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Increased migration of IgA lymphocytes to VIP nerve fibers after DSS-induced colitis

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Summary. Immunoglobulin-positive lymphocytes are present close to vasoactive intestinal polypeptide-positive (VIP^+) nerve fibers in the lamina propria of the intestinal tract, and have an important role in mucosal defense. The number of immunoglobulin A-positive (IgA^+) cells close to the epithelial basement membrane and nerve fibers is increased by the administration of lipopolysaccharides, which induce IgA secretion into the intestinal lumen. The relationship between immunoglobulin-positive lymphocytes and the VIP^+ nerve fibers during inflammation, such as in inflammatory bowel disease, however, is not well known. The morphological relationship between immunoglobulin-positive cells and the basement membrane or the VIP^+ nerve fibers in the colon was examined using double immunofluorescent labeling in an inflammatory bowel disease mouse model created by oral administration of dextran sodium sulfate (DSS). DSS administration induced goblet cell loss, crypt loss, intestinal epithelium deformation and infiltration of inflammatory cells in the mucosa. In the colon, the number and percentage of IgA^+ lymphocytes close to the basement membrane and the VIP^+ nerve fibers in the lamina propria increased after DSS administration, in parallel with the pathologic progress in the inflamed tissue. On the other hand, the percentage of immunoglobulin G-positive (IgG^+) lymphocytes close to the basement membrane and the VIP^+ nerve fibers decreased, although the total number of IgG^+ lymphocytes in the lamina propria increased. We suggest that the immunoglobulin-producing lymphocytes and enteric nerve fibers in the colon normally have a close morphological relationship, and that this relationship is reinforced in a cell-specific manner during inflammation.

Key words: Immunoglobulin A, Immunoglobulin G, Vasoactive intestinal polypeptide, Enteric nervous system, Colon, Dextran sulfate sodium, Inflammatory bowel disease, Ulcerative colitis

Introduction

Immunoglobulin A (IgA) is the main immunoglobulin in the mucosal immune system (Mestecky and McGhee, 1987). Polymeric IgA antibodies produced by plasma cells in the lamina propria of the intestinal tract bind to polymeric immunoglobulin receptors at the base of the epithelium. The IgA and polymeric immunoglobulin receptor complex undergoes endocytosis and vesicular transport to the apical surface of the enterocytes, and is secreted into the lumen (Lamm, 1998). In the mucosa, IgA-producing cells are attracted to the basement membrane around the crypts by chemokines expressed by epithelial cells (Kunkel et al., 2000, 2003), which increase IgA production and secretion. Previous morphological studies demonstrated that plasma cells in the lamina propria are close to the nerve fibers (Gottwald et al., 1997; Crivellato et al., 1998), suggesting that the nervous system interacts with the mucosal immune system in the gastrointestinal tract. Nerve fibers in the large intestine originate from the intrinsic enteric nervous system, comprising the

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Abbreviations: BM^+, close to basement membrane; DSS, dextran sodium sulfate; HCS, histological colitis score; IgA, immunoglobulin A; IgA^+, immunoglobulin A-positive; IgG, immunoglobulin G; IgG^+, immunoglobulin G-positive; IL, interleukin; LPS, lipopolysaccharide; N^+, close to nerve fibers; PBS, phosphate-buffered saline; VIP, vasoactive intestinal polypeptide; VIP^+, vasoactive intestinal polypeptide-positive; $VIPR^+$, vasoactive intestinal polypeptide receptor-positive

submucosal plexus and the myenteric plexus, in addition to the extrinsic nervous system. The neurons in the enteric nervous system contain various neuropeptides, such as vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide, neuropeptide Y and substance P. VIP-positive (VIP^+) nerve fibers are widely distributed in the lamina propria, and are involved in glandular secretion into the intestinal lumen (Furness, 2006). In mice, the submucosal VIP^+ nerve fibers that localize with calcitonin gene-related peptide and neuropeptide Y are close to IgA-positive (IgA^+) lymphocytes in the mouse ileum lamina propria (Shibata et al., 2008). Furthermore, almost all IgA^+ lymphocytes in the ileum lamina propria express the VIPR1 and VIPR2 receptors (Shibata et al., 2008). Therefore, VIP^+ nerve fibers in the intestine lamina propria might have a role in transmitting signals to the IgA^+ lymphocytes (Shibata et al., 2008).

