NOS) and nonmacrophage inducible NOS (Schmidt et al., 1991; Mayer et al., 1990; Yui et al., 1991; Stuehr et al., 1991; Hevel et al., 1991; Evans et al., 1992; Pollock et al., 1991; Bredt et al., 1990, 1991; Nakane et al., 1991; Lamas et al., 1992; Sessa et al., 1992; Janssens et al., 1992; Xie et al., 1992; Lyons et al., 1992; Lowenstein et al., 1992; Geller et al., 1993).

Function of nitric oxide synthase

The substrate for NOS is L-arginine, whose guanidine group is oxidized in a process consuming five electrons, resulting in the formation of NO and L-citrulline (Figure 1).

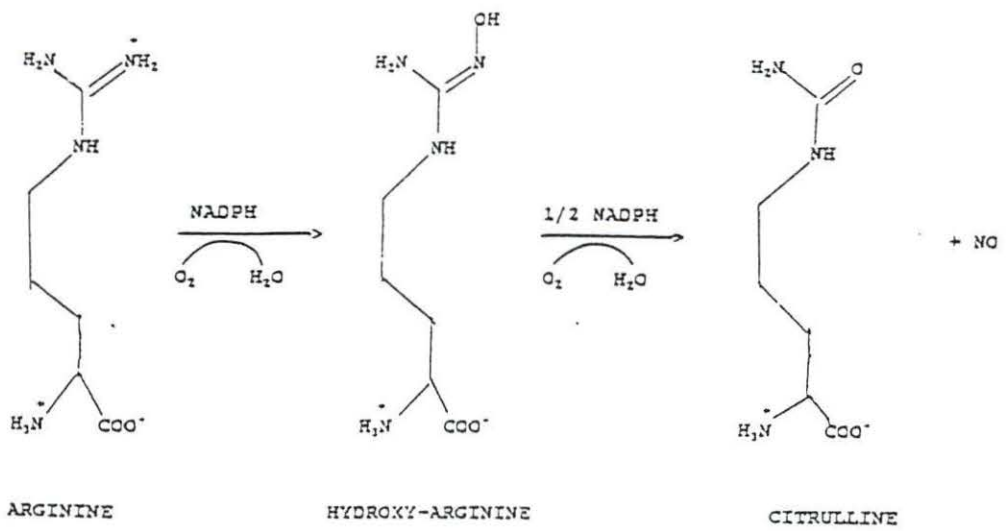

Figure 1. Biosynthesis of nitric oxide. Nitric oxide and L-citrulline are the end products of the oxidation reaction of L-arginine, catalyzed by nitric oxide synthase. The reaction involves the intermediate L-hydroxy-arginine, which is tightly bound to the enzyme.

This reaction can be inhibited by structural analogs of L-arginine such as L-Nw-substituted arginines. Although simultaneous application of L-arginine initially reverses this inhibition, some of these agents can cause irreversible inhibition of NOS upon prolonged exposure. The sensitivity to these inhibitors differ slightly among different isoforms of NOS. The brain and endothelial NOS are most potently inhibited by L-

NNA while the most potent inhibiter for macNOS is L-Nw aminoarginine (L-NAA). (Pufahl et al., 1992; Feldman et al., 1993; Moore et al., 1993). NOS has close homology with the enzyme cytochrome P450 reductase (CPR) and employs five redox active cofactors including NADPH, FAD and FMN (Hevel et al., 1991; Bredt et al., 1991). The mechanism of action of CPR where reducing equivalents are relayed from NADPH to heme-containing cytochrome P450 enzymes, is thought to be shared by NOS. Evidence for this is that each NOS monomer in bNOS and macNOS contains one equivalent of iron-protoporphyrin IX (McMillan et al., 1992; White et al., 1992; Stuehr et al., 1992). The involvement of a cytochrome P450 type heme is also suggested by the inhibition of purified NOS by carbon monoxide. NOS is feedback inhibited by NO, which might interact with the heme prosthetic group of NOS. Arginine, the substrate of NOS, also binds to the heme prosthetic group initially, as do the substrates of various cytochrome P450 enzymes (Rengasamy et al., 1993; Assreuy et al., 1993; Rogers et al., 1992; McMillan et al., 1993). One difference between NOS and other mammalian cytochrome P450 enzymes is that NOS is not an integral membrane protein, and its flavin and heme-containing domains are fused in a single polypeptide (Narhi et al., 1986). NOS requires tetrahydrobiopterin (H_4B) for its activity. H_4B is believed to stabilize NOS, as it does not affect the initial rate of NOS and is not recycled (Giovanelli et al., 1991).

The conversion of arginine to NO occurs in two distinct steps. The first step is the oxidation of arginine to Nw-hydroxyarginine (NHA) by

removal of two electrons. This intermediate is usually tightly bound to the enzyme. This hydroxylation step utilizes 1 eq of NADPH and 1 eq of O_2, as do the classical P450 type monooxygenation reactions. The reaction requires calcium and calmodulin as activators. It is accelerated by H_4B and blocked by CO (Klatt et al., 1993; White et al., 1992; Stuehr et al., 1991).

The second step where NHA is converted to NO and citrulline is not fully elucidated. The known facts about this reaction are that it utilizes 0.5 eq NADPH, requires calcium/calmodulin, is accelerated by H_4B and is inhibited by carbon monoxide and arginine analogs (Klatt et al., 1993; White et al., 1992; Stuehr et al., 1991).

Regulation of nitric oxide synthase

The brain and endothelial NOSs, being calmodulin activated enzymes, require calcium as a major regulator. Calcium ion (Ca^{++}) binds to calmodulin and thereby activates NOS. For each isoform of NOS, Ca^{++} becomes available by a different mechanism. For example in the brain, glutamate acting on NMDA receptors triggers Ca^{++} influx, while in endothelial cells Ca^{++} is generated by the phosphoinositide cycle activated by acetylcholine binding to muscarinic receptors. The electron transfer and oxygen activation of NOS are regulated by calcium and calmodulin, while the arginine binding is not (Klatt et al., 1993; McMillan et al., 1993; Pou et al., 1992). Calmodulin antagonists such as

trifluoperazine inhibit the brain and endothelial NOSs (Bredt et al., 1990).

While the macrophage and nonmacrophage inducible NOSs are not regulated by calcium or calmodulin, they do possess calmodulin recognition sites. Calmodulin binds very tightly to these sites unaffected by calcium, unlike neuronal NOS whose binding to calmodulin is dependent on the presence of calcium (Cho et al., 1992; Nathan, 1992).

The inducible form of NOS was first detected in macrophages which, in response to stimulation by interferon-gamma and lipopolysaccharide (LPS), synthesized new NOS proteins over 2-4 hours. Such NOS proteins were not detectable without stimulation (Nathan 1992). Apart from macrophages, numerous other tissues have shown inducible NOS activity in response to endotoxin treatment. The hepatocyte-inducible NOS is considered to be the main form of nonmacrophage-inducible NOS (Geller et al., 1993).

Another regulatory mechanism of NOS is phosphorylation. Brain, endothelial and hepatic-inducible forms of NOS possess consensus sequences for phosphorylation by cAMP dependent protein kinase. Such sequences are not obvious in macNOS. Neuronal NOS can be phosphorylated by several kinases such as cAMP dependent protein kinase, cGMP dependent protein kinase, protein kinase C, and Ca^{++}/calmodulin dependent protein kinase. Phosphorylation by each of these kinases results in decreased catalytic activity. Enzyme regulation occurs at multiple levels, such as direct enzyme activation by Ca^{++}/calmodulin and inhibition of enzyme

activity through phosphorylation by Ca^{++}/calmodulin dependent protein kinase (Bredt et al., 1992; Nakane et al., 1991; Brune et al., 1991).

In addition to enzymatic activity, subcellular distribution of eNOS is also regulated by phosphorylation. This enzyme which is predominantly localized in the plasma membrane (unlike bNOS and macNOS which are cytosolic) translocates to the soluble fractions upon phosphorylation in response to bradykinin. This cytosolic eNOS being catalytically inactive, NO is not generated within the endothelial cell. It is the non phosphorylated NOS in the plasma membrane that generates NO, which is released into the extracellular environment. This may be true for neuronal NOS as well (Pollock et al., 1991; Michel et al., 1993; Hiki et al., 1992).

Neuronal functions of nitric oxide

The main function of NO in the nervous system is believed to be neurotransmission. Using the technique of immunohistochemical staining, it has been discovered that throughout the central nervous system, NOS occurs only in neurons. Although NOS containing neurons represent a small percentage of total neurons, they are widely distributed in the central nervous system (Bredt et al., 1990).

The synthesis of NO is believed to be triggered through stimulation of NMDA receptors by glutamate and similar substances. This NO in turn activates guanylate cyclase (GC), which may be the principle target through which NO exerts its action. That NO may have other targets than

GC, and that other neurotransmitters besides NO may regulate GC, is indicated by the markedly differing localizations of GC and NO (Bredt and Snyder, 1994). NO, through the activation of GC, generates cGMP, whose exact function is as yet unclear. However, a variety of actions exerted by cGMP have been discovered, such as the activation of a serine/threonine protein kinase which is selectively expressed in the cerebellar purkinje cells (Lohmann et al., 1981), regulation of ion channels in visual and olfactory tissue (Nakamura et al., 1987), and modulation of cAMP signalling by regulating the activity of certain cAMP phosphodiesterases (Peunova et al., 1993). There is evidence that NO enhances neurotransmitter release, since NOS inhibitors such as nitroarginine block neurotransmitter release (Zhu et al., 1992). Further evidence of this effect comes from a system using PC12 cells which develop neuronal properties in the presence of nerve growth factor. In these cells, NOS staining and NOS catalytic activity (which is normally absent) appears after eight days of nerve growth factor application. At the same time, a marked enhancement of acetylcholine release in response to depolarization was observed, and this effect was blocked by NOS inhibitors and reversed by excess L-arginine (Hirsch et al., 1993; cited in Bredt and Snyder, 1994, pp.185-186).

Nitric oxide may also influence the differentiation and regeneration of neurons. There is transient expression of bNOS throughout the embryonic nervous system and following neuronal injury, although the

precise role played by NO in these situations is not clear (Bredt and Snyder, 1994).

The neurotransmitter function of NO is relatively clear in the autonomic nervous system. Myenteric plexus in the gastrointestinal pathway contain many NOS neurons (Bredt et al., 1990). The relaxation of smooth muscle which results from depolarization of the myenteric plexus is blocked by NOS inhibitors, indicating that NO is a neurotransmitter in this situation (Desai et al., 1991).

Similar experiments have revealed NO to be the autonomic neurotransmitter in several other tissues such as the adventitial layers of large blood vessels (Bredt et al., 1990), the pelvic plexus and cavernous nerve of penile tissue (Burnett et al., 1992), ganglion cells and fibers in the adrenal medulla (Bredt et al., 1990), and macula densa of the kidney (Wilcox et al., 1992). In spite of its physiological role in the nervous system, excess NO can be neurotoxic. In neurodegenerative diseases like Alzheimers disease and Huntingdon's disease, and in the focal ischemia of vascular stroke, the neurotoxicity is mediated by excess glutamate acting at NMDA receptors (Choi 1988) . Excess NMDA receptor stimulation does not destroy the NOS neurons, as these neurons are resistant to the NMDA induced damage (Dawson et al., 1993). Hence, excess NMDA stimulation would result in the release of excess NO, which is believed to damage other neurons. In cerebral cortical cultures, 60-90% of the neurons die upon exposure to NMDA while NOS neurons remain undamaged. This damage can be blocked by treatment with NOS inhibitors,

removal of arginine from the medium, or scavenging NO using hemoglobin, which indicates that the primary damaging agent may be NO (Dawson et al., 1993; Koh et al., 1986; Koh et al., 1988; Dawson et al., 1991).

Immune functions of nitric oxide

The primary role of NO in macrophages is protection, which involves the mediation of bactericidal and tumoricidal actions of macrophages in response to inflammatory stimuli like endotoxin. Such stimuli, including interferon-gamma, factor-alpha, interleukin-1, and lipopolysaccharide, are responsible for synthesis of large amounts macNOS from negligible basal levels. The tumoricidal and bactericidal activity in macrophage cultures can be blocked by NOS inhibitors or removal of arginine, indicating that NO is likely to be responsible for this activity (Hibbs et al., 1987; Nathan, 1992). Another possible protective function of NO is inhibition of viral replication, as indicated by the lowering of viral titers in cell cultures upon addition of NOS, and elevation of Coxsackie viral titers in intact rats upon administration of NOS inhibitors (Lowenstein et al., 1993, cited in Bredt and Snyder, 1994; Karupiah et al., 1993).

Cardiovascular functions of nitric oxide

The main substance responsible for endogenous vasodilatation is believed to be NO. Endothelial NOS is activated by an influx of calcium (which binds to calmodulin) triggered by the action of a vasodilator

agent (such as acetylcholine or bradykinin) at the endothelial cell receptors. The NO thus synthesized, activates GC in the adjacent smooth muscle cells. The GC in turn catalyzes the synthesis of cGMP, which brings about the relaxation of smooth muscle possibly through activation of cGMP-dependent protein kinase and phosphorylation of myosin light chains (Bredt and Snyder, 1994).

