Morphometric evaluation of IgA, IgG, and T-cells in duodenal mucosa from normal dogs and dogs with inflammatory bowel disease

by

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GENERAL INTRODUCTION

A variety of disorders including giardiasis, bacterial overgrowth, *Campylobacter* or *Salmonella* infections, food allergy, lymphangiectasia, neoplasia, and inflammatory bowel disease may result in gastroenteritis with inflammatory infiltrates in dogs.[1-4] Inflammatory bowel disease (IBD) refers to a group of idiopathic, chronic gastrointestinal tract disorders, characterized by infiltration of the gastrointestinal tract by inflammatory cells. This cellular infiltrate includes various populations of lymphocytes, plasma cells, eosinophils, and neutrophils that generally are confined to the mucosa. Variable forms of IBD, including lymphocytic - plasmacytic[2,3,5,6], eosinophilic[7,8], and granulomatous enterocolitis[9,10] have been described as having lesions involving the stomach, small intestine, or colon. Inflammatory bowel disease is the most common cause of chronic gastroenteritis (chronic vomiting, small and/or large bowel diarrhea, anorexia, and weight loss) in dogs.

Classification of Inflammatory Bowel Diseases

Idiopathic inflammatory bowel diseases are commonly classified according to the type of mucosal inflammation present and the area of the gastrointestinal tract in which the inflammation predominates. It is important to realize that this classification scheme is an arbitrary system, and is named by

the predominant cell infiltrate present within histopathologic specimens. The archetypal histologic lesion is controversial, and there are inflammatory bowel diseases that do not easily fit into the standard classifications. Indeed, a variable but mixed inflammatory infiltrate in the lamina propria is the salient feature in most histologic specimens.[2,4-6] Canine chronic colitis (ie, lymphocytic - plasmacytic colitis) has similarities to ulcerative colitis and microscopic colitis of humans. Canine granulomatous enteritis resembles Crohn's disease of humans.[9] Current evidence in humans suggests that ulcerative colitis and Crohn's disease are the two ends of a spectrum of idiopathic inflammatory bowel disorders.[11] These two diseases are commonly grouped together under the term inflammatory bowel disease since 5-10% of the cases share overlapping clinicopathologic features, the diseases share certain immunologic abnormalities, and both are idiopathic.[11] Canine lymphocytic plasmacytic enteritis (LPE) is a chronic, idiopathic, inflammatory disease of the small bowel characterized by a mixed inflammatory infiltrate in which lymphocytes and plasma cells predominate. The incidence of LPE is unknown, but recent reports and personal experience suggest that small intestinal LPE is the most common IBD variant affecting the small bowel of the dog.[1-3,6]

Etiopathogenesis

The cause for IBD in animals and humans is not understood, although the histologic and clinical features suggest an underlying immunologic mechanism.[4,11] Increased numbers of inflammatory cells are observed microscopically in the gastrointestinal mucosa of affected patients. Clinically, IBD in humans may be associated with other diseases in which immunologic mechanisms play an integral role (e.g. auto-immune hemolytic anemia). A variety of extra intestinal manifestations, including pericholangitis, chronic active hepatitis, and polyarthritis may accompany IBD in humans and rarely, animals.[12] The traditional mainstays of therapy in IBD involve dietary management and the use of drugs that are known to alter the immune response, such as corticosteroids, azathioprine, and sulfasalazine.[1,2-4] These accumulated observations suggest that immune mechanisms are involved in the disease process; however, there currently is insufficient evidence to classify IBD as a primary immunologic disease.

Recent theories in humans and domestic animals propose that IBD involves host hypersensitivity responses to antigens (bacterial, food, or self-antigens) within the bowel lumen or mucosa.[1,2-4,11] Possible causes of antigenic exposure include alterations in the mucosal permeability barrier and defective immunoregulation of gut-associated lymphatic tissue. Mucosal inflammation

resulting from any cause potentially can increase intestinal permeability.[13,14] Humans with IBD have increased bowel permeability, raising suspicion that inflammation - mediated breakdown in the mucosal barrier can contribute to the perpetuation of inflammatory lesions. Recently, it has been shown that relatives of people with IBD also have increased bowel permeability.[14] This raises the probability that disordered intestinal permeability is the primary lesion in IBD, and that the intestinal inflammation is the result of chronic excessive antigenic exposure.

Abnormalities in the suppressor function of gut-associated lymphatic tissue (GALT) is currently thought by many to be the cause of the hypersensitivity response characteristic of IBD.[11,15,16] It has been proposed that the defect in this system allows the escape of a reactive clone of lymphocytes which may incite immune responses against various gut antigens. The ensuing immune response would induce significant gastrointestinal inflammation, which in turn would increase bowel permeability, inundating the lamina propria with additional antigen of a variety of types. The net result is that the suppressor function of the GALT to a variety of antigens is eventually overwhelmed, resulting in a cycle of further inflammation, further permeability increases, and eventual overt tissue damage.

Cellular recruitment during active disease in humans is facilitated by the expression of specific binding molecules, ELAM (endothelial-cell-leukocyte-

adhesion molecule), on endothelial cells of the bowel.[17] Neutrophils are attracted by increased concentrations of chemotactic leukotrienes (especially leukotriene B_4) as well as formylated bacterial peptides (such as FMLP) and other phlogistic factors which are present in increased concentrations in IBD. Recruitment of additional inflammatory cellular constituents (lymphocytes, macrophages, mast cells) occurs in response to activation by leukotrienes, cytokines, and other inflammatory mediators. This inflammatory process results in the production of an autoamplifing stimulatory network of cytokines, eicosanoids (including leukotriene B_4), and oxygen-free radicals that damage tissue. Therefore, specific or generalized luminal stimuli may lead to sustained stimulation of immune and inflammatory processes which cause mucosal injuries. Clinical signs in affected animals are directly attributable to gastrointestinal inflammation and its effects on gastrointestinal permeability, motility, nutrient absorption, and the vomiting center.

Clinical Signs of Inflammatory Bowel Disease

There is no age, sex, or breed predisposition for the development of canine IBD. Most cases of IBD occur in middle-aged and older animals; however, a recent clinical study found that 27% of dogs and cats having IBD were 2 years of age or less.[18] All breeds may be affected with specific disease

syndromes recognized in the Boxer, Ludenhund, and Basenji canine breeds.[1,4] Lymphocytic - plasmacytic enteritis is one of many primary enteropathies affecting German Shepherd dogs. Clinical signs are variable and reflect the site of the disease and the extent of mucosal damage. Predominant clinical findings include chronic vomiting, diarrhea, anorexia, weight loss, tenesmus, hematochezia, mucoid stools, and abdominal pain.[1-5,18] Signs of gastrointestinal ulceration-erosion (melena, hematemesis) have been recently reported in dogs having endoscopic lesions and histologic infiltrates of IBD.[19] In general, clinical signs have a cyclical rather than progressive clinical course.

Diagnosis of Inflammatory Bowel Disease

Inflammatory bowel disease is a diagnosis of exclusion and should be differentiated from other disorders causing gastrointestinal inflammation. Criteria which support a diagnosis of IBD include the following: clinical signs of persistent gastrointestinal disease; failure or inadequate clinical response to dietary trials using hypoallergenic or controlled diets; performance of a thorough data base which excludes metabolic disease and other primary gastrointestinal causes; and a histologic diagnosis of inflammatory cellular infiltrates and failure to demonstrate other causes of gastroenteritis.[18,20] Definitive diagnosis requires gastrointestinal biopsy obtained by endoscopic procedures or by laparotomy.

Mucosal biopsy confirms a diagnosis of IBD and provides information for prognosis and individualization of therapy. Endoscopy is preferred since it is inexpensive, fast, and minimally invasive. Targeted biopsies for cytologic and histologic evaluation may be obtained. The expanding use of gastrointestinal endoscopy in veterinary clinical practice has led to increased numbers of biopsy specimens being submitted to pathology services for histologic review.[21,22] The small size of these biopsy specimens and the lack of objective histologic criteria contribute to the difficulty in diagnosing IBD in animals.

Histopathology of Human Inflammatory Bowel Disease

Normal human lamina propria lymphocytes. The normal human intestinal lamina propria contains a wide variety of lymphocytes. B cells are scarce in the intraepithelial compartment but common in the lamina propria. In all species studied, including normal dogs and cats, IgA-containing B cells are the predominant B-cell subtype.[23,24] In "normal" human mucosa, 70-90% of the B-cells are IgA-containing cells, including plasma cells and their immediate precursors.[25,26] Smaller numbers of IgG, IgM, and IgE-containing lymphocytes have also been identified. T-cells with helper, suppressor, and cytotoxic functions are also present, with the majority being of the helper subset. The relative numbers of B and T-cells and other leukocytes in the gastrointestinal

mucosa differ with antigenic stimulation, the age of the animal, and the sampling site within the intestine (ie, oral versus aboral). In human adults, B cells, T-cells, and null cells have been reported to make up 25%, 50%, and 25% of the lamina propria lymphoid pool, respectively.[27] In diseased mucosa the number of leukocytes in the lamina propria increases. The IgA-containing cells usually remain predominant, but the relative numeric increase is largest for the IgG-secreting cells.[28]

The immunopathology of human IBD. The large number of lymphocytes and plasma cells observed in humans with ulcerative colitis and Crohn's disease is consistent with an immunological mechanism in these diseases. The inflammatory infiltrate, especially the immunoglobulin containing cells in the gut, has been the subject of a number of quantitative studies.[29-32] All of these investigators used an immunofluorescence technique, but their quantitative methods varied. In most studies, an increase of IgA and IgG-containing cells was found in inflammatory bowel disease. Skinner and Whitehead[30] and Baklien and Brandtzaeg[32] showed that in active ulcerative colitis and Crohn's disease the increase was greatest for IgG and IgM-containing cells. Only a few authors have looked at the inflammatory infiltrate with the immunoperoxidase technique although this technique has the advantage of permanence of the sections and is more suitable for morphometric study than immunofluorescence on frozen sections. Using this methodology, Otto and Gebbers[33] found marked increases

of IgG-containing cells in patients having ulcerative colitis. Similarly, Rosekrans *et al*[31] showed a relatively large increase in the IgG-containing cells in ulcerative colitis and increases in IgG and IgM-containing cells in Crohn's disease. These same authors used morphometric techniques to quantitate IgA-, IgG-, IgM-, IgD-, and IgE-containing cells in colonic mucosa from endoscopically procured biopsies. The number of IgM-containing cells in the lamina propria per mm of colonic mucosa length was found to be of discriminatory value in differentiating the pathology of Crohn's disease from ulcerative colitis. In contrast, the numbers of mucosal T-cells and the distribution of mucosal T-cell subsets in patients with Crohn's disease and ulcerative colitis has not differed from normal controls.[34] Other histologic criteria found to be of discriminatory value for the differentiation of ulcerative colitis from acute non-specific colitis include distortion of crypt architecture, a villus surface, crypt atrophy, and increased lamina proprial cellularity.[35] Of these features, the subjective assessment of cell number has the most inter-observer variability and is felt by many to be an unreliable means to diagnose IBD.[36]

Histopathology of Canine Inflammatory Bowel Disease

Statement of the problem. Highly discriminant histopathologic criteria for diagnosing canine IBD have not been published. Endoscopy, with mucosal

biopsy procurement, remains the procedure of choice in evaluating dogs having chronic gastroenteritis. Biopsy interpretation allows the clinician and the pathologist to establish a diagnosis, therapy, and prognosis. Biopsy results should ideally distinguish inflammation from neoplasia, should detect and characterize severe inflammatory diseases, or should suggest that mucosal histology falls within the broad range of "normal" such that alternatives to inflammatory diseases are investigated.[37] At present, biopsy interpretation is entirely subjective and varies greatly from investigator to investigator. Discordant results from erroneous biopsy interpretation should be avoided since misdiagnosis, inappropriate therapeutic recommendations, lack of clinical responsiveness by the patient, and the need for additional diagnostic testing will occur.