Increased secretion of IgA into the intestinal lumen following intraperitoneal administration of lipopolysaccharides (LPS) was recently reported (Hisajima et al., 2005). LPS are large molecules located on the outer membrane of Gram-negative bacteria that act as mouse B cell mitogens, which induce B cell multiplication (Stavnezer, 1996) and B cell differentiation into plasma cells. Our laboratory also reported that the number of IgA^+ cells close to the basement membrane in the ileum lamina propria increases 1 h after intraperitoneal LPS injection, and that the number of cells close to both the basement membrane and the nerve fibers significantly increases 6 h after LPS injection (Hisajima et al., 2005). These findings suggest that migration of the IgA^+ lymphocytes to the nerve fibers is as important as migration to the epithelial basement membrane for lymphocyte secretion of IgA.

Ulcerative colitis is an inflammatory bowel disease characterized by chronic inflammation in the colon. The pathogenesis of inflammatory bowel disease remains unknown, although genetic and environmental factors have been reported (Fiocchi, 1998). Previous studies reported changes in the number of IgA-containing cells (Brandtzaeg et al., 1974; Skinner and Whitehead, 1974; Baklien and Brandtzaeg, 1975; Meuwissen et al., 1976; Rosekrans et al., 1980; MacDermott et al., 1986; Badr-el-Din et al., 1988; Wu et al., 1989), the number of immunoglobulin G (IgG)-containing cells (Brandtzaeg et al., 1974; Skinner and Whitehead, 1974; Meuwissen et al., 1976; Rosekrans et al., 1980; Scott et al., 1983; van Spreeuwel et al., 1985a,b; MacDermott et al., 1986; Badr-el-Din et al., 1988; Strober et al., 2002), and IgA production and secretion (Brandtzaeg et al., 1974; Brandtzaeg and Korsrud 1984; van Spreeuwel et al., 1985a; MacDermott et al., 1986; Badr-el-Din et al., 1988; Wu et al., 1989; Cicalese et al., 1995) in active and quiescent ulcerative colitis. On the other hand, abnormal distribution of the enteric nervous system, especially of the VIP^+ nerve fibers and substance P-positive nerve fibers, is observed in the inflammatory bowel disease mucosa (Koch et al., 1987; Kimura et al., 1994; Lee et al., 2002). The relationship between the B cell system and the enteric nervous system in inflammatory bowel disease, however, remains unclear.

The administration of dextran sulfate sodium (DSS) solution to rodents induces colitis. The inflammation induced by the sulfated polymer DSS is involved in producing a dysfunctional barrier in intestinal epithelial cells (Cooper et al., 1993) by increasing colonic mucosal permeability (Kitajima et al., 1999). Long-term administration of DSS is widely employed to model human ulcerative colitis because it causes inflammatory reactions and ulceration of the entire colon. Chronic colitis caused by multiple cycles of DSS administration is induced in a T cell-dependent manner (Dieleman et al., 1998; Kabashima et al., 2002). On the other hand, oral administration of 5.0% DSS for 7 and 8 days induces acute colitis in a T cell-independent manner (Dieleman et al., 1994; Stevceva et al., 2001). Thus, DSS-induced colitis is mediated by different mechanisms, such as innate and adaptive immunity at different phases of the disease (Yoshihara et al., 2005).

The primary aim of the present study was to evaluate changes in the relationship between IgA^+ lymphocytes and VIP^+ nerve fibers during inflammation in a mouse DSS-induced model of colitis. We compared the data of the acute colitis model and that of the chronic colitis model to elucidate whether migration of the IgA^+ lymphocytes to the nerve fibers is also important in chronic or subacute inflammation. We also examined the relationship between IgG^+ lymphocytes and VIP^+ enteric nerve fibers in the same models, because IgG^+ lymphocytes play a significant role in inflammatory bowel disease (Brandtzaeg et al., 2006).