The hypotension in septic shock is thought to be mediated by NO, as evidenced by the reversal of hypotension upon administration of NOS inhibitors (Kilbourn et al., 1990; Hotchkiss et al., 1992). In ischemic cardiovascular conditions NO may play a protective role (mechanism unknown) as indicated by the reduction of myocardial infarct size and decrease of mortality in ischemia and reperfusion of splanchnic artery upon administration of NO donors (Siegfried et al., 1992; Carey et al., 1992).

NO can inhibit platelet aggregation and adhesion by mechanisms which are unclear, but which may involve effects on GC, phospholipase C or ADP ribosylation (Durante et al., 1992; Zhang et al., 1992).

Mechanism of action of nitric oxide

A major compound binding site of NO is iron, both heme and non-heme. The action of NO on many enzymes is mediated by binding to the iron in the enzyme. For example in GC, NO binds to the iron in heme at the active site, which alters the enzyme's conformation and augments catalysis. In several other enzymes such as NADH-ubiquinone

oxidoreductase, NADH:succinate oxidoreductase, cis-aconitase and iron-sulfur enzymes, NO binds to non-heme iron (Nathan, 1992; Bredt and Snyder,1994). By binding to the non-heme iron of ribonucleotide reductase, NO inhibits DNA synthesis (Kwon et al., 1991). The iron in ferritin can also be bound by NO, resulting in the liberation of the iron and lipid peroxidation (Reif et al., 1990). Other known mechanisms of NO are S-nitrosylation of numerous proteins (Lipton et al., 1993), and incorporation of NAD into glyceraldehyde 3-phosphate dehydrogenase thereby inhibiting its activity and depressing glycolysis (Zhang et al., 1992).

NO, being a free radical, can damage DNA by base deamination, which is the main mechanism of NO neurotoxicity (Zhang et al., 1993; Wink et al., 1991). DNA thus damaged leads to poly-ADP-ribosylation of nuclear proteins, thereby depleting NAD from the cell. Resynthesizing NAD requires very large amounts of ATP. This process continues until the cell dies from energy depletion (Zhang et al., 1993).

Nitric Oxide Associated with the Intestine

The principle known function of NO in the mammalian intestine is that of an inhibitory neurotransmitter/neuromodulator in the enteric nervous system. NO is released from the non-adrenergic non-cholinergic (NANC) nerve endings in the gut wall (and in blood vessels), and acts as a smooth muscle relaxant. While application of NO mimics the responses to

NANC inhibitory nerve stimulation, NOS inhibitors reduce electrically evoked NANC mediated relaxation. Also, scavenging of NO using oxyhemoglobin inhibits the effects of both NANC stimulation and externally applied NO. (Nichols et al., 1993).

A basic histochemical technique involving the reduction of a tetrazolium dye in the presence of NADPH has been used to reveal the catalytic activity of constitutive NOS and thereby examine its distribution in the intestine. Such studies in rat and guinea pig intestines have shown NOS activity to be localized in subpopulations of neurons and nerve fibers throughout the gut wall nerve networks and in enteric blood vessels (Nichols et al., 1993).

Apart from the neuronal cells and vascular endothelial cells, another important site of NOS activity (relevant to our study) is the intestinal epithelium. A study by Tepperman et al., 1993, demonstrated the presence of both constitutive and inducible forms of NOS in isolated epithelial cells from rat small intestine. The highest levels of both forms of the enzyme were found in villus cells.

The role of NO in the intestinal epithelium is believed to be physiological regulation of epithelial cell integrity and secretion. Acute microvascular and epithelial cell damage in rat small intestine was found to be exacerbated by intravenous administration of a NOS inhibitor (L-NMMA), which effect was prevented by concurrent administration of a NO donor (S-nitroso-N-acetyl-penicillamine) (Tepperman et al., 1993). In the feline small intestine, NO has been found to modulate epithelial

permeability. In this study, local intra-arterial infusion of a NOS inhibitor resulted in a sixfold increase in mucosal permeability, and this effect was completely reversed upon infusion of a NO donor (Kubes, 1992).

The role of NO in modulating intestinal epithelial permeability is particularly relevant in inflammatory bowel disease, since IBD is characterized by increased microvascular permeability and mucosal barrier dysfunction. The mechanism that initially brings about mucosal leakiness is unclear, and various agents such as reactive oxygen metabolites, vasocongestion and vascular leukocytes have been suspected as potential mediators. Several studies have provided evidence to believe that NO may play a significant role in intestinal inflammation.

In acute intestinal inflammation, NO seems to play a protective role as has been revealed in studies by Aoki et al., 1990, Hutcheson et al., 1990, and Kubes, 1992. Exogenously administered NO was beneficial in ischemia/reperfusion of the intestine (Aoki et al., 1990), and NOS inhibitors including N-monomethyl-L-arginine exacerbated damage to the intestine in endotoxic shock. That NO may play a role in maintaining integrity in the normal intestine was suggested in the study by Kubes 1992, where autoperfused segments of cat ileum were subjected to local intra-arterial infusion of the NOS inhibitor Ng-nitro-L-arginine-methyl ester (L-NAME) for 90 minutes, with concurrent administration of a an exogenous NO donor, sodium nitroprusside (SNP) only during the last 30 minutes. Quantification of epithelial permeability by blood to lumen

clearance of ^{51}Cr labeled EDTA throughout the period revealed an increase in permeability of approximately six times the original level on infusion of L-NAME. This effect was completely reversed by SNP, the NO donor. A later study by Kubes and Payne, 1993, revealed that reperfusion-induced increase in intestinal mucosal permeability can be reduced by NO donors. A 1 hour intestinal ischemia/reperfusion model of feline small intestine was used in this study, and the mucosal barrier integrity was assessed using blood-to-lumen ^{51}Cr EDTA clearance. Another parameter measured in this study was intestinal blood flow and resistance, in order to test whether the effect of NO is mediated through alteration in blood flow. The mucosal permeability was found to be significantly increased on reperfusion after 1 hour of ischemia, and was accompanied by a 50% reduction in the intestinal blood flow. This mucosal barrier dysfunction was reduced by exogenous sources of NO and/or exogenous L-arginine, which did not have any effect on the blood flow. Administration of L-NAME worsened the mucosal barrier dysfunction and further increased the permeability. A significant improvement of the intestinal blood flow was observed upon addition of 8-bromoguanosine 3',5'-cyclic monophosphate which is a permeable analogue of guanosine 3',5'-cyclic monophosphate. However, this compound did not produce any change in the mucosal permeability. The main conclusion of this study was that mucosal barrier disruption induced by reperfusion can be reduced by exogenous sources of NO, independent of alterations in intestinal blood flow.

All of the abovementioned studies indicate that NO plays a protective role in the intestine both in the normal situation and in acute injury. Surprisingly, the effect of NO in chronic bowel inflammation appears to be the exact opposite, as revealed by the following studies. The effects of NO in chronic inflammatory bowel disease were investigated in a guinea pig model of chronic ileitis induced by intraluminal administration of trinitrobenzenesulfonic acid (TNBS) (Miller et al., 1992). The animals with TNBS induced ileitis (as opposed to the sham operated controls) were divided into two groups. One group was treated with oral L-NAME, and another group with D-NAME (the inactive enantiomer of L-NAME), while the remaining group was not treated with NOS inhibitors. Luminal nitrite and protein levels were measured and the inflammatory reaction was monitored over a 30 day period. The animals with TNBS-induced chronic ileitis showed a significant inflammatory reaction characterized by cellular infiltration, mucosal thickening and elevation of myeloperoxidase activity. There was significant mucosal leakage of protein and nitrite, while the mucosa was in a net secretory state as opposed to the net absorptive state observed in the normal intestine. Such changes were much less in the L-NAME treated group but not in the D-NAME treated group, indicating that inhibition of NO synthesis may be beneficial in chronic inflammation. In fact, treatment with L-NAME actually reversed the secretory state of the mucosa to the absorptive state. The endogenous NO production was apparently elevated in proportion to the inflammatory reaction, as indicated by the elevated

nitrite levels in the lavage samples from the TNBS ileitis group and the D-NAME treated group as opposed to the sham operated controls and the L-NAME treated group. Protein concentrations in the lavage fluid showed a five-fold increase in the TNBS group, suggesting a significant disruption of the mucosal barrier during the inflammation.

Apart from such experimental models, elevation of luminal nitrite levels and mucosal barrier disruption leading to protein leakage are characteristic findings in clinical cases of chronic inflammatory bowel disease in humans (Roediger et al., 1986; Choudari et al., 1993). Upon measurement of nitrite levels in rectal dialysates in human subjects, luminal nitrite levels were significantly elevated in patients with acute ulcerative colitis as opposed to the immeasurable levels in control subjects and quiescent colitis patients.

One purpose of our study was to investigate whether there is a similar relationship between NO and chronic intestinal inflammation in canine patients. To determine whether intestinal epithelial NO synthesis is increased in canine inflammatory bowel disease, colonic lavage nitrite levels were measured in IBD patients and healthy controls.

Assessment of Disease Activity

The principle criterion used in this investigation to determine the presence and degree of IBD was histopathological examination of colonic biopsy specimens of each animal. Specimens were examined for classic

signs of chronic inflammation such as cellular infiltration and fibrosis. In addition, the activity of neutrophil myeloperoxidase was measured as a possible marker of disease activity.

Myeloperoxidase (MPO) is a neutrophil enzyme whose function is to convert hydrogen peroxide to hypohalous acids. Hydrogen peroxide is one of the end products of the respiratory reactions of phagocytes and it is formed by free oxygen radicals reacting with themselves. The hypohalous acid produced by neutrophil MPO is hypochlorous acid (HOCl), the function of which is to kill microbes (Powell, 1988).

Since this enzyme is predominantly localized in neutrophils, its activity can be used as a marker for the neutrophil quantity of a given site. Most inflammatory reactions, including human IBD, are characterized by neutrophilic infiltration. MPO activity has been used to assess the degree of inflammatory activity in several previous studies (Miller et al., 1991; Miller et al., 1992; Bradley et al., 1982).

MATERIALS AND METHODS

Animals

Control group: Nine apparently healthy dogs with weights ranging from 36 to 43 pounds were obtained from Laboratory Animal Resources, College of Veterinary Medicine,Iowa State University. There was no specific selection for breed or sex, and the group consisted of four males and five females of varying breeds, namely 1 Collie, 1 Chow, 1 Pointer, 1 German Shepherd, 1 Labrador, 1 Spaniel cross, 1 Greyhound and 2 mongrels.

Test group: Ten dogs presented to the ISU Small Animal Hospital over a period of one year (from August 1993 to September 1994) were used as test animals. These dogs came with a history of chronic diarrhea and weight loss. Upon clinical and laboratory examination IBD was suspected, and endoscopy and mucosal biopsy were performed as further diagnostic procedures. For convenience in identification, these dogs will be referred to using numbers from T1 to T10.

T1: A 4 year old female of Mixed breed, presented with a history of chronic intermittent diarrhea and weight loss for 2-3 months. Clinical examination revealed a thickened descending colon and irregular roughening of the rectum. Watery and mucoid stools with occasional bloodstaining had been observed. Fecal examination revealed whipworm eggs. Small intestinal diarrhea with secondary colitis was suspected. The patient was wormed, and subsequent fecal tests were negative for parasite

ova. Diarrhea had continued since this treatment. Dietary therapy with a hypoallergenic diet[1] and enteric antibiotics produced no improvement. Subsequent feeding of a different type of hypoallergenic diet and treatment with metronidazole (antigiardial drug) had brought about some improvement.

T2: A 3 year old male Terrier, presented with chronic diarrhea, weight loss and vomition. Feces had been observed to be soft and formed, and sometimes stained with fresh blood. Large bowel disease was suspected.

T3: A 21/2 year old female Doberman, presented with chronic diarrhea, vomition and weight loss through a period of 4 months. She had been treated previously with a hypoallergenic diet and metronidazole. The symptoms had subsided upon this treatment, and recurred following attempts to return to an ordinary diet. Appetite was poor at the time of presentation.

T4: A one year old female of Mixed breed, was presented with a history of intermittent vomition and diarrhea. The feces were very watery and occasionally bloodstained. The vomiting usually preceded the onset of diarrhea, and the vomitus was yellowish white in color. Appetite had decreased gradually.

T5: A 5 year old male English Pointer presented with a history of diarrhea and weight loss. The stools were watery, mucoid and sometimes bloodstained. The appetite was good. Large bowel disease was suspected.

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[1] I/D Canned food: Mills Pet Nutrition Inc. Topeka, Kansas.

T6: An eight year old female Beagle presented with a history of chronic diarrhea, tenesmus, hematochezia and urgency of defecation. An acute onset of vomition had started a week prior to presentation. There was pain on abdominal palpation.

