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Evaluation of B and T lymphocytes and plasma cells in colonic mucosa from healthy dogs and from dogs with inflammatory bowel disease

J. STONEHEWER, J. W. SIMPSON, *Department of Veterinary Clinical Studies, Royal (Dick) School of Veterinary Studies, The University of Edinburgh, Small Animal Clinic, Summerhall, Edinburgh EH9 1QH,*
R. W. ELSE, N. MACINTYRE, *Department of Veterinary Pathology, Royal (Dick) School of Veterinary Studies, The University of Edinburgh, Easter Bush, Roslin, Midlothian EH25 9RG*

SUMMARY

The aim of this study was to investigate the subpopulations of lymphocytes in the colonic mucosa of healthy dogs and dogs with inflammatory bowel disease (IBD). Fourteen normal dogs and 13 dogs with IBD were examined. Endoscopic biopsy specimens of colonic mucosa from each dog were stained specifically for pan T lymphocytes (CD3) and pan B lymphocytes (CD79a), and for plasma cells with methyl green pyronin (MGP) stain. Cells were counted by means of a grid and statistical analysis was performed on the data collected. B and T lymphocytes were also counted in the glandular epithelium of normal dogs and dogs with IBD and the normal and abnormal groups compared statistically. Healthy dogs had significantly lower numbers of T cells in the lamina propria and glandular epithelium and significantly lower numbers of B cells in the lamina propria. Significant group differences for plasma cells were not evident. Our results indicate that in IBD a chronic cellular immune reaction is present in the diseased gut involving increased numbers of B and T lymphocytes.

IDIOPATHIC inflammatory bowel disease (IBD) particularly lymphocytic-plasmacytic colitis is considered to be among the commonest causes of large bowel disease in the dog (Burrows 1992). The diagnosis is made on histopathological analysis of mucosal biopsy specimens with the morphological abnormality detected being an increase in the number of inflammatory cells in the lamina propria (Jergens et al 1992, Leib et al 1989). The diagnosis of IBD by assessing infiltration of inflammatory cells is problematic as colon tissue may normally contain variable numbers of mucosal leukocytes (Zeroogian and Chopra 1994). This fact along with the subjectivity involved in assessing changes in lamina proprial leukocyte populations and inherent inter-pathologist variation has led to the production of grading systems for use in both the human and veterinary fields (Jenkins et al 1988, Roth et al 1990, Seldrenijk et al 1991, Thompson et al 1985).

Local intestinal immunity in human beings is largely mediated through B and T lymphocytes in the intestinal mucosa (Strober and James 1986). In the human intestine, B cells are most common in the lamina propria whereas T cells are predominantly intraepithelial (Fell et al 1996, Hirata et al 1986).

The pathogenesis of inflammatory bowel disease is poorly understood (Korelitz and Sohn 1985, Shorter and Kirsner 1985). Although IBD is associated with several immunological perturbations, there is no direct evidence indicating whether these alterations in the immune system are primary manifestations of disordered immunity or secondary abnormalities resulting from inflammation in the gastrointestinal tract (Shorter et al 1976). There is a growing confidence that new studies will yield information defining the immunopathogenesis of IBD (Markowitz and Daum 1991, Shorter and Shephard 1975). A great deal of work has been done in the investigation of lymphocyte subpopulations in the human colon. To our knowledge there has only been two limited studies involving the *normal* canine colon (Vaerman and Heremans 1969, Willard et al 1982). Our study aims to define some of the subpopulations of leukocytes in the colon of normal dogs and those with IBD.

MATERIALS AND METHODS

Dogs

Fourteen normal dogs (group 1) and 13 dogs with IBD (group 2) were examined. Group 1 were composed of a population of stray dogs euthanased for behavioural reasons. All were healthy at the time of euthanasia and had no history of gastrointestinal disease. They had all been previously wormed with a pyrantel, febantel, praziquantel mix (Drontal Plus®, Bayer plc, Bury St. Edmunds, Suffolk). Euthanasia was performed using an intravenous injection of Pentobarbitone Sodium (Euthatal®, Rhône Mérieux Limited, Harlow, Essex). Multiple mucosal biopsy samples were collected from the ascending, transverse and descending colon using standard non-serrated pinch biopsy forceps (Biopsy forceps FB21K, KeyMed) within 15 minutes of death. Multiple colotomy incisions were made along the length of the colon and mucosal samples were collected via these incisions using the biopsy forceps. All group 1 biopsy samples were interpreted as histopathologically normal. Represented breeds comprised of (in rank order) German Shepherd Dogs and their crosses (six), Pit Bull Terriers (four), Labrador crosses (two) and Greyhounds (two). The mean age was 4·1 years.