Diagnostic criteria for canine IBD. Lymphocytic plasmacytic enteritis currently is defined by intestinal histopathology: too many lymphocytes and plasma cells in the lamina propria.[2,5,6] Additional published descriptions have included villus atrophy, dilatation of villus lacteals, necrosis of the superficial enterocytes, and mucosal edema and/or fibrosis.[6] As with IBD in humans, investigator bias plays a major role in attempting to evaluate biopsy specimens in which there is only a subjective increase in lymphocytic-plasmacytic proprial cellularity without other evidence of mucosal inflammation. Earlier investigations of canine lymphocytic-plasmacytic enterocolitis were based on

subjective increases in mucosal immunocyte numbers (criteria for determining this increase or its magnitude were not determined) which were semi-qualitatively[2,3,5] or semi-quantitatively[6] scored as mild, moderate, or severe. More recently, another investigator[38] has proposed a grading scheme to describe mucosal changes in lymphocytic-plasmacytic colitis patients based on proprial leukocyte numbers, attenuation or loss of surface epithelium, and increased numbers of intraepithelial leukocytes. Unfortunately, the orientation of biopsy specimens and the method used for counting proprial leukocytes were not defined, thus limiting the application of the scheme by other investigators.

The inherent subjectivity in determining increased cellularity and the extensive interobserver variation has led to the assessment of epithelial/glandular alterations as criteria for diagnosis of IBD in humans.[39] A series of endoscopic biopsy specimens from dog and cat patients with gastrointestinal clinical signs were recently evaluated using similar criteria for severity of lesions.[18,20] In this study, **mild** IBD lesions were those with cellular infiltrates but without architectural distortion; **moderate** lesions had cellular infiltrates accompanied by mucosal epithelial immaturity and/or solitary epithelial necrosis; and **severe** IBD lesions consisted of cellular infiltrates accompanied by multifocal epithelial necrosis or extensive architectural distortion, such as fibrosis. These data would suggest that reliance upon subjective increases in

lamina proprial cellularity without other evidence of mucosal inflammation or epithelial injury is inappropriate for a histologic diagnosis of canine IBD.

Hypothesis. We hypothesize that lamina propria cellularity within small intestinal biopsy specimens obtained endoscopically cannot be reliably used as the primary histologic criteria for diagnosing canine IBD. We propose that quantitative morphometric analysis of selected mucosal cellular populations (e.g. IgA, IgG, and T-cells) be performed to objectively compare lesions of IBD with other inflammatory mucosal disorders and normal small intestinal tissue in the dog. This will allow definative comparison of intestinal cellularity between dog groups, and should tell us whether a diagnosis of canine IBD can be made on the basis of lamina propria cellularity alone. To our knowledge these studies have not been previously performed in the dog.

An Explanation of the Thesis Organization

The following manuscript (paper) will demonstrate our objectives, experimental methodology, and results. The paper is preceded by the general introduction and literature review, and the paper will be followed by a general summary highlighting the results and conclusions of this study. References cited in the general introduction and literature review will follow the general summary. Suggestions for additional research in the areas of canine

inflammatory bowel disorders will also be included in the summary section following the paper.

PAPER : MORPHOMETRIC EVALUATION OF IgA, IgG, AND T-CELLS IN DUODENAL MUCOSA FROM NORMAL DOGS AND DOGS WITH INFLAMMATORY BOWEL DISEASE

Morphometric evaluation of IgA, IgG, and T-cells in duodenal mucosa from normal dogs and dogs with inflammatory bowel disease

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SUMMARY

A histologic diagnosis of inflammatory bowel disease (IBD) is usually based on increased proprial leukocyte numbers. Duodenal biopsy specimens obtained endoscopically from 28 dogs were evaluated for lamina propria cellularity using morphometric analysis. Dogs were classified in 3 groups: I, IBD group based on histologic criteria of epithelial/glandular alterations (n = 11); II, nonspecific gastroenteritis (n = 8); III, normal group (n = 9). Paired contiguous villi were serially evaluated for their distribution of IgA, IgG, IgM, T-cells, and macrophages using avidin-biotin methods. Mucosal mast cells were also assessed. Data (cell counts) were analyzed using a two stage Gamma-Poisson mixture model. The numbers of IgM cells, mast cells, and macrophages were too low to perform reliable statistical analysis. Significant differences in the villus distribution of IgA, IgG, and T-cells within individual dog groups were not observed. Group II had higher IgA counts and was significantly different than both Group I and Group III dogs. Significant group differences for IgG cells were present with the Group I dogs having the lowest cell counts. T-cells were the predominant proprial cell observed in all groups. Significant group differences for T-cells were observed with Group III dogs having higher counts as compared to Group I and Group II dogs. These results indicate that increased lamina propria cellularity cannot be reliably used to distinguish canine IBD from other intestinal disorders.

INTRODUCTION

The gastrointestinal tract (GIT) immune system is one of the largest immunologic organs of the body, containing a vast supply of lymphocytes and immunoglobulin-producing cells.[1] Broadly speaking, the function of the GIT immune system is to protect the body from the deleterious effects of the material present within the intestinal lumen. Local immunity is largely mediated through B and T cells in the intestinal mucosa. T cells are predominantly found as intraepithelial leukocytes which reside between the intestinal epithelial cells.[2] In contrast to this intraepithelial compartment, B cells are most common in the lamina propria and are composed primarily of immunoglobulin (Ig) A-producing cells.[3] The intestinal immune system in healthy and diseased human beings has been previously reviewed.[4,5]

The local immune system of the canine GIT has only recently been evaluated with regard to cellular numbers and distribution of Ig-producing cells. Previous studies have evaluated selected populations of Ig-containing cells (primarily IgA cells and IgM cells) in the small[6-10] and large[10,11] intestines of normal dogs. These investigations indicate that the GIT in healthy dogs is immunologically similar to that of the healthy human being. In both species, IgA cells are the predominant cell type while IgG cells and IgM cells are less plentiful.[6-11] Data describing the numerical range of T-cells and their distribution within the normal canine GIT has not been published.

There is a need to further define and critically compare the GIT in healthy and diseased dogs, since the histologic assessment of such tissue is part of the clinical evaluation of dogs with chronic gastroenteritis. Idiopathic inflammatory bowel disease (IBD), particularly lymphocytic-plasmacytic enteritis, is considered by many investigators to be the most prevalent cause for chronic gastrointestinal signs in dogs.[12-14] The expected morphologic lesion is increased numbers of mixed inflammatory cells in the lamina propria.[12,13,15,16] The difficulty in making a histologic diagnosis of IBD from intestinal tissues which normally contain a complement of mucosal leukocytes is well recognized. Indeed, the inherent subjectivity in determining increased proprial cellularity and the extensive interobserver variation have lead others to propose alternative grading systems in human[17] and canine[18,19] patients suspected to have IBD. These data would suggest that reliance upon subjective increases in lamina propria cellularity alone is an unreliable way to diagnose canine IBD.

The purposes of this study were (1) to apply criteria of quantitative morphometric analysis to aid in the histologic interpretation of small intestinal mucosal biopsy specimens obtained using endoscopic techniques; (2) to define the vertical distributions of IgA-, IgG-, IgM-containing cells, T-cells, macrophages, and mast cells in the small intestinal mucosa; and (3) to objectively compare the lamina propria cellularity of IBD with other mucosal disorders and normal intestinal tissue in the dog.

MATERIAL AND METHODS

Dog Groups

Mucosal biopsy specimens were obtained endoscopically from the small intestine in 28 dogs. Normal and diseased dogs (Table 1) were classified in 3 groups by clinical and histologic criteria. All tissues were reviewed by 2 experienced pathologists and a histologic diagnosis was assessed by consensus.

IBD Group (Group I) - This group consisted of 11 dogs diagnosed with IBD according to specific criteria.[18,19] Criteria for selection included clinical signs of persistent gastroenteritis of greater than 2 weeks' duration; inadequate or no clinical response to control diets fed exclusively for at least 3 weeks; thorough diagnostic evaluation; histologic diagnosis of mucosal cellular infiltrates with epithelial/glandular alterations; and failure to demonstrate other causes of gastroenteritis. Signs of chronic gastroenteritis must have been minimally responsive to prior drug treatments.

Nonspecific Gastroenteritis Group (Group II) - This group contained 8 dogs which had clinical signs of chronic gastroenteritis but without histologic lesions of IBD. Mucosal biopsies previously had been interpreted as normal. Signs of small bowel diarrhea, vomiting, anorexia, and/or weight loss predominated and had persisted for more than 2 months in 88% of all dogs.

Normal Dogs (Group III) - A group of 9 adult mixed-breed dogs comprised the normal (control) group. Each dog was judged to be healthy on the basis of physical examination and thorough pre-endoscopy laboratory evaluation (CBC, serum biochemical analysis, complete urinalysis, and three negative fecal examinations for endoparasitic ova and *Giardia* sp [using zinc-sulfate centrifugal flotation techniques]). All dogs were fed a balanced commercial canine maintenance ration[a] and offered free choice water for 4 weeks prior to endoscopy. None of the dogs had diarrhea or other gastrointestinal signs and biopsy specimens were interpreted as normal.

Endoscopy

Animals were prepared for endoscopy (duodenoscopy) by withholding food for 12-24 hours. All dogs were anesthetized with thiopental sodium, orotracheally intubated, and maintained on halothane-oxygen anesthesia. Duodenoscopy was performed by use of a flexible endoscope[b] which had an accessory channel diameter of 2.0 mm. Multiple mucosal biopsy specimens were procured with standard serrated jaw pinch forceps[c] from the descending duodenum. Care was taken to avoid mucosa adjacent to or overlying normal lymphoid aggregates. Mucosal specimens were placed on formalin-soaked biopsy sponges prior to tissue processing.

Histology and Immunohistochemistry

Tissue specimens were fixed in neutral-buffered 10% formalin for 12-24 hours, processed by routine paraffin techniques, sectioned at 6 μm, and stained with hematoxylin and eosin. A minimum of 6 biopsy specimens from the descending duodenum and multiple serial sections were examined. For inclusion in the study reported here, it was required that at least one biopsy specimen from each dog be optimally oriented such that the crypt epithelium lay perpendicular to the mucosal surface. Additionally, these specimens must have had at least 2 villi (preferably contiguous) which were of adequate height and width to permit morphometric evaluation. Following selection of properly oriented paraffin blocks, the tissues were resectioned at 3μm and placed on glass slides.

The avidin-biotin immunoenzymatic staining method[20] was used to identify IgA-, IgG-, and IgM-containing cells, T-cells, and macrophages in fixed tissues as follows: The unstained sections were sequentially deparaffinized in 2 changes of xylene, 3 changes of 100% ethanol, 2 changes of 95% ethanol, 70% ethanol, and rehydrated in water. The slides were placed in a methanol-hydrogen peroxide solution (9 parts of methanol plus 1 part of aqueous 30% hydrogen peroxide) for 20 minutes, washed twice with TRIS, and placed in 37° C preheated TRIS for 15 minutes. The slides for identification of IgA-, IgG-, and IgM-cells were placed in a solution of 0.1% trypsin plus 0.1% calcium chloride

at 37° C for 5 minutes, immediately washed twice in cold TRIS, and then incubated for 20 minutes in a 5% solution of normal goat serum. A 1:1000 rabbit anti-canine IgA[d] was applied for 60 minutes, a 1:2000 rabbit anti-canine IgG[e] or 1:3000 rabbit anti-canine IgM[f] antibody was applied for 30 minutes, the slides were washed twice in TRIS, washed in TRIS plus 1% normal goat serum, and then incubated for 30 minutes with 1:200 biotinylated goat anti-rabbit antibody.[g]

For identification of T-cells, the slides were placed in a solution of 0.125% pronase at 37° C for 5 minutes, washed twice in cold TRIS, and incubated for 20 minutes in a 5% solution of normal goat serum. A 1:200 rabbit anti-human T-cell (CD3)[h] antibody was applied for 30 minutes, the slides were washed twice in TRIS, washed in TRIS plus 1% normal goat serum, and then incubated with a 1:200 biotinylated goat anti-rabbit antibody for 30 minutes. Macrophages were identified by placing slides in a solution of 0.25% pronase at 37° C for 2 minutes, the slides were twice washed in cold TRIS, and incubated for 20 minutes in a 5% solution of normal horse serum. A 1:25 mouse anti-human macrophage[i] antibody was applied for 60 minutes, the slides were washed twice in TRIS, washed in TRIS plus 1% normal horse serum, and incubated for 30 minutes with a 1:200 biotinylated horse anti-mouse antibody.[j]

The secondary antibodies were washed off all slides by three sequential rinses in TRIS. A 1:200 solution of HRP streptavidin was applied for 45

minutes, washed off twice with TRIS, and washed with sodium acetate buffer. A substrate chromogen solution of AEC (2 ml of sodium acetate buffer to which 3 drops of 0.4% AEC and 3 drops of aqueous 1% hydrogen peroxide were added) was applied and incubated until the desired color intensity developed. The AEC was washed off with water, the slides were washed with sodium acetate buffer, washed with water, counter stained with Shandon hematoxylin, washed again with water, and cover slipped. An Astrablau stain[21] was applied to identify mucosal mast cells.