T7: A six year old male Charpei, presented with a history of vomition and diarrhea which had started about four weeks prior to admission. The vomitus had initially been yellow with no food, then contained food and mucus, and gradually become foamy and bloodstained. The feces had been initially yellow, and had later become dark and tarry, with higher frequency of defecation. Roundworm eggs had been detected in the feces. Previous treatment with antiparasitics and bismuth salts (mucosal protectant) had brought no improvement in the vomition and diarrhea. The dog was anorectic for ten days prior to presentation, and had lost eight pounds in the past month.

T8: A six year old male of mixed breed, was admitted with a history of bloodstained stools which had started two months prior to presentation. There had been no diarrhea, but fresh blood was observed after defecation. Vomition had occurred occasionally. Appetite was normal. He had been treated with metronidazole for one month, and blood in the feces had not been observed in the seven days prior to admission.

T9: A nine year old male Cocker spaniel, presented with a history of chronic colitis of six months duration. Clinical history included tenesmus and large bowel diarrhea with mucus and fresh blood. Treatment

with sulfasalazine and corticosteroids for two months prior to admission had not been effective.

T10: A twelve year old male of mixed breed, was admitted with a history of intermittent diarrhea of five months' duration. There had been no vomition or weight loss, but the animal had been lethargic and appeared to be in some pain.

Collection of Samples

Animal preparation

The animals of the control group were fasted overnight and three doses of "Golytely"[2] solution, 250 ml each were orally administered at 12 hour intervals.

Anesthesia was induced with sodium thiopental 20 mg/kg intravenously, and maintained by inhalation with halothane-oxygen. The large bowel was washed out by administration of 200 ml of the PEG/electrolyte solution into the colon via a rectal balloon catheter attached to a peristaltic infusion pump[3]. This fluid was left inside for two minutes, and completely withdrawn by reversal of pump action.

The same procedure was followed with the test animals except that they were not given Golytely solution on the day prior to lavage and

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[2]An isotonic solution containing polyethylene glycol (PEG), sodium sulfate, sodium bicarbonate, sodium chloride and potassium chloride.

[3]Harvard Apparatus, Millis, Mass. 02054.

endoscopy. The only process of bowel cleansing used was the washing out immediately before infusion of the lavage solution.

Collection of lavage samples and biopsy specimens

To each animal in the control group, 300 ml of lavage solution was administered using the infusion pump and rectal balloon catheter. This fluid was left inside for 30 minutes before withdrawal. Immediately after withdrawal, lavage fluid was processed as described below.

For the test animals, most of which were smaller in size, 250 ml was used instead of 300, and the time the fluid was left inside the colon ranged from 20 to 25 minutes, in order to minimize the time of anesthesia.

After completion of colonoscopy, several biopsies from the colonic mucosa were taken from each subject. These included both normal looking and grossly abnormal areas in the case of test animals. A flexible fiberoptic endoscope[4] was used for colonoscopy.

Biopsy specimens from each subject were fixed in formalin, embedded in paraffin, sectioned at 6 μm and stained with Hematoxylin and Eosin. Slides prepared from control and test animals were later examined "blindly" by one histopathologist.

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[4]Flexible Videoscope with O.D. of 9.2 mm

Processing of Samples

Immediately after collection, the lavage samples were processed using the method described by Gaspari et al. (1988) with a few minor modifications.

The samples (volumes approximately 150-200 ml) were placed in 50 ml polypropylene centrifuge tubes and centrifuged at 2000xg for 30 minutes in order to remove particulate matter. The supernatants were poured into clean tubes. In order to destroy any protease activity (which would interfere with IgG levels) in the samples, soybean trypsin inhibitor[5] (1 mg/ml) in phosphate bufferred saline (PBS) was added to each tube to a final concentration of 10% v/v. EDTA (1.0 M) in PBS was then added to a final concentration of 5% v/v. The tubes were centrifuged at 550xg for 10 minutes, and the supernatant transferred to new tubes. Phenylmethylsulfonyl fluoride (PMSF), (100 mM) in 95% ethanol, was added to a final concentration of 1% v/v, and the tubes were centrifuged again at 2200xg for 30 minutes. The supernatants were removed into clean tubes, and PMSF was added again to a final concentration of 1% v/v. To these tubes, sodium azide (20 mg/ml) in PBS was added to a final concentration of 1% v/v. The tubes were left at room temperature for 15 minutes, and heat-inactivated calf serum was added to a final concentration of 4% v/v. From these tubes, 7.5 ml samples were taken for the myeloperoxidase assay (which was performed immediately after processing), and the rest were

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[5]Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 (Sigma).

divided into aliquots and stored at -70°c for future assays (nitrite, total protein and IgG).

Assays

Myeloperoxidase activity

Myeloperoxidase activity on the processed samples was measured using the method described by Miller et al., 1991, which is a modification of the original method described by Bradley et al., 1982.

To each 7.5 ml sample of lavage fluid, 0.25 ml of 15% hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer[6] was added (a dilution of 30:1 v:v) in order to negate the pseudoperoxidase activity of hemoglobin. The tubes were sonicated for 15 seconds to disrupt MPO containing cells for release of the enzyme. They were then centrifuged at 20,000xg for 15 minutes, and the supernatant taken for the spectrophotometric reaction.

From this supernatant, a 100 μl aliquot was taken and mixed with 2.9 ml of potassium phosphate buffer (50 mM, pH 6.0) containing 0.167 mg/ml o-dianisidine dihydrochloride and 0.0006% hydrogen peroxide. The % transmittance at 460 nm was recorded spectrophotometrically[7] at the end of each minute for 3 minutes. One unit of myeloperoxidase activity has

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[6]Mallinkrodt Chemical Works.

[7]Beckman DB-GT Grating double beam spectrophotometer.

been defined as that required to degrade 1 μMol of hydrogen peroxide per minute at 25°C.

Nitrite assay

Since we suspected the levels of nitrite in these samples to be quite small, the samples were concentrated 2 to 12 fold by membrane centrifugation[8]. Nitrite levels in the concentrated samples were measured using the method described by Roediger et al., 1986.

One half ml of the sample was placed in a 3.5 ml cuvette and 2.0 ml of distilled water was added. To this, 0.5 ml of 1.6% sulfanilamide in 3.6 N HCl was added. After mixing by inverting the cuvettes, the % transmittance at 540 nm was recorded spectrophotometrically, to determine blank values. Then, 125 μl of 0.1% N-(1-naphthyl) ethylenediamine was added, and the tube was left at room temperature for the color to develop. After 15 minutes the % transmittance at 540 nm was recorded again.

The corresponding optical density values were obtained from conversion tables and the difference in optical density between 0 and 15 minutes was calculated. A standard curve was prepared using sodium nitrite solutions of 0.5, 1.0, 5.0 and 10.0 mMol/0.5ml, and the nitrite levels of the samples were calculated using this curve.

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[8]Centriperp 10 concentrators, Amicon Division, W.R. Grace Co., Beverley, MA 01915.

Total protein assay

Total protein levels in lavage samples were measured with a Bicinchoninic Acid Protein Assay Kits[9]. The principle of this assay is the concentration dependent reduction of Cu^{++} to Cu^{+} by protein. Bicinchoninic acid reacts specifically with Cu^{+}, and forms a purple complex with an absorbance maximum at 562 nm. Protein standards were prepared with bovine serum albumin.

Fifty ml of bicinchoninic acid solution was mixed with 1 ml of cupric sulfate pentahydrate (4%) solution, and 2 ml added to each sample or standard (0.1 ml). Samples were incubated at 37°C for 30 minutes, cooled to room temperature and the absorbance at 562 nm was measured spectrophotometrically.

A standard curve was prepared by plotting the net absorbance at 562 nm against the amount of protein in each standard tube in μg. The protein content in each lavage sample was calculated by comparison with this standard curve.

Immunoglobulin G assay

The assay was performed on lavage samples concentrated by membrane centrifugation as previously described. The method employed for the assay

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[9]Sigma Chemical Co., P.O. Box 14508, St Louis, MO 63178 (Sigma).

was single radial immunodiffusion, using a commercial kit developed for Canine IgG[10].

The kits consisted of plates of agarose gel into which antiserum specific for canine IgG had been incorporated, and four IgG standard solutions containing 225, 450, 900, and 1800 mg/100 ml respectively. Wells of 3 μl volume had been cut in the agar. From each standard IgG solution, 3 μl was placed in a well. In the remaining wells, 3 μl of the lavage samples to be tested were placed. The plates were left at room temperature for 18-24 hours, to allow the diffusion of the solutions in the wells into the surrounding agar.

When the sample antigen reacted with the antibody in the agar, a ring of precipitation was formed around the well, whose diameter was proportionate to the concentration of antigen. The diameters of the rings were measured, a standard curve was prepared with the ring diameters of the IgG standard solutions, and the concentrations of IgG in the lavage samples were calculated by comparison with the standard curve.

Analysis of Data

The data (which included the levels of nitrite, total protein and IgG from each animal) were analyzed using ANOVA (Proc GLM).

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[10]Canine IgG SRID kits from Veterinary Medical Research and Development Inc.

RESULTS

Histology

Histopathological features of IBD were observed in the colonic biopsy specimens from all dogs in the test group (Figures 3,4,5,6,7,8,9,10,11 and 12). These features included infiltration of lymphocytes and plasma cells in all cases and edema of the lamina propria, general or focal fibrosis and goblet cell hyperplasia in some cases. The degree of IBD varied from mild to severe. Two dogs (T5 and T7) showed severe IBD and two dogs (T4 and T6) showed moderate IBD lesions, while all the rest were diagnosed as mild IBD. The biopsies from the control group showed normal histology (Figure 2) except for two cases (C4 and C7) which showed mild IBD lesions.

Recovery Rate

The amount of fluid recovered varied with each animal, but was generally 60% or more of the infused volume. Lower recovery volumes in two control dogs (C4 and C6), and two IBD cases (T2 and T10) were due to leakage around the rectal balloon catheter.

Assays

Myeloperoxidase: Myeloperoxidase activity was not detectable in colonic lavage samples from either control dogs or dogs with suspected IBD.

Nitrite content: Nitrite levels in the lavage fluid of dogs with suspected IBD ranged from 0.02 to 3.75 mMol/ml with a mean of 1.83 mMol/ml. These were significantly higher than those of the control group which ranged from 0 to 1.2 mMol/ml with a mean of 0.158 mMol/ml (Figure 13, Tables 1 and 2).

Total protein content: Total protein concentrations ranged from 228 to 671 mg/dl (mean: 407) in the control dogs and ranged from 162 to 496 mg/dl (mean: 392) in the dogs with suspected IBD. There was no significant difference between the total protein levels in the two groups (Figure 14, Tables 1 and 2).

Immunoglobulin G content: The control group had no measurable IgG. In the test group, the IgG content ranged from 28 to 64 mg/dl, with a mean of 46 mg/dl. One sample in the test group, T5, had no detectable IgG (Figure 15, Tables 1 and 2).

Table 1. Control group: Animal data and values.

Animal	Breed	Sex	Fluid in ml	Fluid out ml	Nitrite mMol/ml	Total Protein mg/dl	IgG mg/dl	Histological diagnosis
C1	Collie	F	300	203	0.126	425	0.0	Normal
C2	GS[a]	M	300	295	0.570	337	0.0	Normal
C3	Chow	F	300	210	0.170	384	0.0	Normal
C4	Greyhound	M	300	72	0.009	613	0.0	Mild IBD
C5	Mix	F	300	200	0.140	365	0.0	Normal
C6	Mix	F	300	41	0.000	280	0.0	Normal
C7	Pointer	F	300	146	0.050	365	0.0	Normal
C8	Labrador	M	250	121	1.200	671	0.0	Normal
C9	Spaniel	M	250	170	0.036	228	0.0	Normal
Mean					0.256	407	0.0	
SD					0.394	145	0.0	

a: German Shepard.

Table 2. Test Group: Animal data and values.

Animal	Breed	Sex	Fluid in ml	Fluid out ml	Nitrite mMol/ml	Total Protein mg/dl	IgG mg/dl	Histological diagnosis
T1	Mix	F	250	200	0.880	468	41.07	Mild IBD
T2	Terrier	M	250	50	3.750	496	38.34	Mild IBD
T3	Doberman	F	250	170	0.021	162	0.0	Mild IBD
T4	Mix	F	250	113	0.305	453	58.52	Moderate IBD
T5	Pointer	M	250	142	2.920	487	28.24	Severe IBD
T6	Beagle	F	250	220	1.190	332	22.53	Moderate IBD
T7	Charpei	M	240	196	1.770	365	55.12	Severe IBD
T8	Mix	M	200	173	3.440	453	64.00	Mild IBD
T9	CS[a]	F	250	240	3.330	382	61.00	Mild IBD
T10	Mix	M	250	96	0.730	425	52.30	Mild IBD
Mean					1.830	392	46.00	
SD					1.408	107	20.40	

a: Cocker Spaniel.