Group 2 consisted of 13 dogs with IBD from which a total of 17 samples were collected [three animals being biopsied on more than one occasion (two animals twice and one, three times)].

All were pure-bred dogs [represented breeds in rank order were the Labrador Retriever (three), Boxer (two), Dachshund (two), Cairn Terrier (one), Jack Russell Terrier (one), Basset Hound (one), Tibetan Terrier (one),

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Weimeraner (one) and Springer Spaniel (one)]. Mean age was 4·5 years.

All were referred to the Royal (Dick) School of Veterinary Studies (R(D)SVS) with a history of large bowel diarrhoea. No abnormalities were detected on routine haematology and biochemistry or routine urinalysis and three faecal samples were negative for endoparasitic ova and culture of pathogenic micro-organisms. All underwent colonoscopy and multiple biopsy samples were collected from the ascending, transverse and descending colon using standard non-serrated pinch biopsy forceps. A diagnosis of IBD was made according to published criteria in all dogs (Roth et al 1990).

Group 2 patient preparation

Group 2 dogs were prepared for colonoscopy by withholding food for 24-36 hours. Multiple warm water enemas were given in the 12-18 hours prior to endoscopy. All dogs were sedated using acepromazine (ACP® injection 2 mg ml^{-1} C-Vet Veterinary products, Leyland, Lancashire) and buprenorphine (Vetergesic®, Animalcare Ltd, Dunnington, York) given by intramuscular injection. Endoscopy was performed by use of a flexible endoscope (GIF-PV10 video endoscope, Olympus Corporation). Multiple mucosal biopsy specimens were procured with standard non-serrated pinch biopsy forceps (Biopsy forceps FB21K, KeyMed) from the ascending, transverse and descending colon.

Specimen preparation

Specimens from group 1 and group 2 dogs were fixed in neutral-buffered 10 per cent formalin for 12 to 24 hours, embedded in paraffin wax, processed routinely, sectioned at 5 μm and stained with haematoxylin and eosin (H&E) for routine histopathological analysis and diagnosis. A minimum of six biopsy specimens from each dog was examined. Sections from all dogs were also stained to identify plasma cells using a standard methyl green pyronin stain (Read 1995) and to identify B cells and T cells using a standard avidin-biotin ABC immunoperoxidase technique (Hsu Su-Ming and Raine 1981). B cells were detected using a commercial mouse monoclonal antibody against human B cells (CD79a) (Mouse anti-human B cell (CD79a), Dako, Denmark). T cells were detected using a commercial rabbit polyclonal antibody against human T cells (CD3) (Rabbit anti-human T cell (CD3), Dako, Denmark). For both B and T cells, three, 3′-Diaminobenzidine Tetrahydrochloride (DAB) was used as the substrate and the specimens were counterstained using Harris haematoxylin.

Quantitation

Cell counting per unit area is meaningless in situations where the cell density is variable due to variable orientation of very small endoscopic biopsy samples. It was therefore decided to count the positively stained cells in two ways. Firstly, in the lamina propria, 20 random areas were chosen as a guide to counting using a grid. Each area was 3025 μm^2 therefore a total area of 60500 μm^2 was assessed. The number of positively stained cells in each area was counted as a percentage of the total number of mononuclear cells. Around 1000 cells were counted in the lamina propria of each specimen. Secondly, in order to count intraepithelial lymphocytes, 10 random glands were chosen and the percentage of positive staining cells in the glandular epithelium was calculated.