Appropriate positive and negative controls demonstrated the specificity of the reaction for each primary antibody. Negative controls consisted of incubating comparable intestinal sections in TRIS/PBS (1:10) instead of primary antibody, with all other incubations and washes unchanged. Positive controls were canine small intestine for the IgA marker, canine reactive lymph node for the IgG and IgM markers, canine spleen, thymus, and lymph node for the T-cell marker, canine lung and liver for the macrophage marker, and canine mast cell tumor for identification of mast cells.

Image Analysis

Images of the intestinal tissues were acquired at the ISU Image Analysis Facility using a Zeiss SEM-IPS image analysis system (Zeiss-Kontron; IBAS version 2.00). The tissues were viewed with a Zeiss axiophot microscope at

6.25 X magnification (2.5 X 2.5 X optivar) for entire villus area measurements and 100 X (40 X 2.5 X optivar) for individual cell measurements. Images of the villi were captured from the immunohistochemistry slides with a Sony DXC-3000A color video camera. The internal scaling feature of the image analysis software was calibrated to measure in millimeters or microns, depending on the magnification in use.

Specimens were coded and reviewed as a batch without knowledge of the dog's identity, of the severity of clinical signs, or of the group from which the dog came. Paired villi from each dog were identified and videoprinted to assure that the same villi were sequentially examined for each of the six cell types. A cursor was used to trace a solid line around the surface epithelium down to and including the superficial aspect of the crypt epithelium of each villus. The area occupied by this tracing was measured in mm^2 and termed the villus area. Three regions were defined within the villus which allowed vertical assessment of cellular distributions: region A = apical villus, region B = mid-villus, and region C = basilar villus (Figure 1). Within each region, three horizontal (from left to right) non-overlapping fields were traced and examined for cell number and cellular area at a magnification of 100 X. The summed area of these traced fields was measured in μm^2 and termed the regional area. Thus, a total of nine fields per villus (18 fields/dog) were evaluated morphometrically.

Countable areas must have contained lamina propria exclusive of epithelium or intestinal glands. Only positive staining cells with an evident nucleus were identified and counted within a field. Cells that were stained but did not completely reside within the field were excluded. For a given cell type, the total number of cells and the area occupied by these cells were recorded for each region. This allowed evaluation of cellular density (number of cells/area) within regions.

Statistical Analysis

Data (cell counts) were analyzed using a two stage Gamma-Poisson mixture model for unequal sample sizes.[22] Maximum likelihood estimates for the mixing distribution parameters were calculated. Likelihood ratio tests selected the appropriate model for comparison of cell counts within regions of each group, and for comparison of cell counts between groups of dogs. Analysis of the group gamma mixing distributions data were used to generate probability density function (PDF) and cumulative density function (CDF) curves for a given cell type. Differences among groups in these estimated distributions represent differences in the tendencies of these groups to contain dogs that exhibit various levels of cell counts.

For example, an estimated Gamma density having large probability mass for small variate values would represent a collection of dogs for which most

individuals are expected to produce small cell counts. This may be seen in figure 7F (e.g. CDF curves for T-cells) where a gamma variate value (e.g. cell count) of 10 would give you probability distributions of 98%, 92%, and 75% in Group III, II, and I dogs, respectively. However, it does not preclude the possibility that the collection may also contain a few individuals that produce high cell counts. In this situation, the tail behavior (shape) of the estimated distributions may provide as much or more information about the situation under study than the more familiar characteristics of mean and variance.

The numbers of mast cells, macrophages, and IgM-containing cells in control and diseased dogs were too low to perform reliable statistical analysis and are therefore excluded from the results.

RESULTS

Abnormalities were not detected during physical examination, or in the laboratory values obtained from the group III (control) dogs. A variety of biochemical abnormalities were observed in the group II (non-specific gastroenteritis) dogs. Mild hypoalbuminemia was present in five dogs; however, two dogs had marked decreases in serum total proteins (both albumin and globulin fractions), cholesterol, and calcium consistent with a diagnosis of protein-losing enteropathy. One dog in group II was free of laboratory abnormalities in spite of a six week history of chronic intermittent vomiting. The clinicopathologic findings in the group I (IBD) dogs have been previously discussed.[19,23] Lamina propria cellularity was considered to be within normal limits in all biopsy specimens obtained from dogs in group III, and in all but two dogs in group II. Dogs 577 and 538 had mild increases in lymphocytic-plasmacytic proprial cellularity which were of insufficient magnitude to warrant a diagnosis of mild IBD. Light microscopic lesion evaluation results from the group I dogs were nearly equally divided between mild (n = 6) and moderate (n = 5) histologic lesions of IBD. In these tissues, epithelial/glandular alterations were accompanied by a variable but mixed lamina propria cellular infiltrate (Table 1).

Paired contiguous villi were available for morphometric analysis in 22 of 28 dogs (79%). Processing artifacts precluded evaluation of adjacent villi in 6

dogs, necessitating evaluation of a second appropriately oriented villus to the right of the first from the same biopsy specimen. For determination of T-cell morphometric parameters, one dog in Group II had only one villus suitable for analysis. IgA- and IgG-containing cells and T-cells were easily distinguishable from other lamina propria cells due to their morphology and cytoplasmic staining. Positive staining cells were mononuclear and typically round to oval with bright red to dark brown cytoplasmic staining adjacent to or surrounding the nucleus (Figure 2). Background staining of other cellular elements and the interstitium was minimal for all cell types.

In all dog groups, IgA cells appeared in large numbers in the basilar lamina propria but cords of IgA cells would extend into the mid- and apical regions of the villus. A more diffuse vertical distribution of IgG cells and T-cells was observed. T-cells were often densely distributed within the lamina propria adjacent to the surface epithelium. Many of the T-cells extended into the surface epithelium itself and were identified as intraepithelial lymphocytes (Figure 3). Considerable variability in the regional absolute cell counts (representing total cell numbers for dog region combinations in paired villi) of IgA cells, IgG cells, and T-cells within dogs from the same group was apparent (Figures 4-6). T-cells were the predominant lymphocyte observed in all groups of dogs.

Results from likelihood ratio tests did not indicate significant differences in the vertical distribution of IgA-containing cells within individual groups of dogs. Comparison of IgA cell counts between groups of dogs revealed no significant difference between group I and group III dogs; however, group II dogs were significantly different (e.g. had higher cell counts) than both group I and group III dogs. Although dogs from all three groups had relatively small cell counts, analysis of PDF and CDF plots indicated that dogs having higher IgA cell numbers were most likely to fall within the non-specific gastroenteritis category (Figures 7A, B). The vertical distribution of IgG-containing cells from dogs of each group were not significantly different. However, there was an apparent group effect with regard to IgG cell numbers as all three groups were significantly different. This group difference was most apparent in dogs having relatively low cell counts (Figures 7C, D).

Significant differences in the vertical distribution of T-cells within each of the 3 dog groups were not observed. T-cell numbers within each group did however vary, and all 3 groups were significantly different. Review of the PDF and CDF plots (Figures 7E, F) readily demonstrated these group differences and the tendency for group III dogs to have higher T-cell counts.

DISCUSSION

Endoscopic examination with procurement of mucosal biopsy specimens is a fundamental procedure in the diagnosis of dogs with chronic gastroenteritis. Inflammatory bowel disease is among the most common clinical diagnoses for these animals, and the expected histologic lesion is lymphocytic-plasmacytic enteritis.[12,13,15,16] This expanding use of gastrointestinal endoscopy has lead to an increased number of biopsy specimens being submitted to pathology services for histologic review.[24,25] Pathologists are now requested to examine multiple, minute, and less well oriented tissue specimens which are prone to a variety of tissue and/or processing artifacts. Furthermore, mention has been made of the need to appreciate the wide range of normal characteristics occurring in canine intestinal mucosal biopsies.[26,27] These considerations are important if meaningful correlation of abnormal histology and intestinal function is to be made. In the case of canine IBD, the diagnostic method used most often has been subjective evaluation of lamina propria cellularity, which relies heavily on the experience and bias of the pathologist.[12-14,16,27]

Quantitative morphometric analysis can clearly complement the qualitative description obtained by tissue examination under light microscopy. Morphometric methods do provide a definition of the normal range of lamina propria cellularity and can identify mild abnormalities in patients in which conventional histologic interpretation has rendered a normal diagnosis. In

human medicine, a variety of semi-automated and automated methods have been designed to assess the number of inflammatory cells in biopsy specimens. Separate studies, one using a morphometric point-counting method[28] and the other performed with a specialized automated image processor[29], have applied morphometry to colonic biopsy specimens obtained from normal and diseased patients. The authors demonstrated an increased cellular density in patients with microscopic colitis syndrome as compared to healthy controls and patients having other causes for chronic diarrhea. Other investigators[30] have shown that quantitative assessment of jejunal biopsy specimens produced less intra- and interobserver variability than simple subjective analysis.

Quantitative methods for assessing canine intestine cellular populations have been previously applied to full-thickness biopsy specimens obtained from normal dogs. In three separate semi-quantitative studies[6,7,9], the authors demonstrated that IgA-, followed by IgM- and IgG-containing cells, were the predominant immunocytes in the small intestinal mucosa. Unfortunately, the regions of the intestinal mucosa examined (ie, villus versus subvillus lamina propria) and the methods used for counting proprial leukocytes were not fully defined. Willard et al[8,10,11] reported that both IgA- and IgM-containing cells have variable vertical and horizontal distributions in the canine GIT. These authors used standardized mucosal areas to quantitate IgM cells in the top half of the subvillus lamina propria[8] and to assess IgA cells within four vertically defined

villus zones.[10] There is only one published report in the use of endoscopically retrieved biopsies to evaluate populations of Ig-producing cells (plasma cells) in the colonic mucosa of normal dogs.[31] Comparative data for small intestinal endoscopic biopsies has not been previously reported.

We used a modification of Willard's method[10] to evaluate duodenal biopsy specimens obtained endoscopically from normal and diseased dogs. Morphometric analysis allowed us to accurately quantitate and to objectively compare IgA, IgG, and T-cell distributions within and between dog groups. Analysis of this data revealed several important findings: (1) that T-cells were the predominant villus lymphocyte in each group; (2) that there was considerable variability in the lymphocyte cell counts within individual groups of dogs; (3) that regional differences in the vertical distribution of cells within dog groups were not present; (4) that group differences in villus cell counts were present; and (5) that increased lamina propria cellularity of IgA, IgG, and T-cells cannot be reliably used to differentiate histologic lesions of IBD from normal biopsy specimens and from biopsy specimens obtained from dogs having chronic nonspecific gastroenteritis.

Our results concerning the vertical distribution of IgA cells in the villus lamina propria were similar to findings in a previous report.[10] Although these authors found nonuniformity in the distribution of IgA cells, this difference could be explained by the use of a different counting method which additionally

counted cells in a suprasubmucosal lamina propria zone. When one compares cell counts from the upper 3 villus zones of this earlier investigation (corresponding to the 3 villus regions used in this study), differences in vertical distribution of IgA cells were not statistically significant. In the present study, there was a trend for group II dogs to have higher IgA cell counts, and group differences were apparent when comparing these dogs to dogs in groups I and III. We did not find significant differences in villus IgA counts in IBD dogs as compared to healthy controls. This finding suggests that there is a difference in the nature of the inflammatory infiltrate of IBD in dogs and human beings. Mucosal concentrations of IgA cells in the colonic lamina propria of human beings with IBD are often increased[32,33]; however, relative decreases in IgA cell populations may occur and are attributable to marked increases in proprial IgG and IgM-containing cells.[34]

The observation of a uniform vertical distribution of canine IgG cells in the duodenum has not been previously reported. Prior work counting numbers of IgG cells has emphasized the evaluation of horizontal (ie, duodenum → jejunum → ileum →colon) rather than vertical distributions of this immunocyte in the canine GIT. Only one investigation has commented on the villus distribution of these cells. In a semi-quantitative study on intestinal mucosa from two dogs, Vaerman and Heremans[6] found that IgG_{2c}-type cells were infrequently observed in the villi but were most numerous in the deeper lamina propria. Further work

evaluating the distributions of IgG cells in the healthy and diseased canine GIT is warranted to determine whether fundamental differences exist.