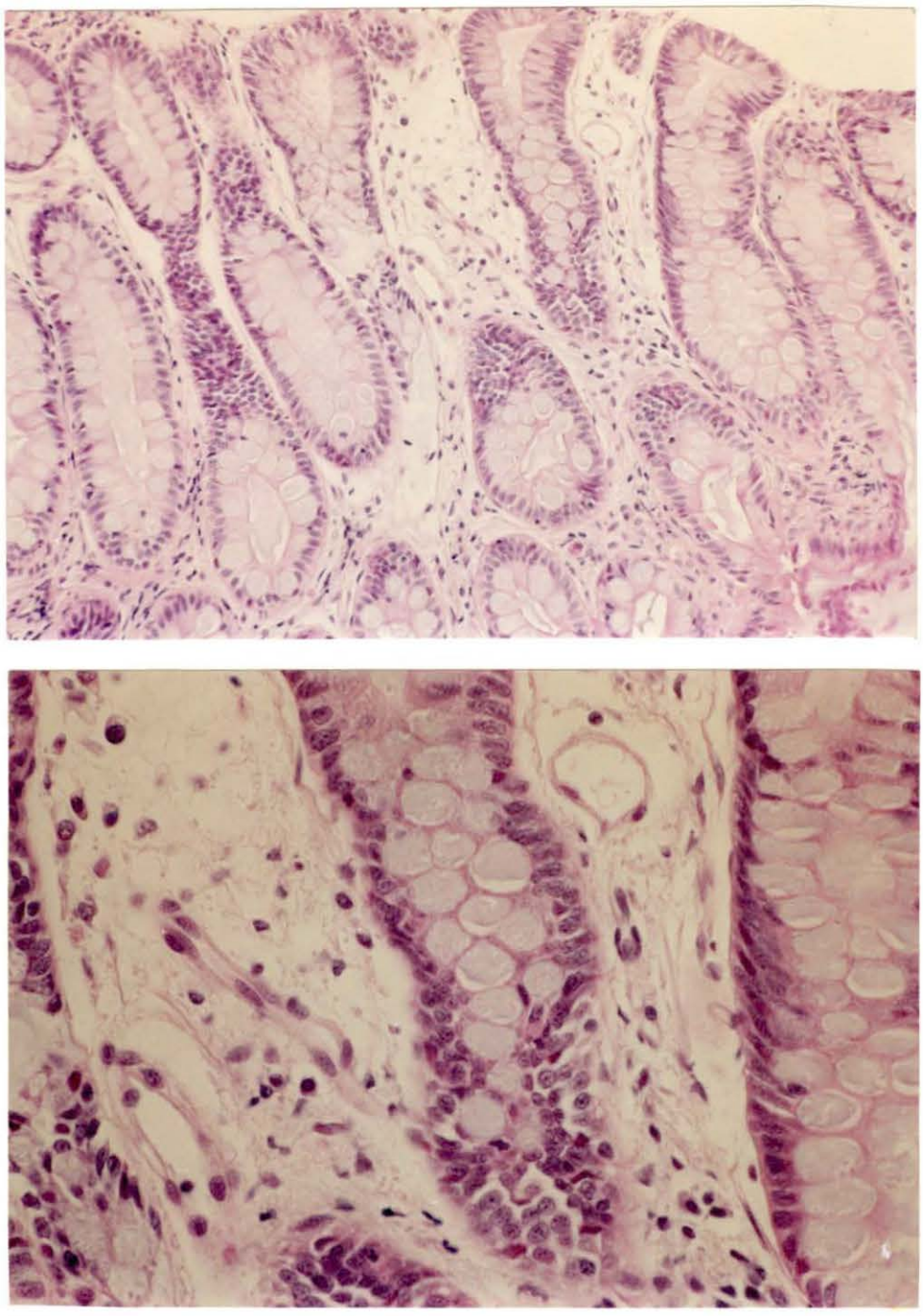

Figure 2. Colonic biopsy from a control animal. Histopathological diagnosis: Normal. (No significant lesions).
Top: Hematoxylin and Eosin. (x 160)
Bottom: Hematoxylin and Eosin. (x 400)

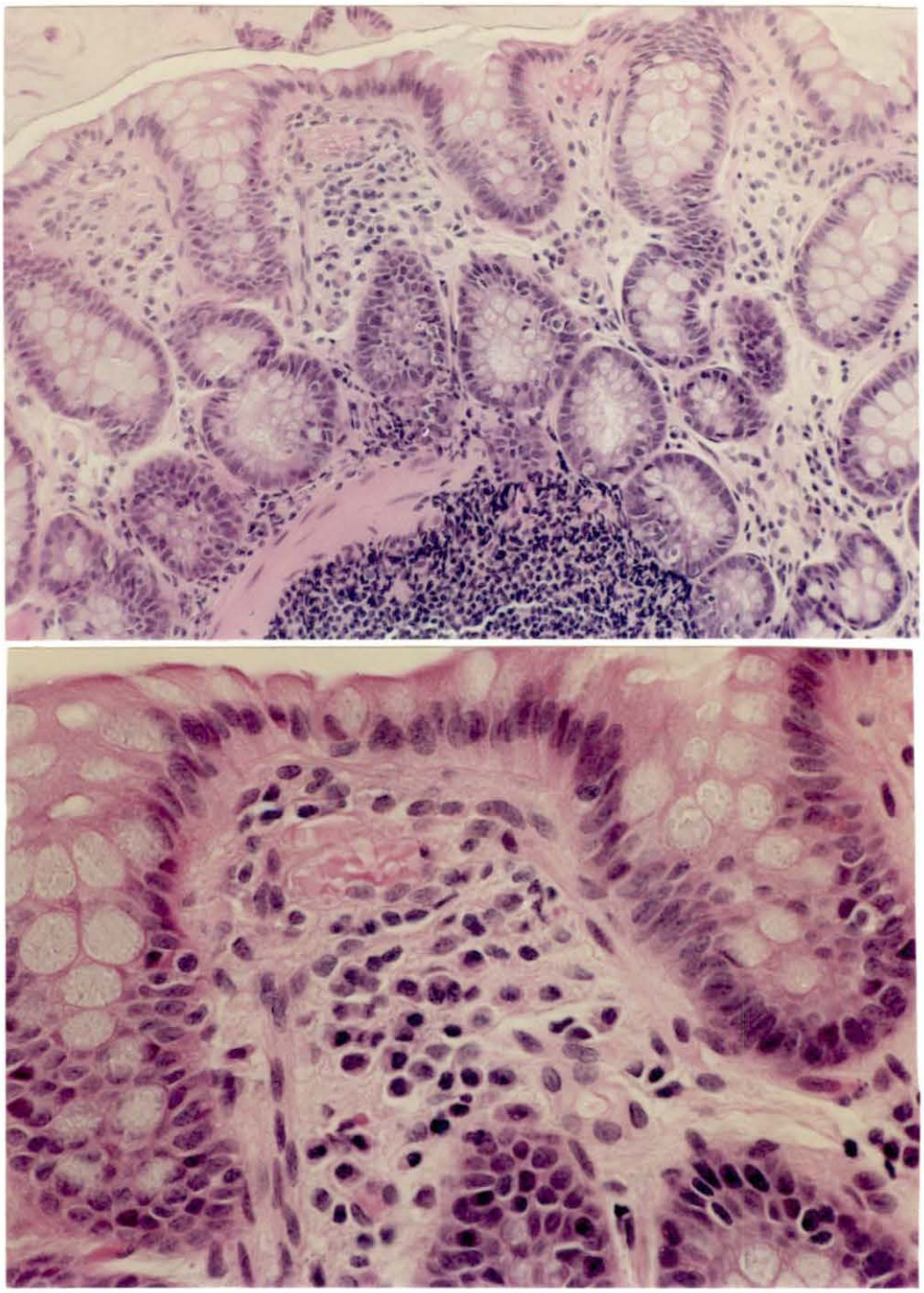

Figure 3: Colonic biopsy from test animal #1. Histopathological diagnosis: Mild IBD, characterized by infiltration of lymphocytes and plasma cells.
Top: Hematoxylin and Eosin (x 160).
Bottom: Hematoxylin and Eosin (x 400).

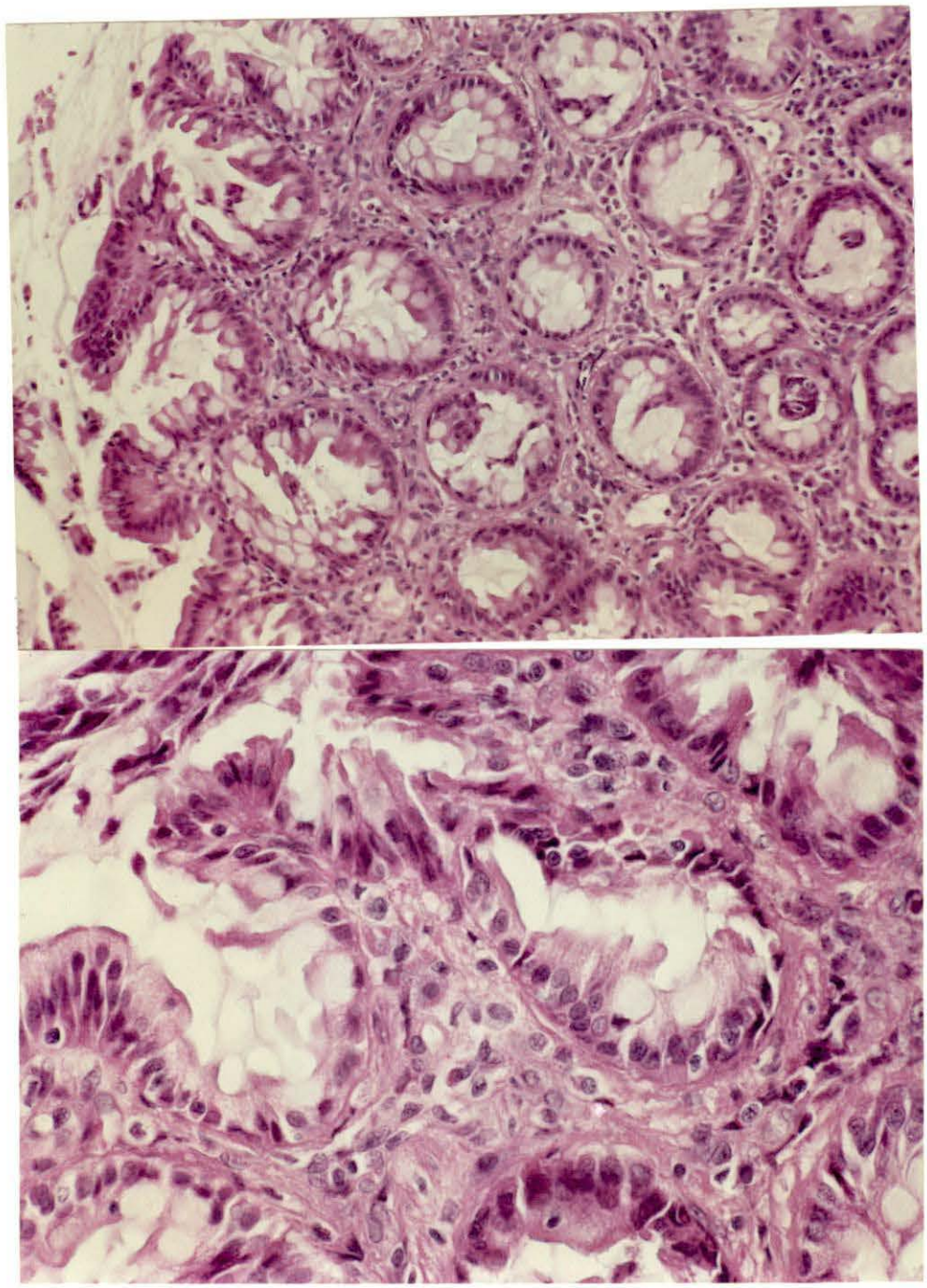

Figure 4: Colonic biopsy from test animal #2. Histopathological diagnosis: Mild IBD, characterized by infiltration of lymphocytes and plasma cells.
Top: Hematoxylin and Eosin (x 160).
Bottom: Hematoxylin and Eosin (x 400).