TABLE 1: Percentage of CD3, CD79a and MGP positive cells (+/– standard deviation) in biopsy specimens

Lamina propria	Number of samples	CD3	CD79a	MGP
Group 1 (controls)	14	7·61 +/– 2·58	1·25 +/– 0·98	26·2 +/– 9·52
Group 2 (IBD)	17	13·72 +/– 4·71	7·29 +/– 3·36	29·58 +/– 7·15
Glandular epithelium	**Number of samples**	**CD3**	**CD79a**	**MGP**
Group (controls)	14	3·8 +/– 1·75	0	Not done
Group 2 (IBD)	17	9·71 +/– 3·40	0	Not done

Statistics (Minitab for Windows®, Minitab Inc)

Cell counts were analysed for normality using a Ryan Joiner analysis. As all groups were normally distributed, two sample T tests were performed on the data.

RESULTS

The results of this study are shown in Table 1. Lamina proprial and glandular epithelial cellularity was within normal limits (Roth et al 1990) in all colonic biopsy specimens from the group 1 dogs. Results of microscopic evaluation of lesions from group 2 dogs were all histologically classified as large intestinal IBD. In these tissues, mixed cellular infiltrate in the lamina propria (predominantly lymphocytes and plasma cells) was often accompanied by epithelial or glandular alterations.

T lymphocytes

Lamina propria (Fig 1). Cell counts from both groups 1 and 2 were found to be normally distributed using a Ryan Joiner analysis. There were significantly more CD3 positive cells in group 2 than in group 1 (P=0·0001) using a two sample T test.

Glandular Epithelium (Fig 2). Cell counts from both groups 1 and 2 were found to be normally distributed using a Ryan Joiner analysis. There were significantly more CD3 positive cells in group 2 than in group 1 (P=0·0001) using a two sample *t* test.

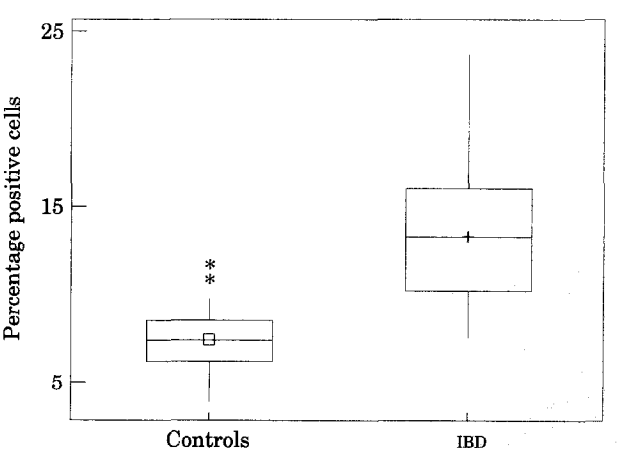

FIG 1: Counts of cells staining positively for CD3 in the *lamina propria* (controls vs IBD dogs)

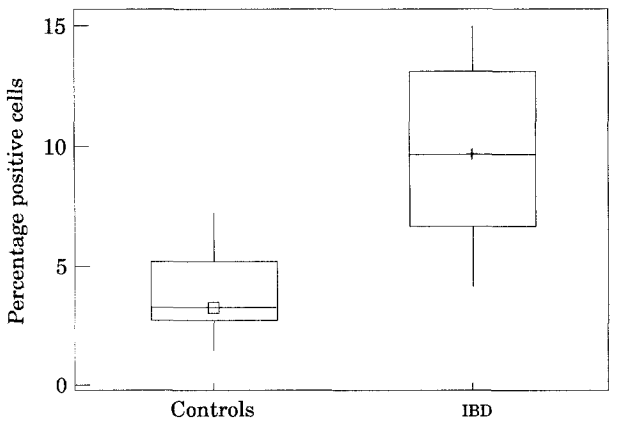

FIG 2: Counts of cells staining positively for CD3 in the glandular epithelium (controls vs IBD dogs)