Comparison of IgG cells between dog groups showed an apparent group effect as all groups were significantly different from each other. This group difference was most obvious in dogs having relatively low (< 10 cells/villi) total IgG cell counts. In evaluating the CDF curve for IgG cells, it was seen that (1) group II dogs tended to have higher IgG cell counts as compared to both group I and group III dogs and (2) that group III dogs tended to have greater IgG cell counts as compared to group I dogs. The significant difference between the IgG counts in IBD dogs and the other two dog groups is noteworthy. Most studies in human beings show that compared to normal tissue, specimens from IBD patients have increased numbers of IgA-, IgM-, and particularly IgG-containing cells.[33,35] The increase in IgG cells in the colonic mucosa appears to be dependent on the degree of inflammation and does not differ significantly in ulcerative colitis and in active Crohn's disease.[34] The present study shows that villus IgG cell counts in dogs with IBD were significantly lower than IgG counts in normal dogs.

To our knowledge, T-cell distributions have not been reported in the dog. The use of a human T-cell (CD3) marker provided reproducible recognition of T lymphocytes in formalin-fixed specimens. Similar to IgA and IgG cells, a uniform vertical distribution of T-cells was observed in this study. These cells

were the most numerous and densely distributed immunocyte in all groups of dogs. This finding parallels observations in human adults where T-cells, B-cells, and null cells have been reported to make up 50%, 25%, and 25% of the lamina propria lymphoid population respectively.[36] Many of the canine T-cells were located in lamina propria adjacent to the surface epithelium and they extended into the epithelial cells as intraepithelial lymphocytes. The reason for the occurrence of T-cells nearest to the intestinal lumen is unknown. Possibly these cells were reacting to antigenic stimulation. If T-cells were responding to luminal antigens, it might be expected that they would migrate from other portions of the lamina propria toward the surface epithelium. Intraepithelial lymphocytes may[37] or may not[38] be increased in dogs with intestinal inflammation, and their precise role in gastrointestinal immunity and disease remains poorly defined. The numbers of intraepithelial lymphocytes in biopsy specimens were not assessed in this study.

Differences between dog groups in villus T-cell numbers were apparent in our study. The trend was for group III dogs to have higher T-cell counts then both group I and group II dogs. Group I dogs tended to have the lowest T-cell counts. In comparing T-cell PDF and CDF curves to those for IgA cells and IgG cells, it becomes evident that analysis of T-cell data provided the most discriminating information to differentiate IBD dogs from normal dogs. This finding differs from that reported in persons which showed no significant

difference in mucosal T-cell populations between patients with ulcerative colitis or Crohn's disease and healthy controls.[39]

In this study, lamina propria cellularity was not increased in the IBD dogs as compared to normal dogs and to dogs having other causes for chronic gastroenteritis. Histopathologic guidelines for biopsy interpretation of canine lymphocytic-plasmacytic enteritis have previously included villus atrophy, dilatation of villus lacteals, necrosis of superficial epithelium, and increased numbers of mixed inflammatory cells in the lamina propria.[12,13,16] Similar criteria have been proposed and found to be of discriminatory value in the differentiation of ulcerative colitis in humans from acute self-limited colitis.[40] However, of these features, the assessment of lamina propria cellularity had the most inter-observer variability and was felt to be an unreliable means of diagnosing IBD.[41]

A variety of IBD histologic grading schemes have been advocated by veterinary investigators; most subjectively based on the semi-qualitative[12-14] or semi-quantitative[16] assessment of proprial cellularity. Since the methods used for counting proprial leukocytes were not defined, other investigators may find difficulty in the application of such schemes to aid in the histologic interpretation of IBD. One author has recently applied more objective quantitative criteria for grading endoscopic colonic biopsies from dogs with lymphocytic-plasmacytic colitis.[26] The results of this study showed that over

40% of dogs having a clinical diagnosis of chronic colitis had no detectable increases in the overall cellularity of the lamina propria. These combined experiences in human and dog patients would indicate that the interpretation of mucosal lymphocyte populations is controversial, and that additional evidence of mucosal injury should be present before a definitive diagnosis of IBD is made. We[18,19] and others[17] have proposed criteria of epithelial/glandular alterations as a more reliable and objective means to assess the histologic lesions of IBD. These epithelial changes may include distortion of crypt architecture, infiltration by neutrophils, epithelial immaturity or necrosis, and/or evidence of architectural distortion such as fibrosis. The clinical utility of this grading scheme in dogs with IBD has been previously reported.[19,23]

It is concluded that techniques of morphometric analysis can be used to evaluate the canine GIT immune system from endoscopically-derived biopsy specimens. Our results indicate that there is considerable variability in small intestinal lymphocytes in healthy and diseased dogs, that T-cells are the predominant villus lymphocyte in dogs, that there is uniformity in the vertical distributions of IgA, IgG, and T-cells in all tissues examined, and that intestinal tissues from healthy and diseased dogs will vary in their mucosal content of IgA, IgG, and T-cells. Analysis of T-cell numbers in biopsy specimens may provide the most discriminating information to differentiate normal from diseased intestinal tissue in the dog. Even so, we were unable to show that

increased lamina propria cellularity of IgA, IgG, or T-cells is a stable characteristic in the histopathologic assessment of canine IBD. This finding suggests that dissimilarities exist in the nature of the inflammatory infiltrate in canine and human patients having IBD. Further work evaluating canine GIT immunity from endoscopic biopsies is warranted to facilitate our understanding of IBD and other intestinal diseases.

LEGENDS

Table 1 Clinical and histologic summary of dogs with persistent gastroenteritis. Key to cellular infiltrate: L = lymphocytes, P = plasma cells, E = eosinophils, N = neutrophils.

NSL = no significant lesion; IBD = inflammatory bowel disease.

Key to groups: Group I = dogs with IBD; Group II = dogs with nonspecific gastroenteritis; Group III = healthy (control) dogs.

Figure 1 Schematic representation of the 3 villus regions used for evaluating lamina propria cellularity.

Figure 2 Canine duodenum stained for IgA activity. Dense staining of the apical cytoplasm of IgA-containing cells is apparent. X40.

Figure 3 Canine duodenum demonstrating a dense infiltrate of T-cells near the surface epithelium. Note the numerous intraepithelial lymphocytes. X40

Figure 4 Histograms showing absolute cell counts for cell type IgA in 3 groups of dogs.

Figure 5 Histograms showing absolute cell counts for cell type IgG in 3 groups of dogs.

Figure 6 Histograms showing absolute cell counts for T-cells in 3 groups of dogs.

Figure 7A-F Probability density function (PDF) and cumulative density function (CDF) curves in 3 groups of dogs for individual cell types. These curves describe the estimated probability distributions of the random variables which in turn determine the distributions of observed cell counts. On the basis of cell counts, the probability of a dog belonging to each group can be estimated by measuring the area under the PDF curve

or by measuring 1 minus the area under the CDF curve. Interpretation of these curves is facilitated by considering the probability distributions to represent the *tendency* of dog groups to contain individuals with *tendencies* to produce certain types of cell counts.

Figures 7 A,B PDF (A) and CDF (B) curves for cell type IgA.

Figures 7 C,D PDF (A) and CDF (B) curves for cell type IgG.

Figures 7 E,F PDF (A) and CDF (B) curves for T-cells.

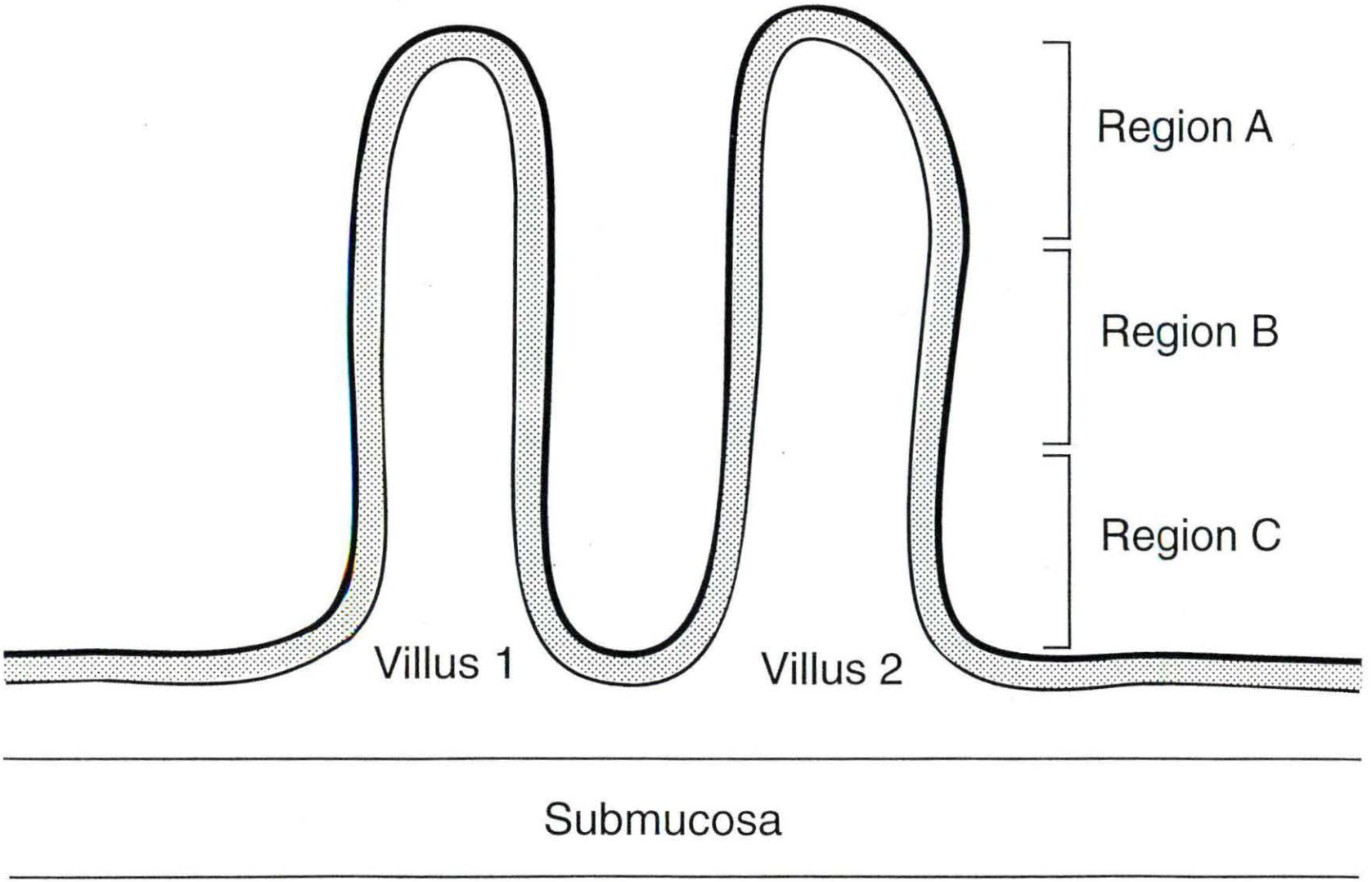
Table 1. Clinical and histologic summary of dogs with persistent gastroenteritis. Key to cellular infiltrate: L = lymphocytes, P = plasma cells, E = eosinophils, N = neutrophils. NSL = no significant lesion; IBD = inflammatory bowel disease. Key to groups: Group I = dogs with IBD; Group II = dogs with nonspecific gastroenteritis.