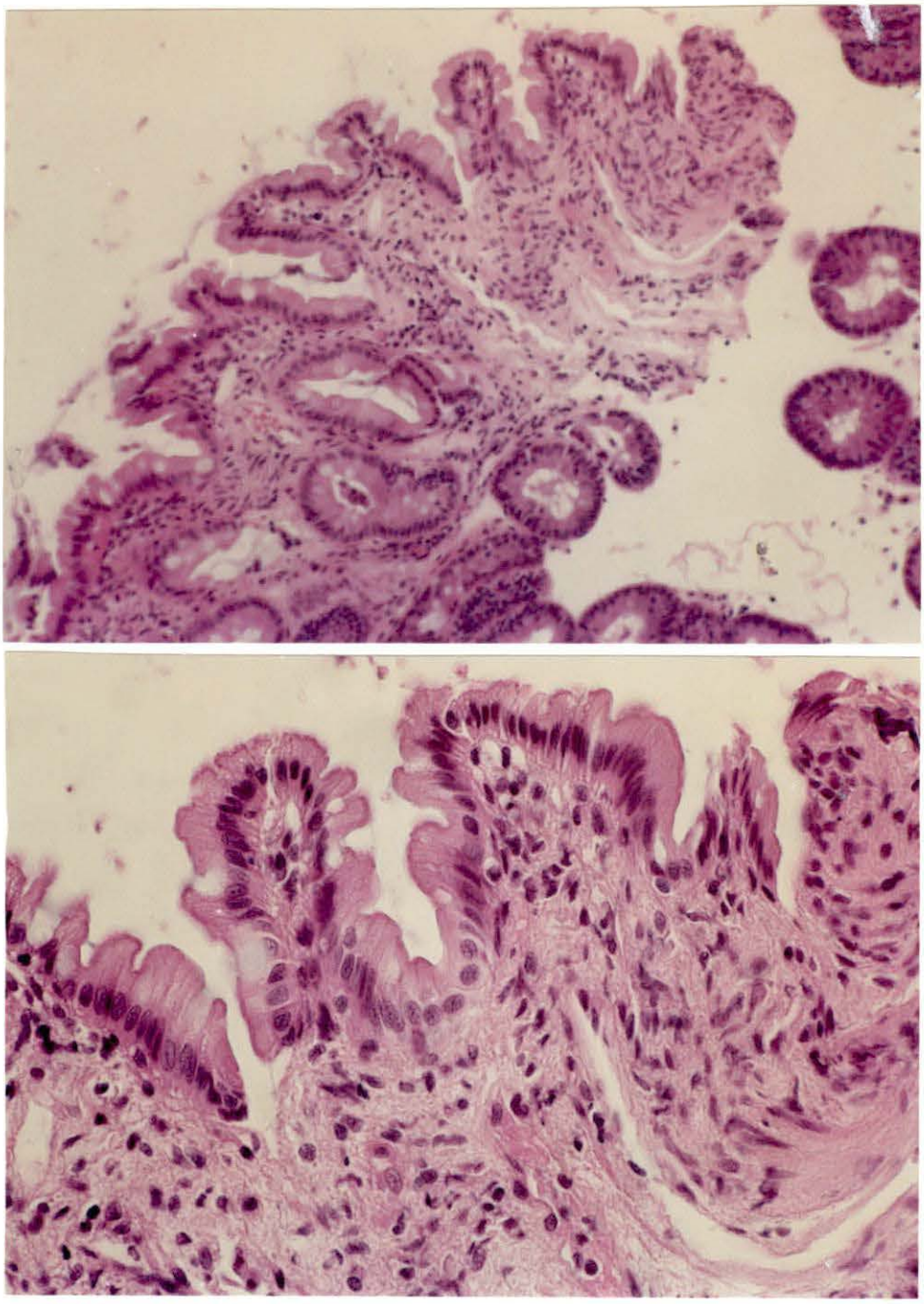

Figure 5. Colonic biopsy from test animal #3. Histopathological diagnosis: Mild IBD, characterized by mucosal edema and infiltration of lymphocytes and plasma cells.
Top: Hematoxylin and Eosin (x 160).
Bottom: Hematoxylin and Eosin (x 400).

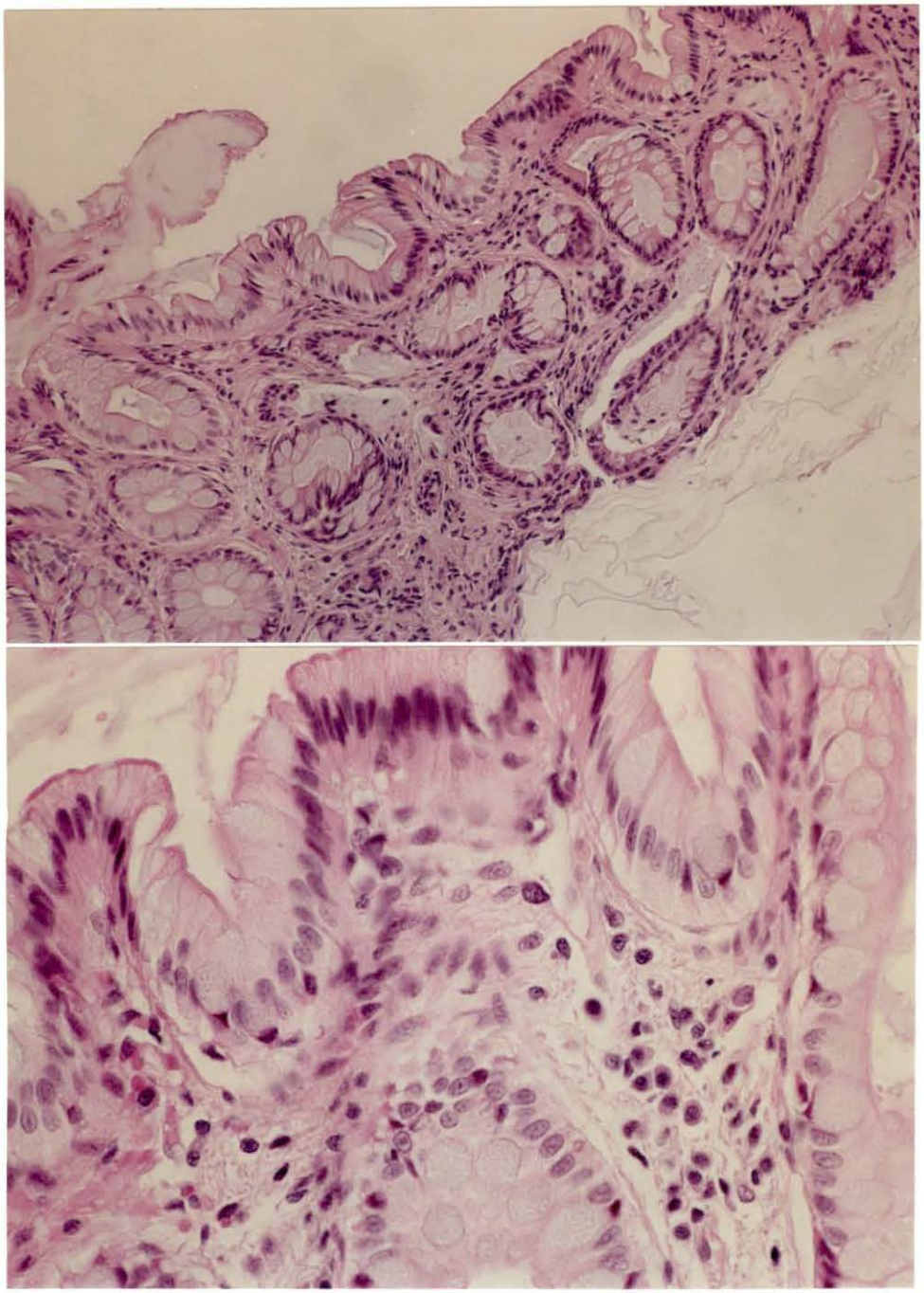

Figure 6: Colonic biopsy from test animal #4. Histopathological diagnosis: Moderate IBD, characterized by infiltration of lymphocytes and plasma cells, glandular immaturity and mucosal edema.
Top: Hematoxylin and Eosin (x 160).
Bottom: Hematoxylin and Eosin (x 400).

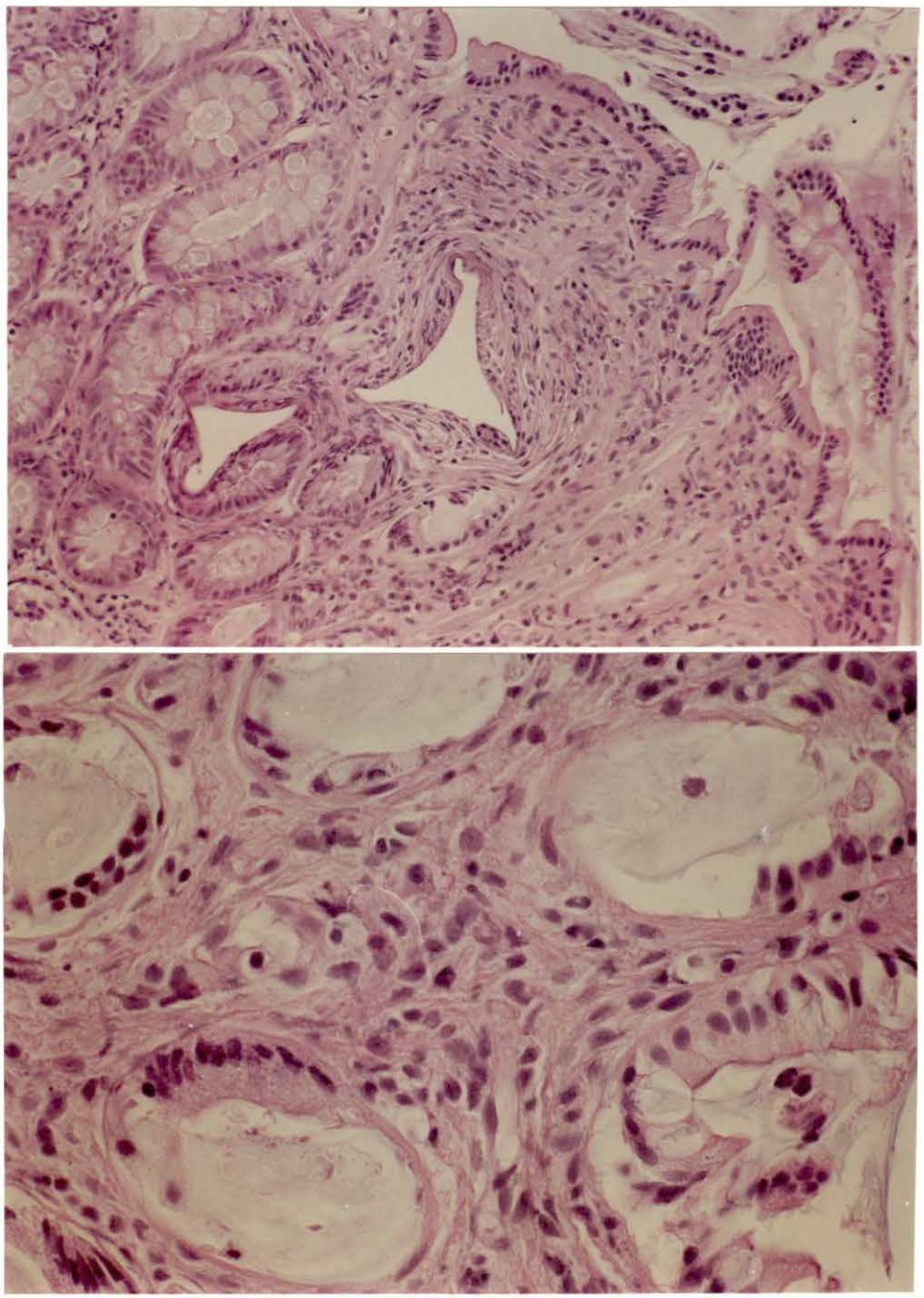

Figure 7: Colonic biopsy from test animal #5. Histopathological diagnosis: Severe IBD, characterized by infiltration of lymphocytes and plasma cells, depletion of goblet cells and fibrosis of lamina propria.
Top: Hematoxylin and Eosin (x 160).
Bottom: Hematoxylin and Eosin (x 400).