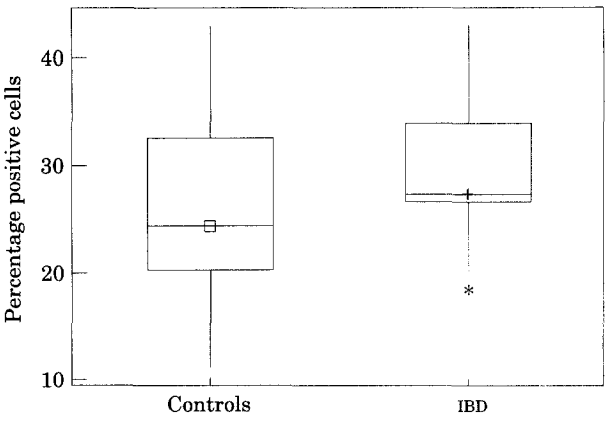

FIG 4: Counts of cells stained with MGP (plasma cells) in the *lamina propria* (controls vs IBD dogs)

B lymphocytes

Lamina propria (Fig 3). Cell counts from both groups 1 and 2 were found to be normally distributed using a Ryan Joiner analysis. There were significantly more CD79a positive cells in group 2 than in group 1 (P=0·0001) using a two sample *t* test.

Glandular epithelium. There were no CD79a positive cells found in the glandular epithelium of either groups 1 or 2. It can therefore be concluded that B lymphocytes either were not present or occurred in very small numbers in the glandular epithelium of the dogs we examined.

Plasma cells

Lamina propria (Fig 4). Cell counts from both groups 1 and 2 were found to be normally distributed using a Ryan Joiner analysis. There was no significant difference between the number of plasma cells in each group using a two sample *t* test (P<0·05).

Glandular epithelium. MGP positive cells were not counted in the glandular epithelium due to excessive background staining of mucin. Different staining methods were attempted but the problem was persistent.

DISCUSSION

Although there have been numerous studies into the subsets of lymphocytes in the human colon there have only been two to our knowledge in the canine colon and these only investigated cells in the *normal* canine colon

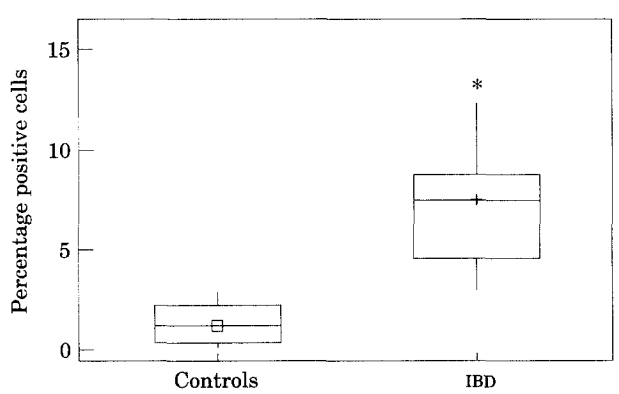

FIG 3: Counts of cells staining positively for CD79a in the *lamina propria* (controls vs IBD dogs)

(Vaerman and Heremans 1969, Willard et al 1982).

The increasing popularity of endoscopy as a means of diagnosing gastrointestinal disease in dogs and human patients means that the pathologist is being faced with the challenge of making a histopathological diagnosis on very small sections of tissue. There is inevitable inter-pathologist variation depending upon their experience and bias (Leib et al 1989). Although there have been several attempts at producing quantitative and morphological grading systems in the human (Jenkins et al 1988, Seldrenijk et 1991, Thompson et al 1985) and veterinary (Roth et al 1990) fields in order to introduce greater objectivity, the diagnosis of Inflammatory Bowel Disease remains largely subjective based on evaluation of the cellularity of the lamina propria (Jergens et al 1992, Leib et al 1989).

Inflammatory Bowel Disease, in particular lymphocytic plasmacytic colitis is considered to be one of the commonest causes of large intestinal disease in the dog (Burrows 1992). Its pathogenesis is poorly understood. Theories put forward include infectious, dietary, vascular, neuromotor, psychosomatic and immune-mediated factors (Sacher et al 1980, Shanahan 1993b). Although the disease may well be multifactorial, immunological research appears to have yielded the most meaningful information in human studies (Kirsner and Shorter 1982).

It was for these two reasons (lack of diagnostic accuracy and poor knowledge of pathogenesis) that this study was carried out.