Dog/Group	Sex	Age in years	Clinical signs	Morphologic description	Histologic diagnosis
577 / II	M	2.5	Chronic vomiting Weight loss	LP cellular infiltrate	Normal tissue
154 / II	SF	2	Ascites Chronic diarrhea	NSL	Normal tissue
300 / II	SF	2	Chronic vomiting	NSL	Normal tissue
328 / II	SF	3	Chronic diarrhea Weight loss	NSL	Normal tissue
347 / II	CM	4	Chronic diarrhea Weight loss	NSL	Normal tissue
776 / II	M	1	Chronic diarrhea	NSL	Normal tissue
115 / II	SF	2	Chronic diarrhea Hematemesis	NSL	Normal tissue
538 / II	CM	1	Chronic vomiting Weight loss	LPE cellular infiltrate	Normal tissue
940 / I	CM	11	Chronic vomiting	LPE cellular infiltrate Glandular immaturity	Moderate IBD

Table 1. Continued

361 / I	CM	5	Chronic vomiting Weight loss Abdominal pain	LP cellular infiltrate Glandular immaturity	Moderate IBD
288 / I	SF	6.5	Chronic diarrhea Weight loss Anorexia	LPN cellular infiltrate	Mild IBD
339 / I	SF	7	Chronic vomiting Chronic diarrhea	LPE cellular infiltrate Glandular immaturity	Moderate IBD
609 / I	M	2	Chronic diarrhea Weight loss	LPE cellular infiltrate	Mild IBD
873 / I	SF	9	Chronic vomiting Chronic diarrhea	LPE cellular infiltrate Glandular immaturity	Moderate IBD
326 / I	M	7	Chronic vomiting Weight loss	LPE cellular infiltrate	Mild IBD
409 / I	M	6	Chronic vomiting	LPE cellular infiltrate	Mild IBD
736 / I	SF	2	Severe melena Anemia	LP cellular infiltrate Glandular immaturity	Moderate IBD
51A / I	SF	8	Chronic vomiting Abdominal pain	LPE cellular infiltrate	Mild IBD
23A / I	F	4	Chronic diarrhea Weight loss Ascites	LPE cellular infiltrate Mild lacteal dilatation	Mild IBD

Figure 1. Schematic representation of the 3 villus regions used for evaluating lamina propria cellularity.


Region A
Region B
Region C
Villus 1
Villus 2
Submucosa

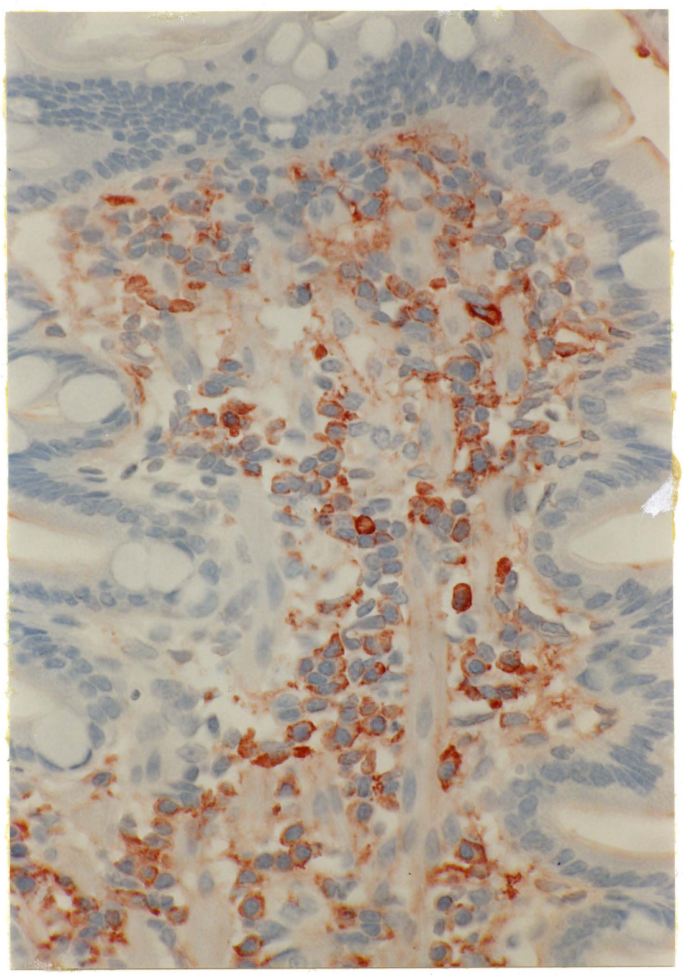


Figure 2. Canine duodenum stained for IgA activity. Dense staining of the apical cytoplasm of IgA-containing cells is apparent. X40.

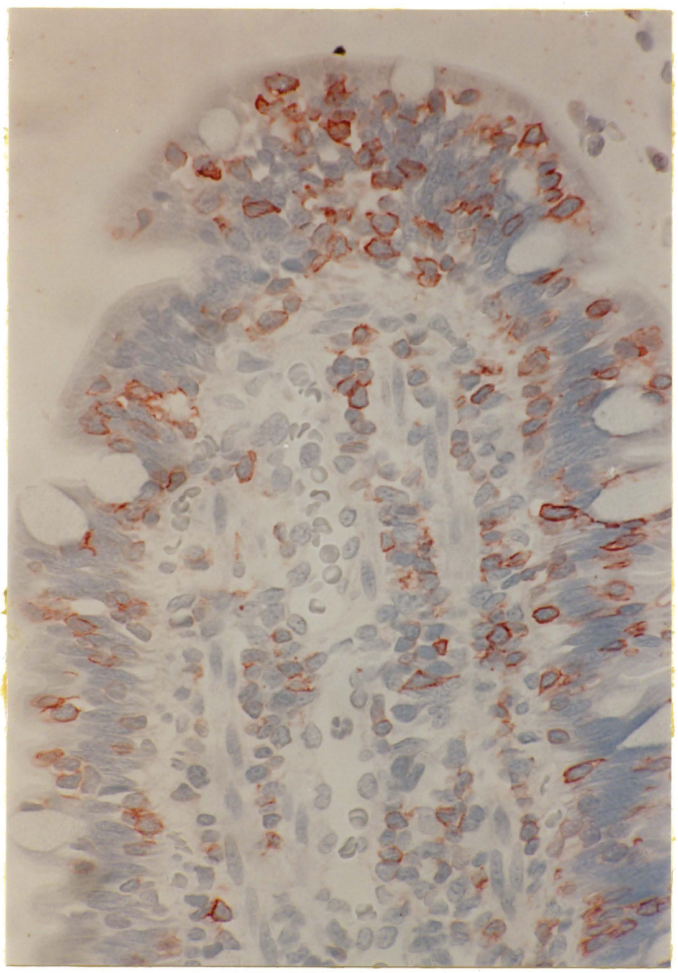


Figure 3. Canine duodenum demonstrating a dense infiltrate of T-cells near the surface epithelium. Note the numerous intraepithelial lymphocytes. X40.

Figure 4. Histograms showing regional absolute cell counts for cell type IgA in 3 groups of dogs. Values in the histograms represent total cell numbers for dog region combinations in paired villi.

OBSERVED COUNTS FOR CELL TYPE IgA

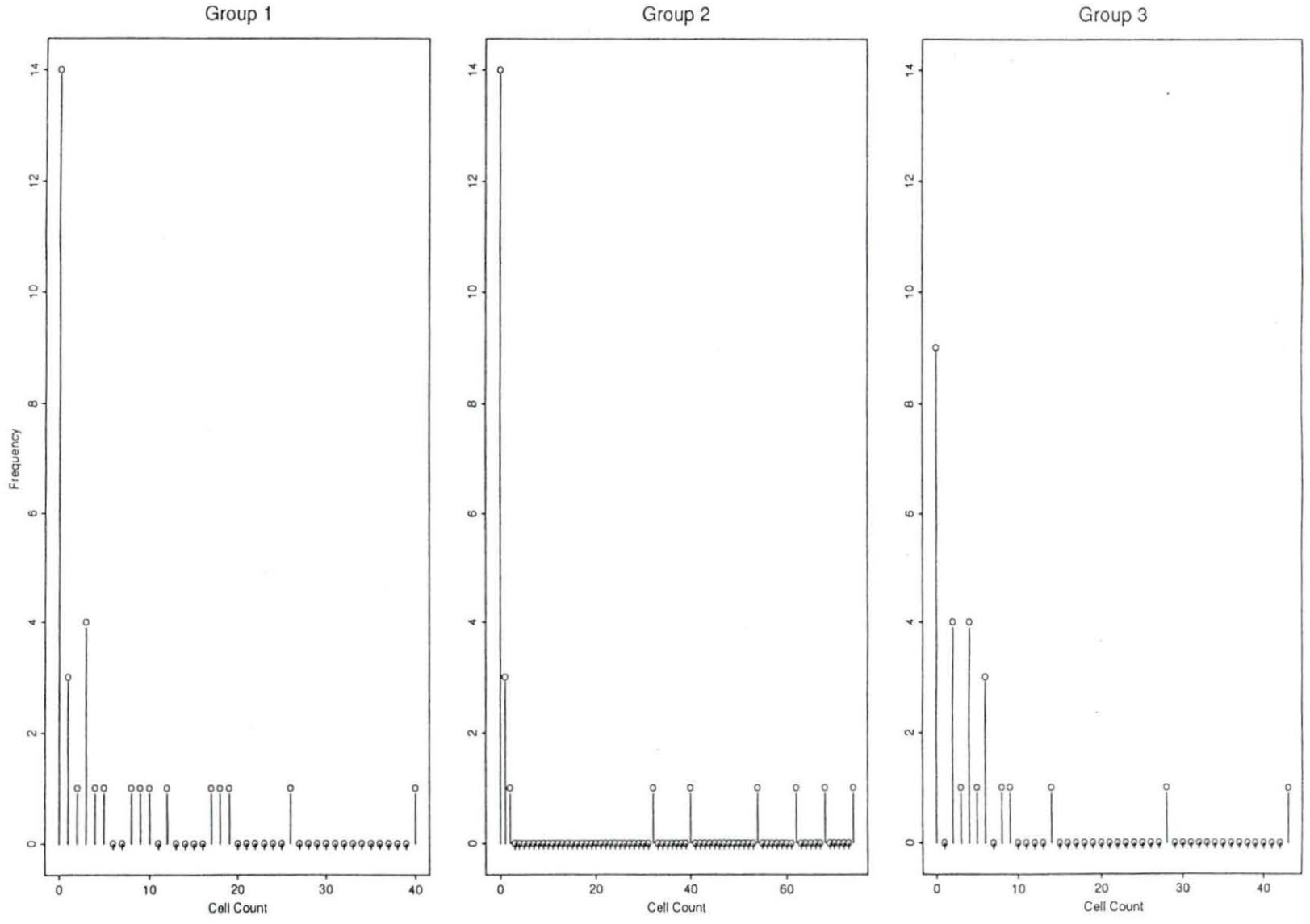


Figure 5. Histograms showing regional absolute cell counts for cell type IgG in 3 groups of dogs. Values in the histograms represent total cell numbers for dog region combinations in paired villi.

OBSERVED COUNTS FOR CELL TYPE IgG

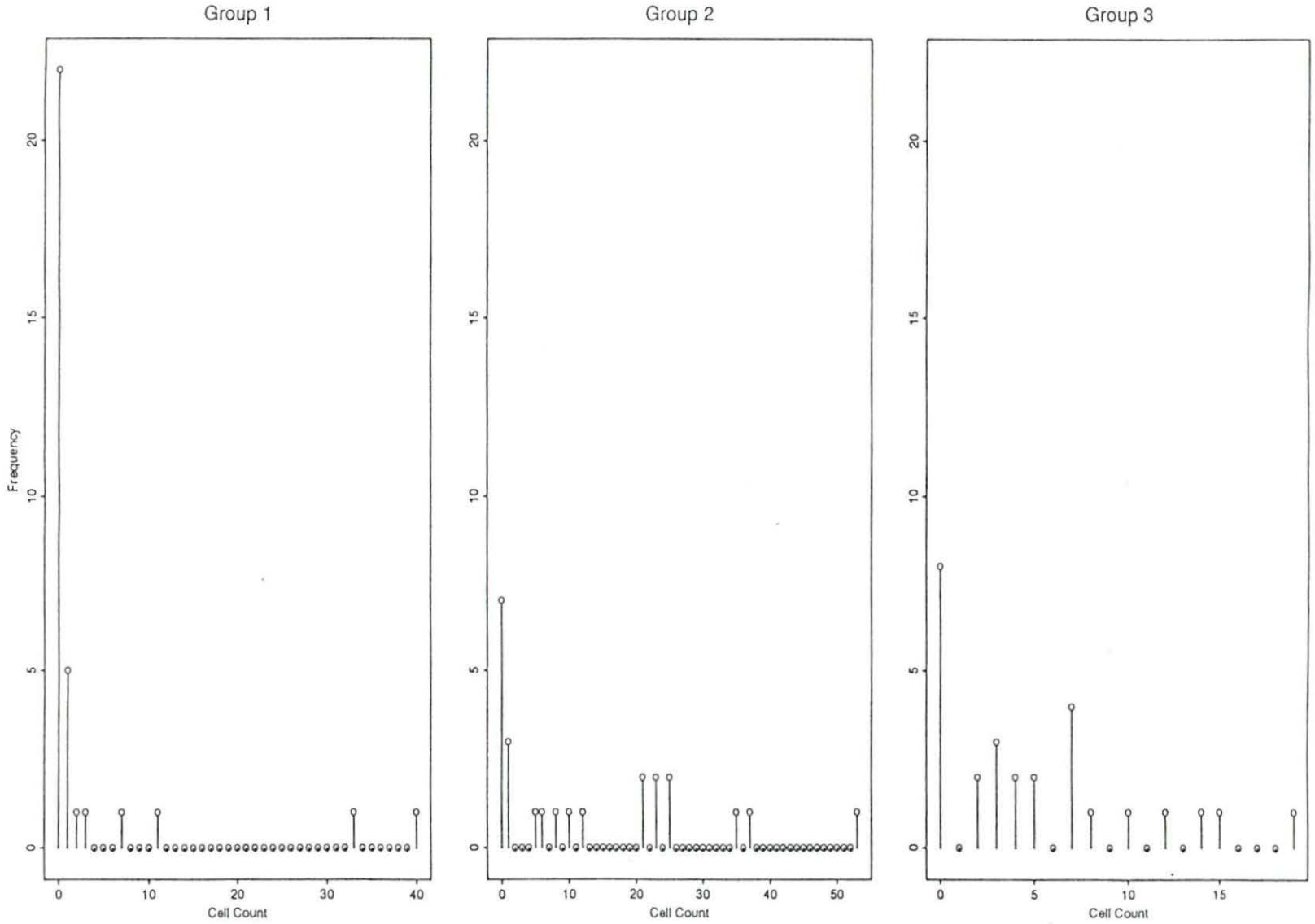


Figure 6. Histograms showing regional absolute cell counts for T-cells in 3 groups of dogs. Values in the histograms represent total cell numbers for dog region combinations in paired villi.