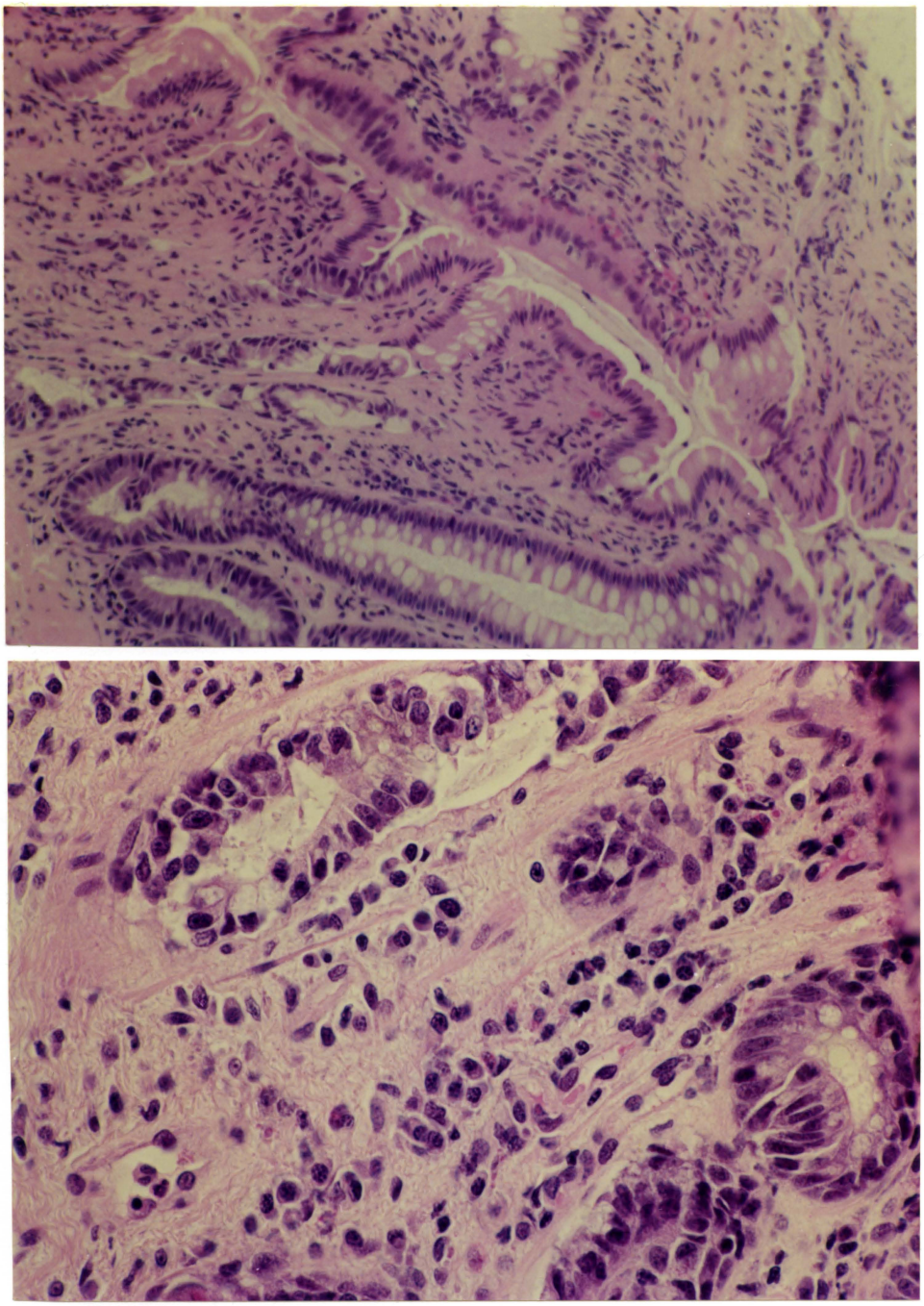

Figure 8: Colonic biopsy from test animal #6. Histopathological diagnosis: Moderate IBD, characterized by infiltration of lymphocytes and plasma cells.
Top: Hematoxylin and Eosin (x 160).
Bottom: Hematoxylin and Eosin (x 400).

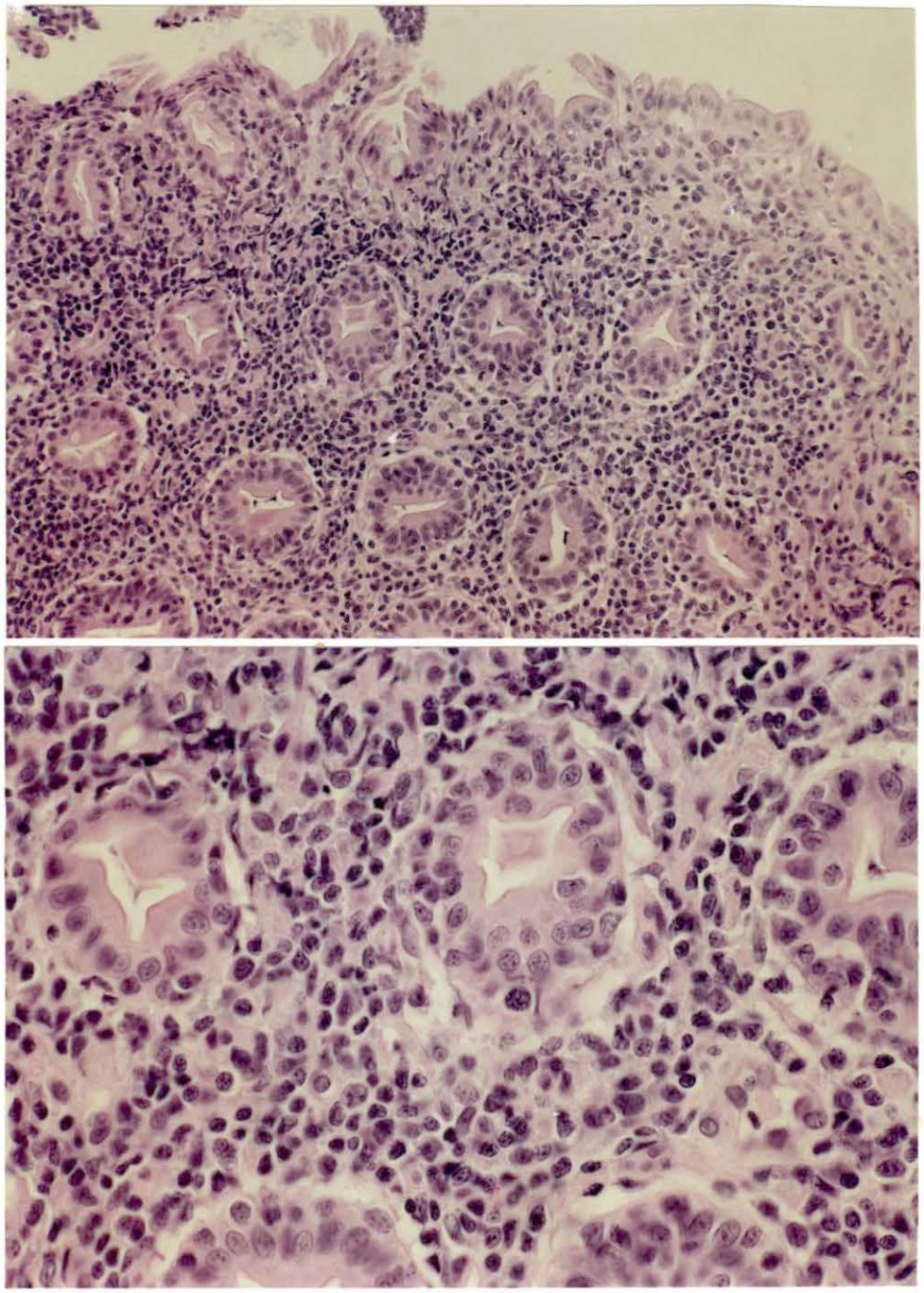

Figure 9: Colonic biopsy from test animal #7. Histopathological diagnosis: Severe IBD, characterized by severe depletion of goblet cells and excessive infiltration of lymphocytes and plasma cells.
Top: Hematoxylin and Eosin (x 160).
Bottom: Hematoxylin and Eosin (x 400).

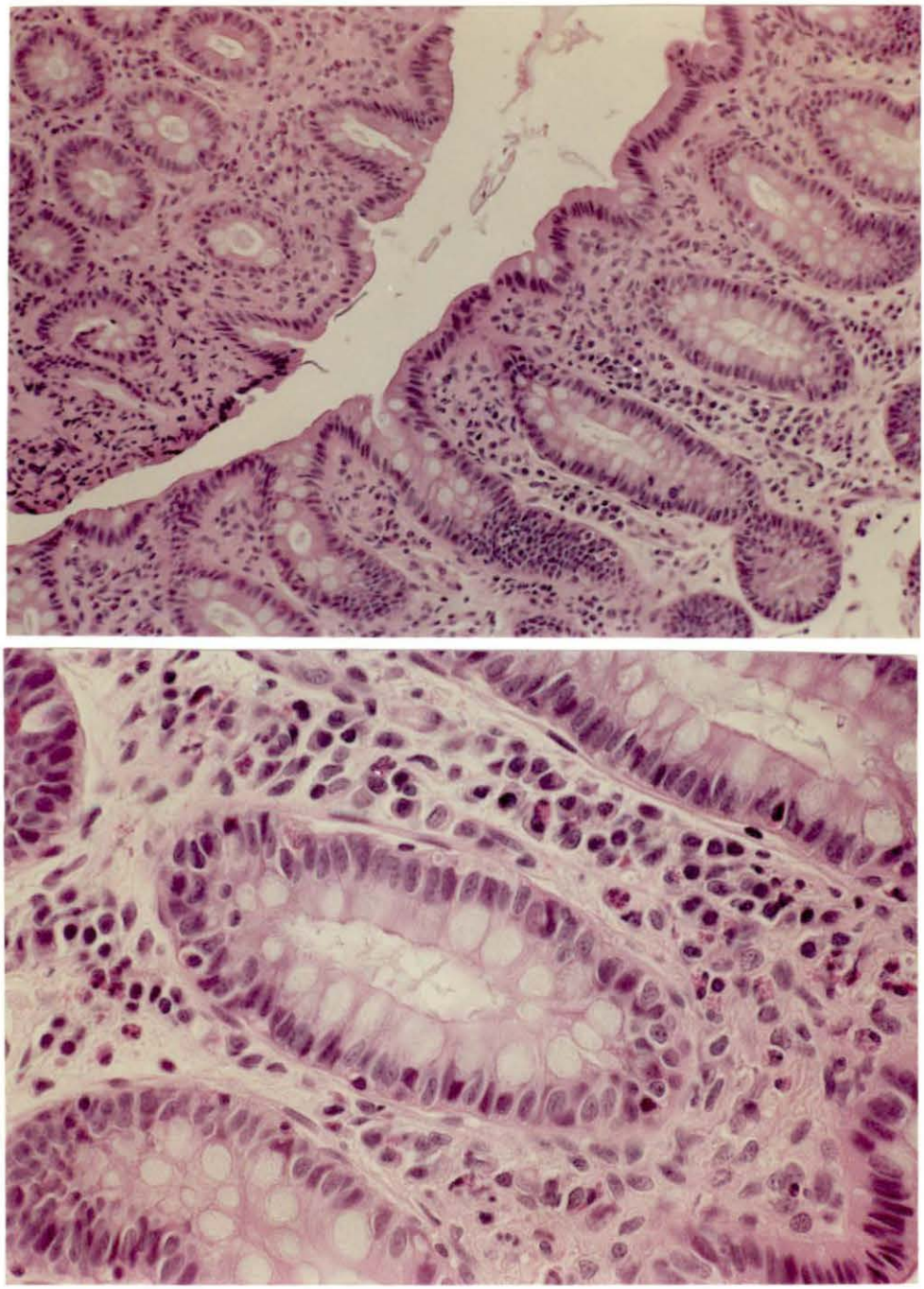

Figure 10: Colonic biopsy from test animal #8. Histopathological diagnosis: Mild IBD, characterized by infiltration of lymphocytes, plasma cells and eosinophils.
Top: Hematoxylin and Eosin (x 160).
Bottom: Hematoxylin and Eosin (x 400).

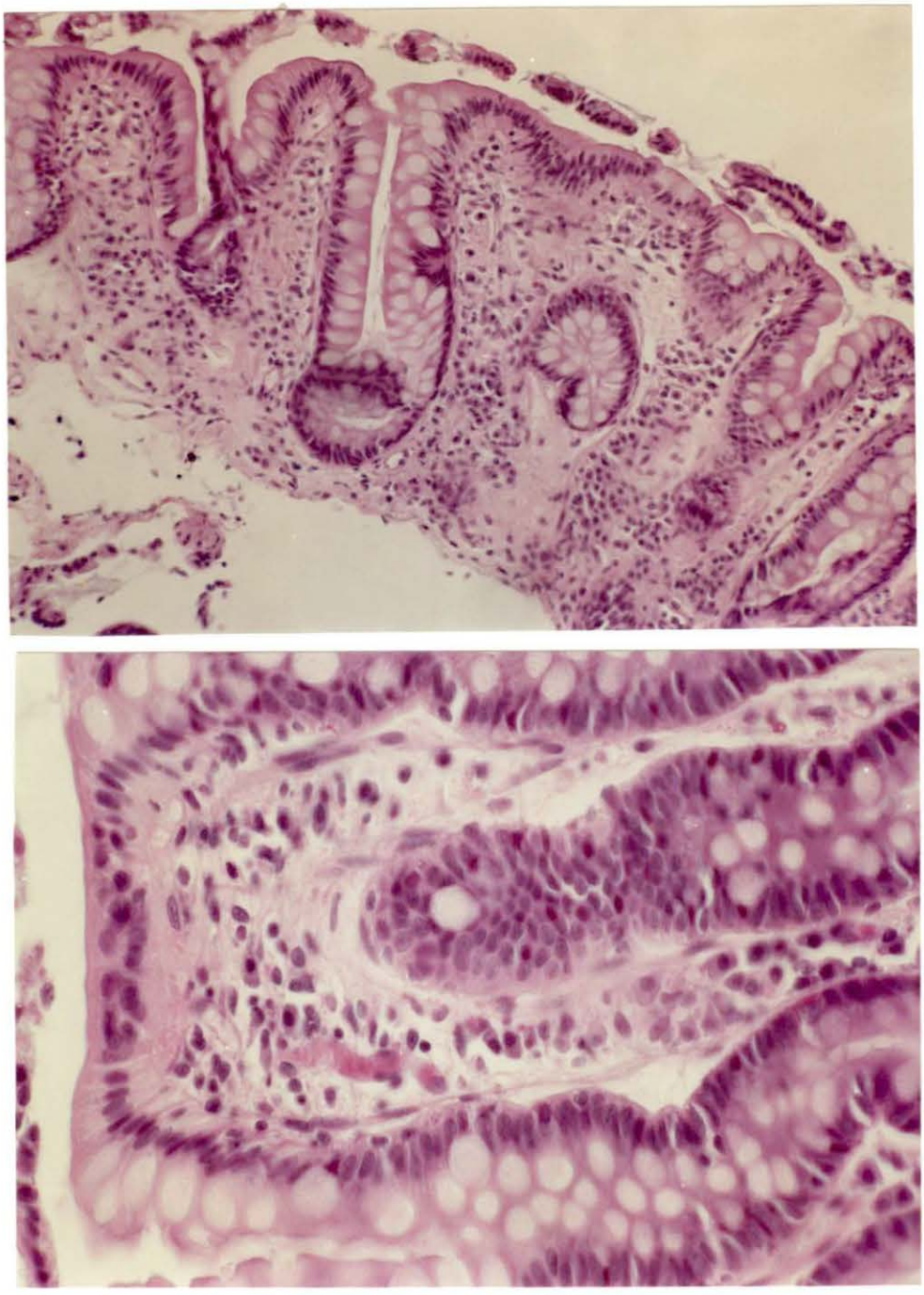

Figure 11: Colonic biopsy from test animal #9. Histopathological diagnosis: Mild IBD, characterized by infiltration of lymphocytes and plasma cells, and goblet cell hyperplasia.
Top: Hematoxylin and Eosin (x 160).
Bottom: Hematoxylin and Eosin (x 400).