In our study, there was slight variation in the methods used in sampling groups 1 and 2. Normal controls (group 1) were sampled post mortem via multiple colotomy incisions using endoscopic biopsy forceps (Biopsy forceps FB21K, KeyMed). Group 2 dogs, on the other hand, were prepared for sampling by withholding food and multiple enemas and colonoscopy was performed to collect the samples. Although it could be argued that colonoscopy could be more traumatic than colotomy to the area sampled and iatrogenic lesions could be created by the endoscope, the cellular changes we were looking at are chronic in nature and therefore unlikely to be altered by endoscopy. There were no problems with fixation artefact due to the rapid fixation of tissue in both groups.

Footnotes for graphs:
Line and symbol at median
Bottom of box at first quartile (Q1)
Top of box at third quartile (Q3)
Whisker at top = Q3 + 1·5 (Q3-Q1)
Whister at bottom = Q1-1·5 (Q3-Q1)
Asterisks symbolise outliers lying outwith the lower and upper limits

There are two methods of quantitation of lamina proprial and intraepithelial cells. One is to count the number of cells per unit area of mucosa and the other is to count the number of positively stained cells per 100 mononuclear cells in the lamina propria or per 100 epithelial cells in the glandular epithelium (Hirata et al 1986).

In the lamina propria, due to the difficulties of orientating small endoscopically collected biopsy samples, the individual cell densities in the lamina propria were variable from specimen to specimen. It was therefore decided to quantitate the number of positive cells per 100 mononuclear cells rather than per unit area. Around 1000 cells were counted in the lamina propria of each specimen.

The epithelial region is morphologically and functionally different from the lamina propria region. We suggest that the intraepithelial lymphocyte (IEL) immune role is directed towards the epithelial cell (Hirata et al 1986). Thus we chose to quantitate the number of IEL/100 epithelial cells.

However, it is appreciated that the method of quantitation of lymphocytes and other cells in histological sections must be considered arbitrary and the 'correct' method of quantitation has not been established.

It was not within the remit of this study to look at neutrophils, macrophages or 'null' cells or to further subclassify T cell subsets or immunoglobulin-containing plasma cells. Additional evaluation of these cell types and morphometric analysis would be a useful further study.

T lymphocyte distributions in large intestinal biopsy specimens have not previously been reported in dogs. In this study, the use of a human T cell (CD3) marker provided reproducible recognition of canine T lymphocytes in formalin-fixed specimens. The CD3 marker is a lineage marker which is exclusive to the T cell line. The CD3 complex is a set of five polypeptides which is closely associated with the T-cell antigen receptor (TCR) to give the T-cell receptor complex (TCR-CD3 complex) (Roitt et al 1996). In both normal and diseased dogs T cells were the most numerous of the lymphocyte subgroups examined and were densely but variably distributed in the lamina propria. This finding parallels the many studies done in adult human beings in which the number of T cells has been reported to be double the number of B cells in the lamina propria (Berk 1985). In IBD, the numbers of T cells in the lamina propria were significantly elevated ($P=0{\cdot}0001$). This is in common with some reports from the human literature (Magne 1992, Markowitz and Daum 1991, Meuwissen et al 1976) where markedly increased T cell populations were found in the colonic lamina propria of patients with IBD. However, one report observed increased numbers of T cells only in the deeper layers of the colon (Strickland et al 1975) and one study found no difference in the numbers of T lymphocytes in the colon of IBD patients compared to controls (Hirata et al 1986).

T cells play a central role in organising the intestinal inflammatory infiltrate in IBD (Shanaharn 1993a). They are the major immunoregulatory immune cell population in the intestinal mucosa and due to their ability to respond specifically to local antigens and to secrete a large variety of lymphokines (Roitt et al 1996), they orchestrate the destructive reaction in the inflamed mucosa in IBD in human beings (Anderson 1992).