OBSERVED COUNTS FOR CELL TYPE T

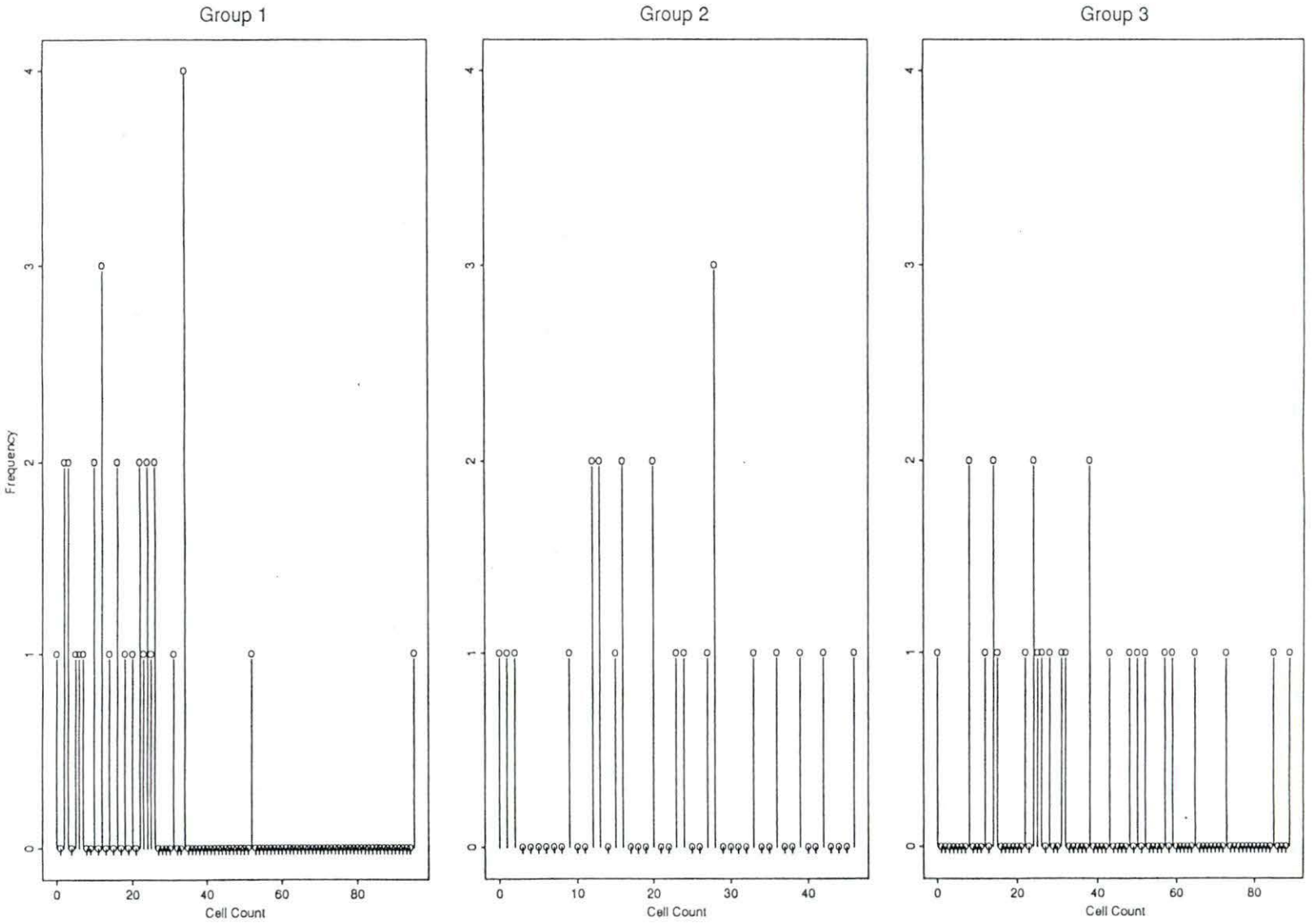


Figure 7A. Probability density function (PDF) curves in 3 groups of dogs for cell type IgA. On the basis of cell counts, the probability of a dog belonging to each group can be estimated by measuring the area under the PDF curve.

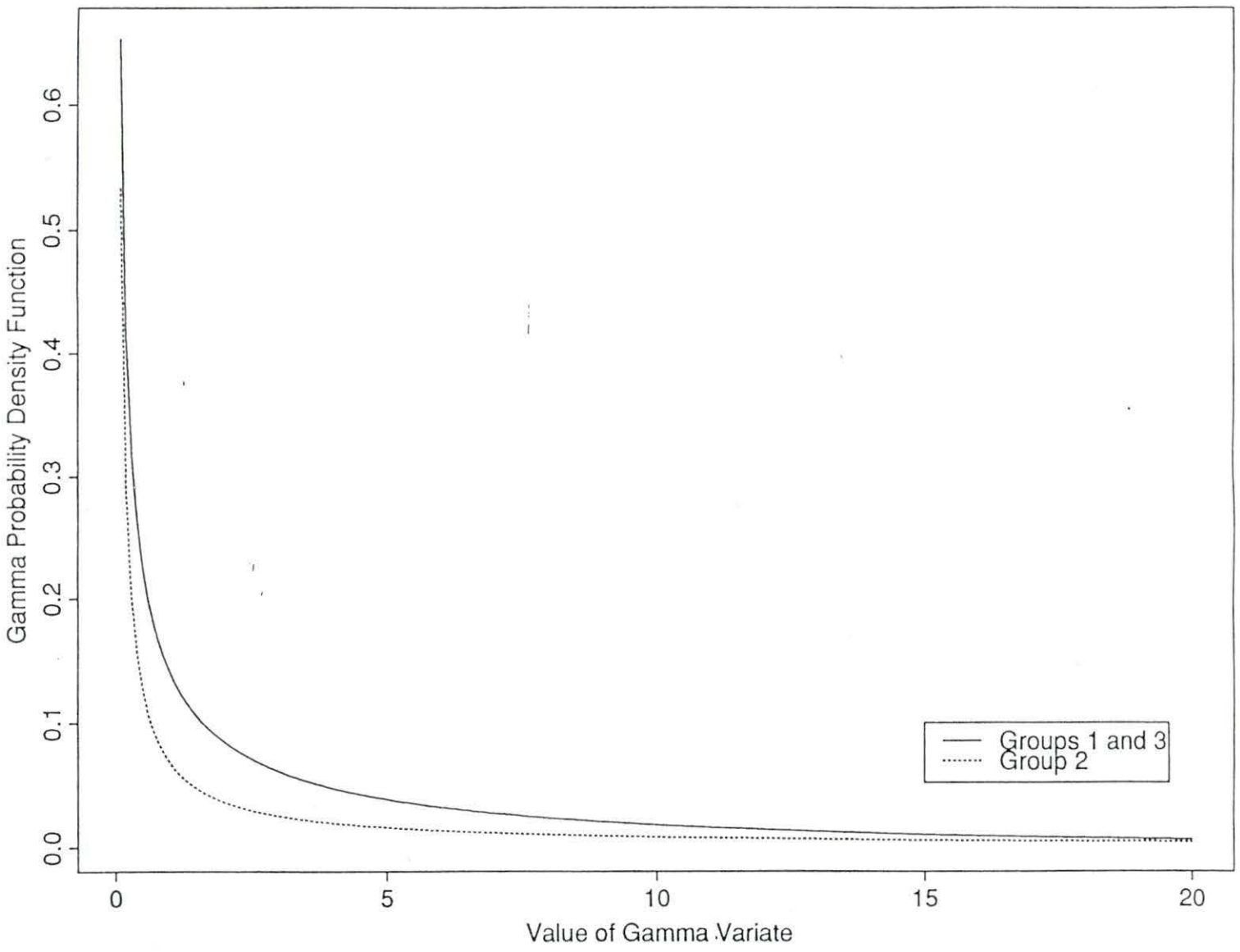

ESTIMATED GAMMA PDF'S FOR CELL TYPE IgA
Gamma Probability Density Function
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
5
10
15
20
Value of Gamma Variate
Groups 1 and 3
Group 2

Figure 7B. Cumulative density function (CDF) curves in 3 groups of dogs for cell type IgA. On the basis of cell counts, the probability of a dog belonging to each group can be estimated by measuring 1 minus the area under the CDF curve.

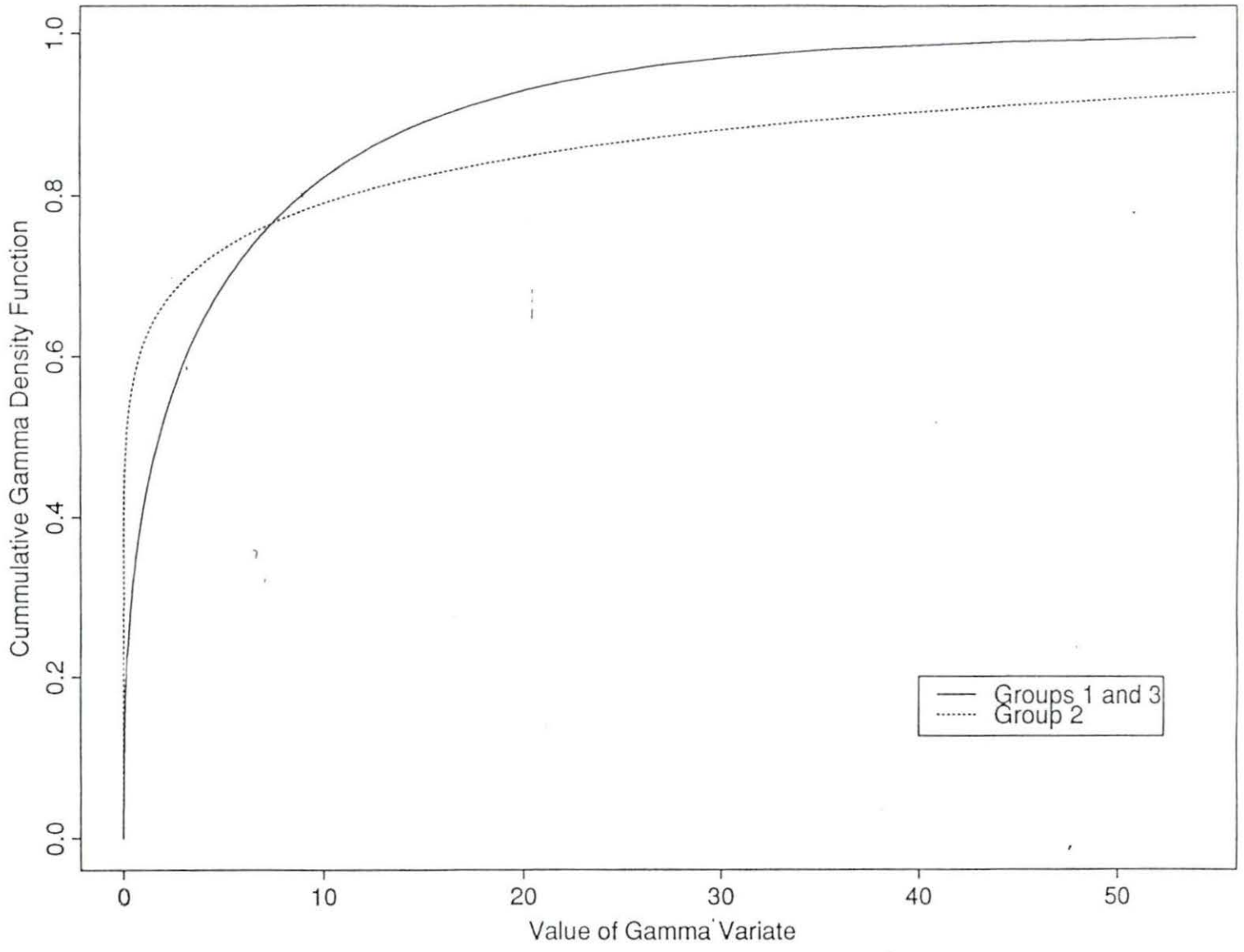

ESTIMATED GAMMA CDF'S FOR CELL TYPE IgA
Cummulative Gamma Density Function
0.0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
50
Value of Gamma Variate
Groups 1 and 3
Group 2

Figure 7C. Probability density function (PDF) curves in 3 groups of dogs for cell type IgG. On the basis of cell counts, the probability of a dog belonging to each group can be estimated by measuring the area under the CDF curve.