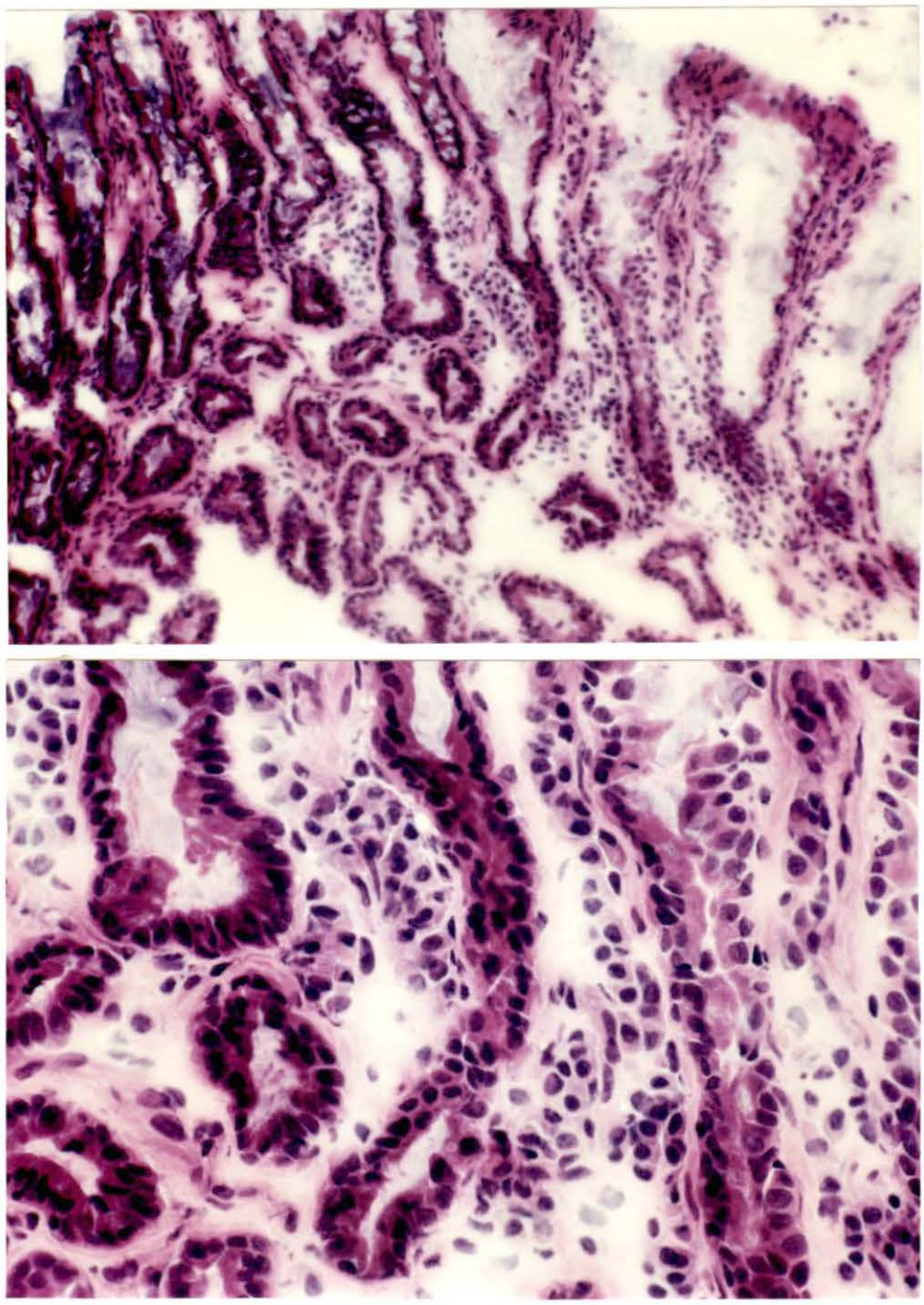

Figure 12. Colonic biopsy (frozen section) from test animal #10. Histopathological diagnosis: Mild IBD, characterized by infiltration of lymphocytes and plasma cells.
Top: Hematoxylin and Eosin (x160).
Bottom: Hematoxylin and Eosin (x 400).

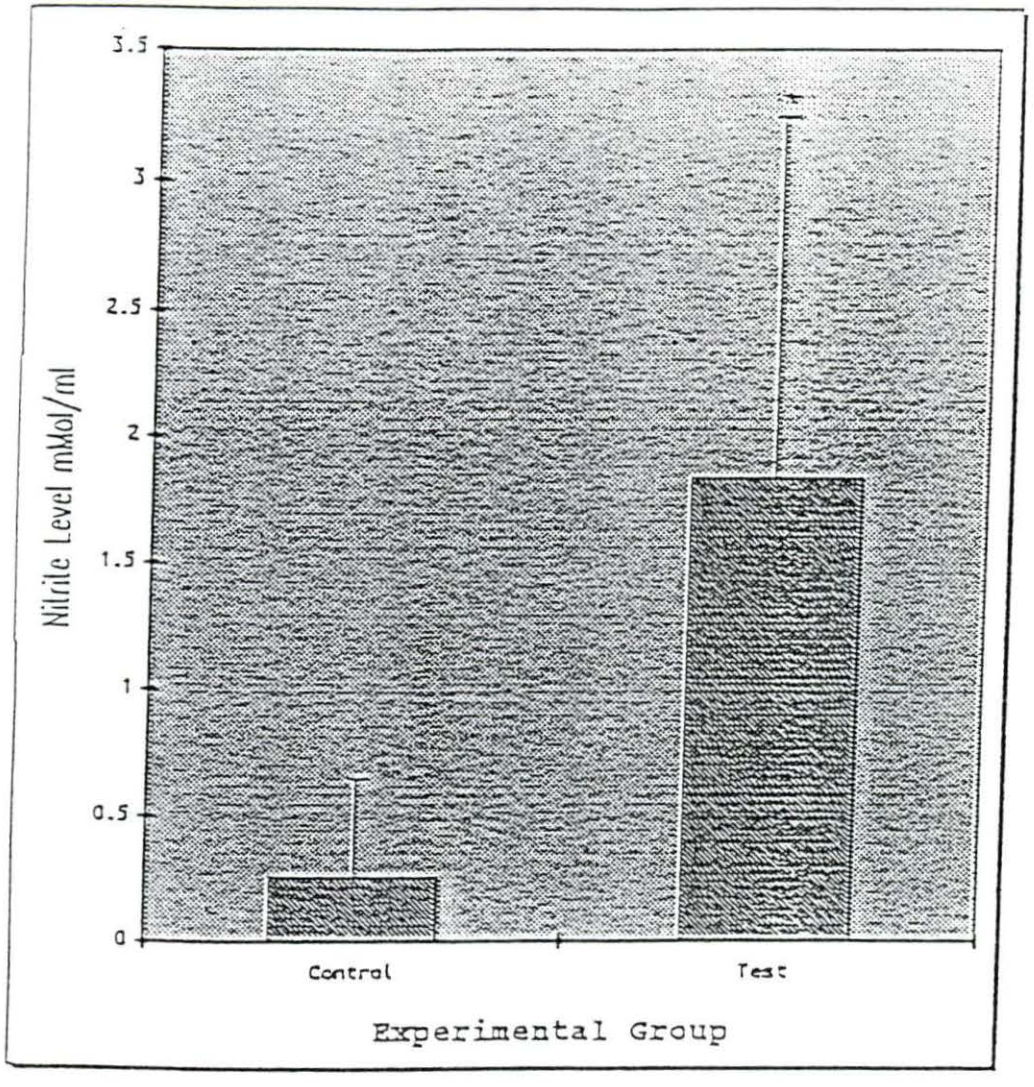

Figure 13. Nitrite concentrations in colonic lavage samples from nine control dogs (control group) and ten dogs diagnosed with IBD (test group). Values are mean ± SD. * = $p < 0.05$.

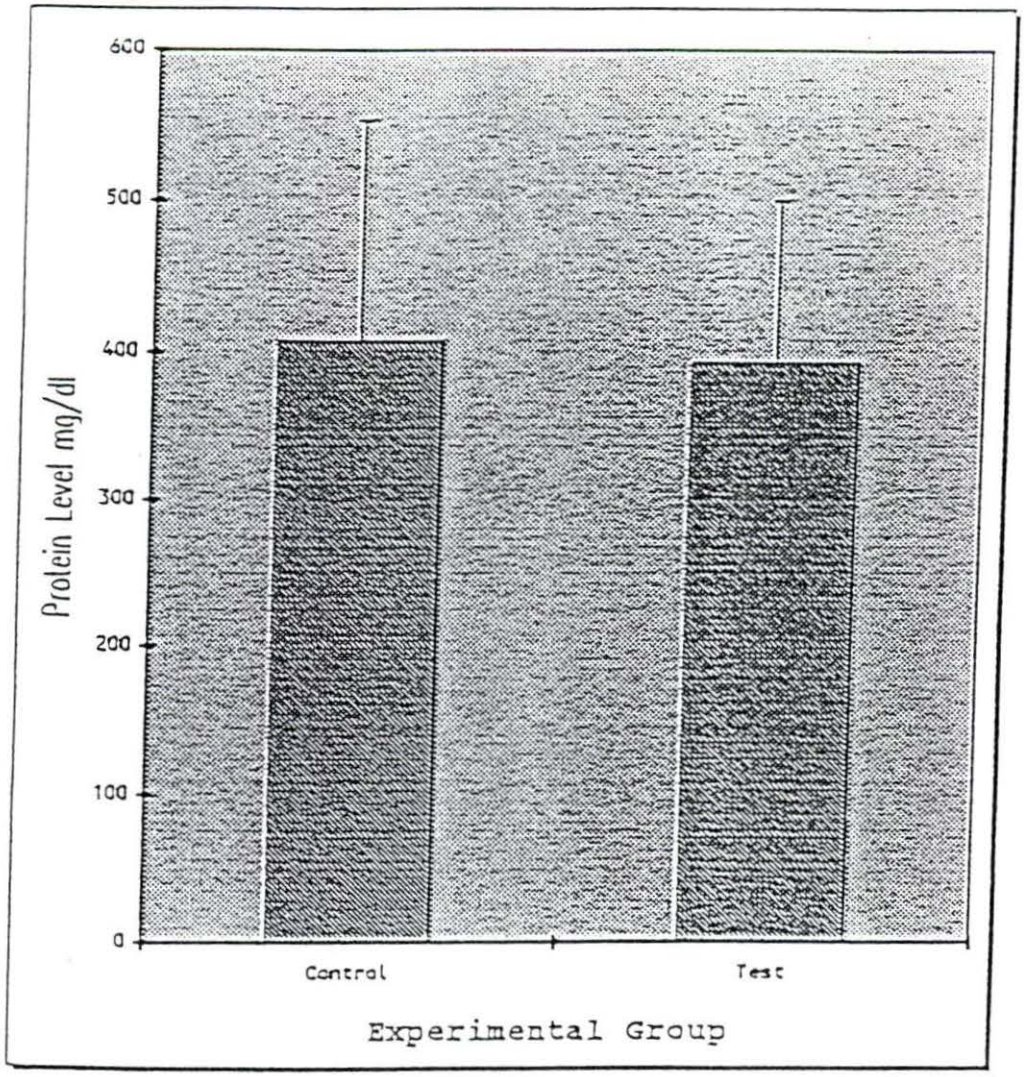

Figure 14. Total protein concentrations in colonic lavage samples from nine control dogs (control group) and ten dogs diagnosed with IBD (test group). Values are mean ± SD.