In our study, T cells were also significantly elevated in the glandular epithelium of the colon of IBD positive dogs compared with controls ($P=0{\cdot}0001$). In fact they were the only lymphocyte subset found in the colonic glandular epithelium in this study. The reason for the presence of T cells nearest to the intestinal lumen is unknown. They are probably derived from the lamina propria. In human beings they are considered to be a morphologically, phenotypically and functionally heterogenous lymphoid cell population although in general it is considered that the majority are T lymphocytes [very few B cells are found in the intraepithelial compartment (Van der Heijden and Stok 1987)] and most are of the suppressor/cytotoxic phenotype (Bienenstock 1984, Harty and Leibach 1985, Brandtzaeg et al 1989, Carman et al 1986). It has been suggested in the dog that T cells migrate from the lamina propria towards the lumen in response to antigenic stimulation (Jergens et al 1996) and at least in man may return to the lamina propria and circulation after antigen priming to act in a sentinel role (Guilford et al 1996, Berk 1985). There is, however, growing evidence that the primary role of the intraepithelial T cell is immunosuppression (Brandtzaeg et al 1989). Furthermore this immunosuppressive function may be important in the development of oral tolerance (Brandztaeg et al 1989, van Garderen et al 1991). The precise role of intraepithelial lymphocytes in gastrointestinal immunity still remains poorly defined.

Our findings with respect to T cells indicate that a substantial proportion of the inflammatory infiltrate in IBD consists of T lymphocytes. Our findings are in agreement with various human studies (Meuwissen et al 1976, Strickland et al 1975, Clancy 1976). The significance of this chronic T cellular immune reaction in man and other species remains to be elucidated and therefore further analysis of the morphology and function of T lymphocytes and T lymphocyte subpopulations is required in order to define more accurately cellular immune phenomena in IBD.

B cell numbers and distribution have not been reported in the colon of canine IBD patients before. In our study a pan B cell marker (CD79a) produced reproducible recognition of canine B lymphocytes in formalin-fixed specimens. IgM is associated with the CD79a molecule on the B cell surface to form the B cell antigen receptor complex (BCR) (Roitt et al 1996).

In the lamina propria, the proportion of B cells was significantly elevated in diseased dogs compared to normal dogs ($P=0{\cdot}0001$). B cells are common in the lamina propria of normal dogs (Willard et al 1982). They also occur in increased numbers in human IBD patients compared with normal human patients (Eade et al 1980). In fact some studies indicate that B cells are more numerous than T cells in colitis patients (Strober and James 1986) and that the majority of lymphocytes in the lamina propria of positive IBD patients are B cells (Blakemore 1994). The elevation in B lymphocytes in the colonic lamina propria of Crohn's disease patients is well recognised (Hirata et al 1986). Human studies have shown that B cells are activated in Crohn's disease and Ulcerative colitis (Deusch and Reich 1992). We did not subclassify the immunoglobulin subclasses of mucosal B cells. In humans, B cells of the IgG class predominate in the mucosal lamina propria (Strickland et al 1975)

In the glandular epithelium, no B cells were found in either normal or diseased animals. This is in common with human studies (Hoang et al 1992) although in some studies a few B cells were found in the intraepithelial lymphocyte population (Guilford et al 1996, Hirata et al 1986, Van der Heijden and Stok 1987, Cerf-Bensussan et al 1983, Selby et al 1981, 1984) but in one of these studies this appeared to be due to artifactual staining of B cells (Hirata et al 1986).

The only conclusion that can be made is that there appears to be a participation of humoral immunity in IBD in

the dog. Further studies are needed to yield more information regarding immunoglobulin subtypes. This would be an important future study in canines.

Although there were more plasma cells in the lamina propria of IBD dogs than normal dogs, the difference between groups was not significant in this study. This is an interesting result as it is generally considered that the number of plasma cells in the lamina propria of human patients with IBD is greater than in normal patients (Keren et al 1984). However, to our knowledge there are no studies in which the methyl green pyronin stain has been used to examine plasma cells in the canine gastrointestinal tract. There is a report in human patients which found no increase in IgA-containing plasma cells in Crohn's disease (Green and Fox 1975) and one in dogs in which no increase in IgA or IgG-containing plasma cells was seen in the small intestine of IBD positive patients (Jergens et al 1996). We recognise the need to subclassify plasma cells according to immunoglobulin type for future studies to be more meaningful (Halliwell 1975, Hart 1979).

In summary, our results indicate that a substantial proportion of the inflammatory infiltrate in IBD in dogs consists of T and B lymphocytes with a reasonable proportion of the T lymphocytes being intraepithelial in type. The significance of this immune reaction remains to be elucidated and therefore further analysis of the morphology and function of T and B lymphocytes and plasma cells and of their subpopulations is required in order to define more accurately cellular immune phenomena in IBD.

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