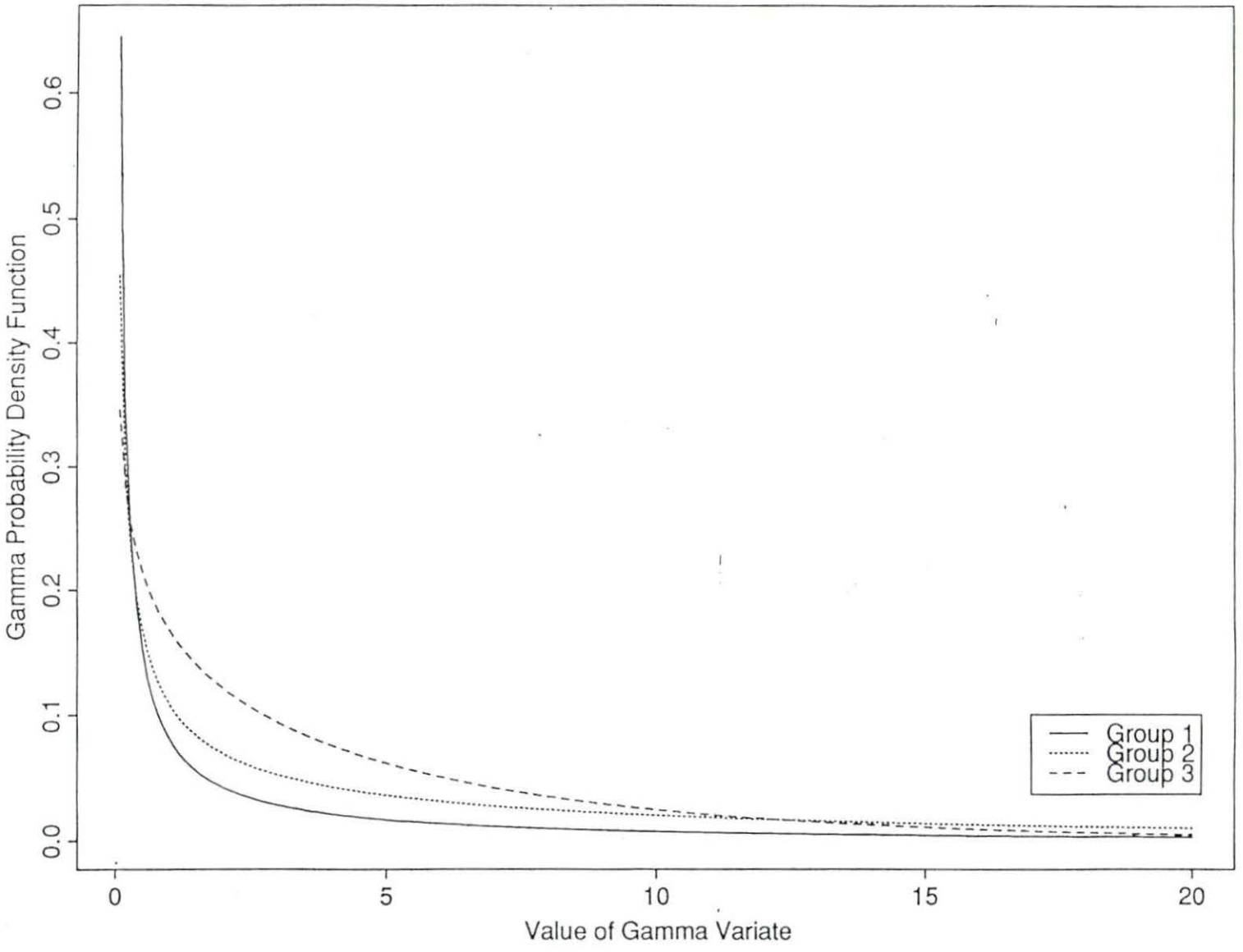

ESTIMATED GAMMA PDF'S FOR CELL TYPE IgG
Gamma Probability Density Function
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
5
10
15
20
Value of Gamma Variate
Group 1
Group 2
Group 3

Figure 7D. Cumulative density function (CDF) curves in 3 groups of dogs for cell type IgG. On the basis of cell counts, the probability of a dog belonging to each group can be estimated by measuring 1 minus the area under the CDF curve.

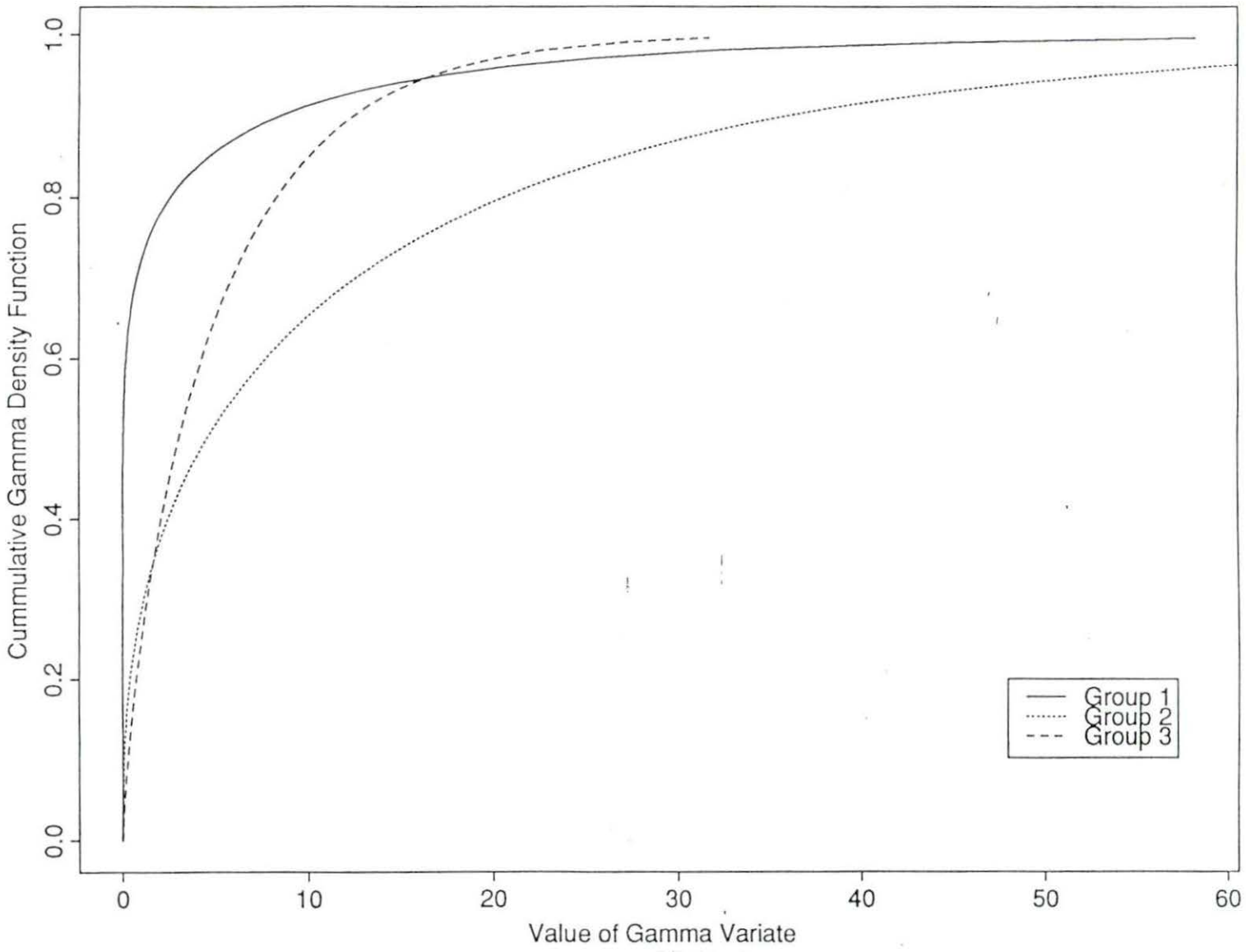

ESTIMATED GAMMA CDF'S FOR CELL TYPE IgG
Cummulative Gamma Density Function
0.0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
50
60
Value of Gamma Variate
Group 1
Group 2
Group 3

Figure 7E. Probability density function (PDF) curves in 3 groups of dogs for T-cells. On the basis of cell counts, the probability of a dog belonging to each group can be estimated by measuring the area under the PDF curve.