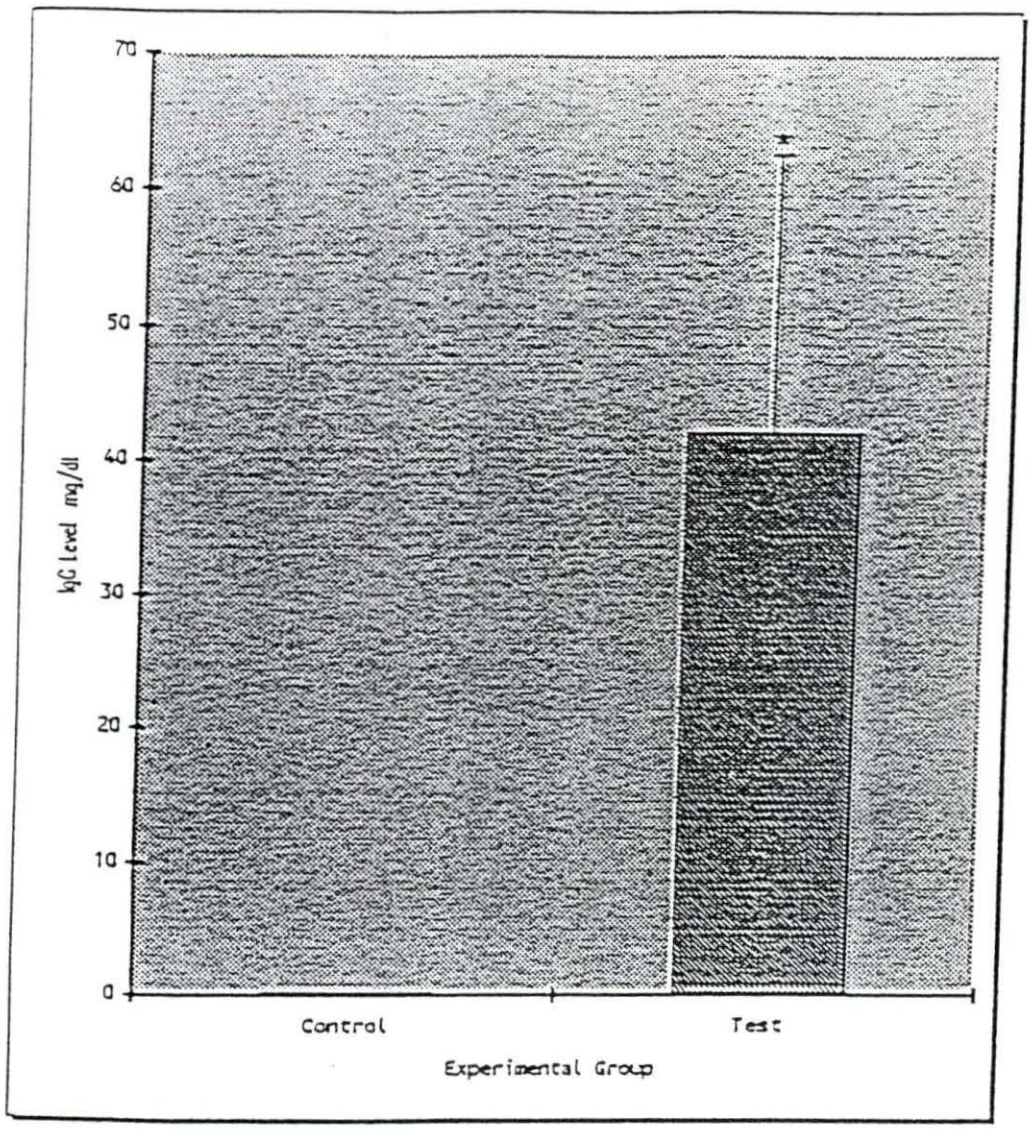

Figure 15. Immunoglobulin G (IgG) concentrations in colonic lavage samples from nine control dogs (control group) and ten dogs diagnosed with IBD (test group).
Values are mean ± SD. * = $p < 0.001$.

DISCUSSION

The purpose of the investigation was to determine whether inflammatory bowel disease is associated with elevated production of nitric oxide and immunoglobulin G in the large intestine. The method of testing the hypothesis was to measure the levels of nitrite and IgG in colonic lavage fluid from healthy control dogs and dogs with suspected IBD. Since nitric oxide is a highly volatile compound, it's levels cannot be measured directly. Nitric oxide concentrations may be determined indirectly by measuring the levels of its end product, nitrite ion. IgG, although susceptible to proteolytic enzymes, was preserved until assay by the use of protease inhibitors. Histopathological examination of colonic biopsy specimens for inflammatory lesions, and measurement of the activity of myeloperoxidase (an enzyme contained in inflammatory cells) in colonic lavage samples, were used to assess the degree of bowel inflammation in each animal.

During colonoscopy, colonic biopsy specimens were obtained from each animal, from both inflamed and normal-appearing areas in the case of suspected IBD patients. Upon histological examination of these specimens, IBD lesions of varying degrees were revealed in all the samples from the IBD group. According to the pathologist's diagnosis, of the ten dogs tested six dogs had mild IBD, two had moderate IBD and two showed severe IBD. The predominant histopathological feature found in all cases was the infiltration of lymphocytes and plasma cells. The degree of infiltration

varied between cases. Eosinophilic infiltration was observed in one animal (T8). One of the cases diagnosed with severe IBD (T5) had extensive fibrosis of the lamina propria, indicating its chronic nature. Focal fibrosis of the lamina propria was observed in one of the cases with moderate IBD (T6). Other features like goblet cell hyperplasia, glandular immaturity and edema of the lamina propria were observed occasionally. All the above findings confirm the histopathological picture of canine IBD as described in the literature (Sherding and Burrows, 1992). In the control group, seven dogs showed normal histology while two were diagnosed to have mild IBD.

Myeloperoxidase (MPO) activity in colonic lavage samples was not detectable in any of the samples tested, (despite immediate processing and analysis upon collection) either from the control animals or from the animals with suspected IBD. This finding contrasts with previous studies where it was concluded that MPO activity is a reliable marker for the inflammatory cell content in a rabbit model of ileitis (Miller et al., 1991), and in cutaneous inflammation in rats (Bradley et al., 1982).

The principle source of MPO in an inflammatory lesion is the neutrophil (Bradley et al., 1982). Hence, the MPO activity may be considered a marker for neutrophil content rather than for the degree of inflammation. In human IBD, MPO activity is a suitable indicator because the inflammatory lesions in ulcerative colitis and Crohn's disease frequently contain elevated numbers of neutrophils. In the clinical cases of the present study, the histopathological examination revealed chronic

inflammatory lesions of varying degrees in all the animals suspected to have IBD. However, these lesions were characterized by predominant infiltration of lymphocytes and plasma cells rather than neutrophils. Eosinophilic infiltration was found occasionally, but the number of neutrophils in these lesions was quite low. This may be the reason for the undetectability of MPO in these samples. Furthermore, neutrophils are characteristic of acute rather than chronic inflammation. Colonic biopsy specimens for this study were obtained at one time only from each animal, so that possible acute flare-ups of the disease may have gone undetected.

In the intestine, nitric oxide can be synthesized both by luminal bacteria and by nitric oxide synthase found in the intestinal epithelial cells. A study with isolated rat intestinal epithelial cells revealed the presence of both inducible and constitutive forms of nitric oxide synthase (NOS), determined by conversion of ^{14}C arginine to citrulline. This conversion was inhibited by incubation with a NOS inhibitor, namely N^G-monomethyl-L-arginine, an analogue of arginine (Tepperman et al., 1993). Several studies in humans and experimental animals have revealed elevated luminal nitrite levels associated with intestinal inflammation, suggesting an elevated production of NO by intestinal epithelial cells under an inflammatory stimulus. Upon analysis of rectal dialysates of human IBD patients, measurable levels of nitrite were found in a majority of active IBD patients, while those with quiescent colitis or the normal control subjects yielded no measurable nitrite (Roediger et al., 1986). Elevated luminal nitrite levels were observed in a guinea pig model of

chemically induced ileitis, and the chronic ileal inflammation was found to be ameliorated upon administration of NOS inhibitors (Miller et al., 1992). In our investigation, colonic lavage nitrite levels in dogs with IBD were significantly higher than those of normal dogs. The mean nitrite level was over seven times higher in the test group than in the control group. This finding supports the conclusions of the above studies, suggesting that canine IBD is also associated with elevated activity of NOS in intestinal epithelial cells. In our study, the IBD group had a higher standard deviation than the test group, in both nitrite and IgG levels. This was to be expected since in the patients we studied the disease had occurred naturally, so that the severity of disease varied among cases.

Intestinal lumen normally contains some proteins, originating from the diet, intestinal secretions, discarded epithelial cells and luminal bacteria. The immunoglobulins found in the intestinal secretions are principally IgA and some IgM, both of which are secreted from the mucosal B lymphocytes (Evans, 1984). IgG is a type of immunoglobulin which is not normally secreted from mucosal surfaces. IgG usually exerts its function within the bloodstream, as do other plasma proteins like albumin. Such proteins do not normally enter the intestinal lumen, since the epithelial barrier between the bloodstream and intestinal lumen is impermeable to these proteins. But, in intestinal inflammation, this mucosal barrier is weakened, allowing the leakage of plasma proteins into the lumen.

Elevated levels of albumin and IgG have been found in the gut lavage fluid from human IBD patients (Choudari et al., 1993; O'Mahoney et al., 1990). In these studies, a specific increase of IgG levels in proportion to the disease activity has been observed, leading to the conclusion that IgG may be a reliable marker for the degree of inflammation in IBD in humans. In our study, while there was no significant difference between the total protein levels in colonic lavage fluid from the two groups, the IgG levels in the diseased animals were found to be markedly elevated when compared to the control group which yielded no detectable IgG. This finding strongly supports the conclusions from the previous studies, suggesting that colonic lavage IgG is a reliable indicator of canine inflammatory bowel disease.

IgG levels were undetectable in all the animals of the control group, including the two animals which showed histopathology indicative of mild IBD. One possible explanation is that the histological lesions in these two animals might have been due to a bowel inflammation other than IBD which is not normally characterized by elevated IgG. This theory is supported by the absence of clinical signs of IBD and the remarkably low levels of colonic nitrite in these two cases.

From the results of this investigation it can be concluded that colonic lavage nitrite and IgG levels are elevated in canine IBD. However, a correlation between the degree of disease and the degree of increase of these compounds cannot be determined. While three cases of mild IBD showed relatively high levels of nitrite and IgG, one case which

showed severe IBD had comparatively low levels of IgG and moderate levels of nitrite. Similarly, a correlation between the nitrite levels and IgG levels was not obvious except in T3 where both were quite low.

A possible reason for this lack of correlation in the nitrite levels with the disease activity might be the varying degree of NO production by colonic bacteria, which was not accounted for in this study. There was no available method for making an allowance for bacterial NO production, and we assumed it to be negligible since reasonably adequate bowel cleansing was done prior to obtaining the lavage samples. However, since there is no known difference between the colonic bacterial populations between normal and IBD subjects, the difference in the mean nitrite levels between the two groups is sufficient to conclude that IBD has a marked influence on the intestinal epithelial NO synthesis.

The role of NO in IBD is not clear. Previous studies have suggested that NO may play a protective role in acute intestinal inflammation. The epithelial permeability in isolated feline small intestine, measured by blood-to-lumen clearance of ^{51}Cr-labeled EDTA, was markedly elevated upon inhibition of NOS, and was restored by administration of exogenous NO (Kubes, 1992). In a feline model of intestinal ischemia followed by reperfusion, the intestinal mucosal barrier disruption was reduced by exogenous NO (Payne and Kubes, 1993). In contrast, NO aggravates chronic inflammation so that inhibition of NOS is beneficial in such situations. In a guinea pig model of chemically induced chronic ileitis, oral

administration of a NOS inhibitor ameliorated the intestinal damage, suggesting a harmful role for NO which is synthesized in large amounts in this situation (Miller et al., 1992). To determine what role NO plays in canine IBD, further studies need to be conducted using NOS inhibitors.

The conclusion of the present investigation is that there is a significant increase in colonic lavage nitrite and IgG levels in canine IBD, and that these parameters may be used as indicators of the presence of IBD. This may be a valuable diagnostic approach, since colonic lavage is an easy and non-invasive procedure and the measurement of nitrite and IgG is rapid and convenient. The quantification of IgG by radial immunoassay is a particularly easy and accurate method, and may be used as a quick diagnostic marker for canine IBD. The elevation of nitrite levels indicate increased activity of intestinal epithelial NOS in canine IBD. Following further studies to determine what role NO plays in the inflammatory process, pharmacologic modification of intestinal NOS activity may serve as a valuable therapeutic approach to canine IBD.

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