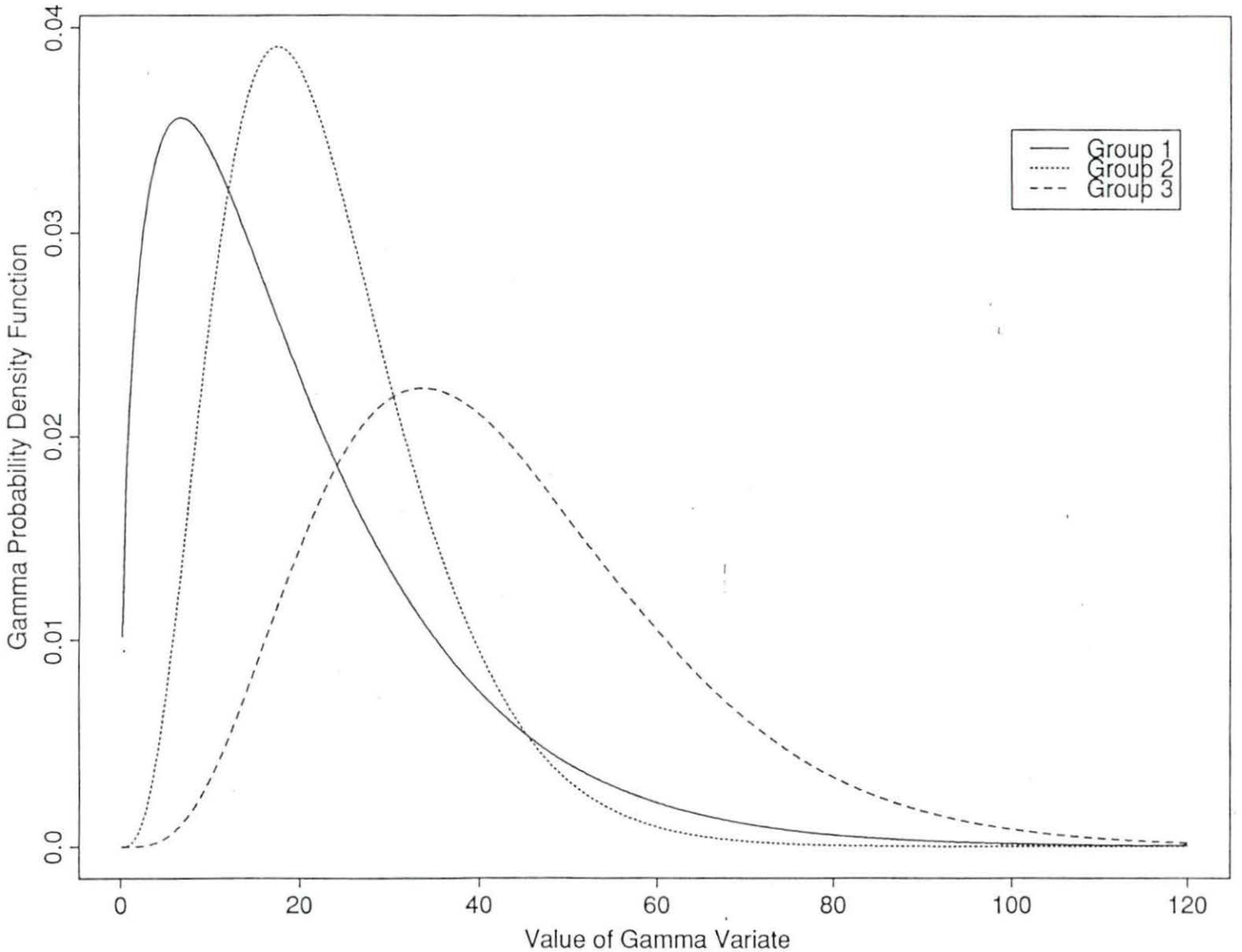
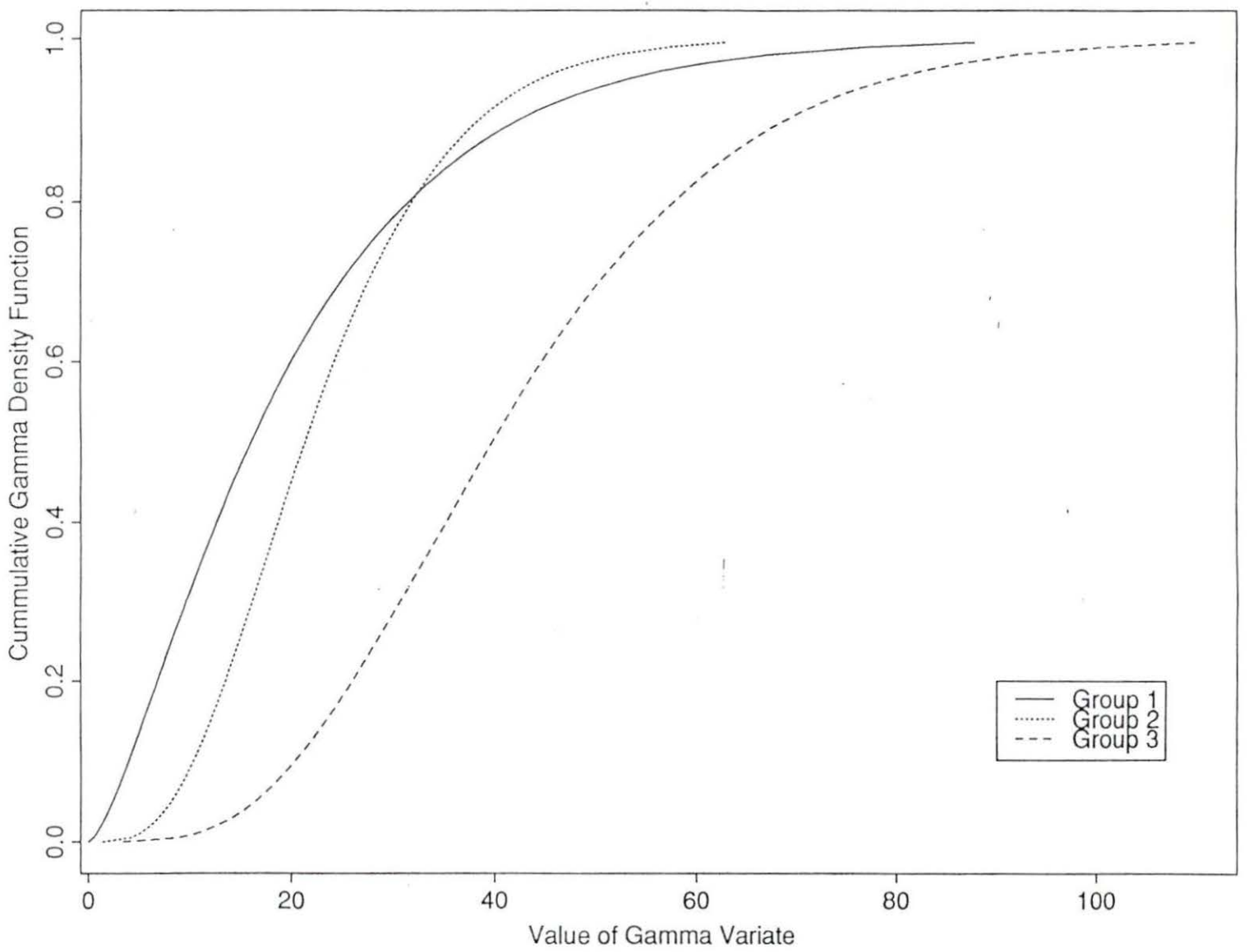

ESTIMATED GAMMA PDF'S FOR CELL TYPE T
Gamma Probability Density Function
0.0
0.01
0.02
0.03
0.04
0
20
40
60
80
100
120
Value of Gamma Variate
Group 1
Group 2
Group 3

Figure 7F. Cumulative density function (CDF) curves in 3 groups of dogs for T-cells. On the basis of cell counts, the probability of a dog belonging to each group can be estimated by measuring 1 minus the area under the CDF curve.


ESTIMATED GAMMA CDF'S FOR CELL TYPE T
Cummulative Gamma Density Function
0.0
0.2
0.4
0.6
0.8
1.0
0
20
40
60
80
100
Value of Gamma Variate
Group 1
Group 2
Group 3

FOOTNOTES

a Science Diet® Canine Maintenance, Hill's Pet Products, Topeka, KS.

b Olympus CV-1 Videoscope, Olympus Corp, Lombard, IL.

c Model FB-15K Alligator jaws biopsy forceps, Olympus Corp, Lombard, IL.

d Rabbit anti-canine IgA, ICN ImmunoBiologicals, Lisle, IL.

e Rabbit anti-canine IgG, Bethyl Laboratories, Montgomery, TX.

f Rabbit anti-canine IgM, Pel-Freez, Rogers, AR.

g Biotinylated goat anti-rabbit antibody, Vector, Burlingame, CA.

h Rabbit anti-human T-cell (CD3), Dako, Carpinteria, CA.

i Macrophage, HAM 56, Dako, Carpinteria, CA.

j Biotinylated horse anti-mouse antibody, Vector, Burlingame, CA.

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GENERAL SUMMARY AND DISCUSSION

The work described here characterizes the distributions of selected lymphoocytes (ie, IgA cells, IgG cells, and T-cells) in the intestinal lamina propria of healthy dogs, in dogs having histologic lesions of IBD, and in dogs with chronic gastroenteritis but no histologic lesions of IBD. Despite the limitation of the small size of the tissue specimens obtained through gastrointestinal endoscopic procedures, we were able to objectively quantitate lamina propria cellularity using morphometric techniques. The first objective of our study required that we design a suitable morphometric method to evaluate lamina propria cellularity in duodenal biopsy specimens. This task proved more difficult than originally thought in that numerous endoscopic specimens (and therefore dogs) were excluded from this study for a variety of reasons. These reasons included: (1) biopsy specimens which were of insufficient size for appropriate evaluation, (2) specimens which were poorly oriented and which precluded examination of the whole villus, (3) endoscopic specimens which contained significant crush and/or processing artifacts, and (4) specimens which were procured near or adjacent to intestinal lymphoid aggregates. The light microscopic review of the remaining tissue specimens from both diseased and healthy control animals resulted in the three canine groups which we evaluated and compared morphometrically.

We adapted Willard's method of quantitating lamina propria cellularity in full-thickness biopsy specimens to our endoscopic specimens, with an emphasis on counting cellular populations in the villus lamina propria only. This modification was necessary since most endoscopic intestinal biopsy specimens contained superficial mucosa which may not extend down to the muscularis mucosae. Once the villus regions to quantitate cells were defined and standardized, we initiated pilot studies to assess the reproducibility of villus cell counts within individual dogs.

The second objective of our study was to identify appropriate canine specific markers (ie, primary antibodies) which could identify antigens in formalin-fixed tissues. The clinical utility of these markers in identifying immunoglobulin-containing cells in formalin-prepared tissues is well recognized. Considerable trial and error in immunohistochemical staining methods resulted in the identification of excellent human primary antibodies which could reproducibly recognize macrophages and T-cells (CD3) in our canine tissues. The CD3 marker (ie, marker of the CD3 complex associated with the T-cell antigen receptor present on most T-cells) identified both CD4 and CD8 T-cell subsets. Lastly, we performed quantitative morphometric analysis on such tissues to objectively compare the histologic lesions of IBD with other mucosal disorders and normal intestinal tissue in the dog.

The results of morphologic studies of endoscopic biopsies in dogs were surprising. In contrast to earlier studies where only IgA-containing cells were assessed, we showed uniform vertical distributions of IgA, IgG, and T-cells in the healthy and diseased canine GIT. Our morphometric data for IgA cells closely parallels that observed by Willard *et al*. Of particular interest, were the significant differences observed between dog groups for the various cell types. Lamina proprial IgA cells were greatest in dogs having chronic non-specific gastroenteritis from a variety of causes. The number of IgA cells in normal and IBD dogs were not significantly different, but were less than the number in group II dogs. In evaluating the CDF curve for IgG cells, a similar trend is observed with the group II dogs tending to have higher IgG counts as compared to both normal and IBD dogs. These observations for IgA and IgG cells are in contrast to studies in humans which showed increased numbers of IgA and IgG cells in IBD patients.

There are no previous veterinary reports of T-cell distribution in the canine intestine. The use of a human T-cell marker produced excellent results and allowed easy identification of these lymphocytes during image analysis. Similar to normal human adults, T-cells were the predominant cell types found in the lamina propria of normal dogs as well as in the diseased dogs. Most of the T-cells were distributed near the surface epithelium and were often observed as intraepithelial lymphocytes. In contrast to humans, where limited studies have

shown no difference in T-cell numbers between normal and IBD patients, we observed significantly greater T-cell counts in our normal dogs. The reason for this difference could not be determined. It was therefore concluded from our immunohistochemical and morphometric studies that increases in lamina propria cellularity of IgA, IgG, and T-cells are not consistently observed in dogs having histologic lesions of IBD. From the results of this research, it is apparent that dissimilarities in the inflammatory infiltrate of IBD exist between human and canine patients. It is obvious that further work on canine GIT immunity is warranted to understand it better and to compare these observations to the human GIT. Further avenues of investigation of the canine GIT should include: (1) additional evaluation of immunoglobulin-containing cells and T-cells from endoscopic biopsies of the normal canine, (2) the evaluation of lymphocytes from endoscopic biopsies of dogs having chronic idiopathic colitis, and (3) the evaluation of T-cell subsets (ie, CD4 and CD8 cells) in the healthy and diseased canine GIT.

Additional research stemming from the results of this study might include the evaluation of <u>total</u> villus lamina propria cellularity in dogs with IBD in comparison to intestinal biopsies from normal control animals. As stated in the introduction to the thesis, the objectives of this study were to apply criteria of morphometric analysis to mucosal biopsies obtained endoscopically and to objectively compare selected cellular populations (e.g. IgA, IgG, and T-cells)

between groups of dogs. Our experimental design and results of this research substantiate that these objectives were met. It does, however, remain possible that the observed variability in IgA, IgG, and T-cell populations could be influenced by overall lamina propria cellularity (e.g. cells which were not identified by the antibody markers used in this study). To investigate this possibility further, research investigations which assess total villus nuclear densities (e.g. consisting of immunocytes, T-cells, fibroblasts, and proprial smooth muscle cells) in dogs would have to be performed. As recommended by this POS committee, these image analysis studies will be performed prior to publication of this manuscript in a peer-reviewed journal.

The ultimate goal of this research was to provide objective information regarding the histologic criteria for making a diagnosis of canine IBD. These studies have confirmed that increased lamina propria cellularity of IgA, IgG, and T-cells without other evidence of mucosal inflammation is inappropriate evidence for the histologic diagnosis of canine IBD.

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