Materials and methods

Materials and induction of colitis

Thirty-two male ICR mice (age, 7 weeks; body weight, 28–35 g, Charles River Japan, Yokohama, Japan) were used in the study. Twenty-four of the mice received oral administration of DSS (MW36,000-50,000, MP Biomedicals, LLC, Irvine, CA). Of the 24 mice, 16 were given 5.0% DSS in the drinking water for 4 (acute-DSS4) or 7 days (acute-DSS7), and the other 8 were given 2.5% DSS in the drinking water for 7 days, and then switched to regular water for 10 days. In some mice, this cycle was repeated twice (chronic- DSS7x2). After administration, the mice were deeply anesthetized with intraperitoneal injections of pentobarbital (50 mg/kg) following inhalation of isoflurane, and the proximal colon and distal colon were dissected out. Eight mice were immediately anesthetized at 8 weeks (DSS0), and the proximal colon, and distal colon were dissected out. All procedures were performed according to the standards established by the NIH Guide for Care and Use of Laboratory Animals and the protocols were approved by the Yokohama City University School of Medicine Committee for Animal Research.

Sampling

Pieces of the proximal colon and distal colon were fixed in 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer (PB, pH 7.4) for 24 h at 4°C, and immersed in 0.1M PB containing 20% sucrose until they sank. The specimens were embedded in Tissue-TeK (Sakura, Tokyo, Japan), and frozen in a methylbutane-liquid nitrogen bath. They were then cut in a cryostat (CM 3050 S, Leica, Nussloch, Germany) into 8 μm thick sections, and mounted on gelatinized glass slides.

Histology and histological colitis score

Some proximal colon and distal colon sections were stained with hematoxylin and eosin to study histological changes. The preparations were examined under a bright field microscope (Eclipse 600; Nikon, Tokyo, Japan). The histological colitis score (HCS), described by Yoshihara et al. (2005) and Kihara et al. (2003) with modifications (Table 1), was used to grade the intestinal inflammation. All scores were obtained in a blind fashion by a pathologist.

Immunofluorescent labeling

To examine the morphological relationship between immunoglobulin-producing lymphocytes and VIP^+ nerve fibers, the sections were first incubated for 24 h at 4°C in a mixture of goat polyclonal antibody against mouse IgA (1:4000; Southern Biotechnology Associates Inc., Birmingham, AL), or goat polyclonal antibody against mouse IgG (1:1000; Southern Biotechnology) and rabbit polyclonal antibody against VIP (1:300; ImmunoStar Inc., Hudson, WI).

To examine the expression of neuropeptide receptors in IgA^+ and IgG^+ lymphocytes, the preparations were first incubated for 24 h at 4°C in goat polyclonal antibody against mouse IgA (1:4000; Southern Biotechnology) or goat polyclonal antibody against mouse IgG (1:1000; Southern Biotechnology) and mouse monoclonal antibody against VIP receptor 1 (VIPR1; 1:1000; Exalpha Biologicals Inc., Maynard, MA) or mouse monoclonal antibody against VIP receptor 2 (VIPR2; 1:1000; Exalpha). The anti-VIPR1 antibody recognizes a 122-134 amino acid sequence (CGLDNDKRAASSLDEQ) of human VIPR1 receptors (Goetzl et al., 1994), whereas anti-VIPR2 antibody recognizes a 105-122 amino acid sequence of human VIPR2 receptors, according to the supplier (Exalpha). After rinsing several times with phosphate-buffered saline with 0.3% Triton-X (PBST, pH 7.4), the sections were incubated for 2 h at room temperature in a mixture of $F(ab')_2$ fragment donkey anti-goat IgG conjugated with Cy2 (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA), and $F(ab')_2$ fragment donkey anti-rabbit IgG conjugated with Cy3 (1:300; Jackson), or $F(ab')_2$ fragment donkey anti-mouse IgG conjugated with Cy5 (1:400; Chemicon Inc., Temecula, CA). Cy3 images were artificially colored using Photoshop software (Adobe, San Jose, CA).

All sections were pre-incubated in 10% normal donkey serum and 2% bovine serum albumin in 0.1 M PBST (pH 7.4) for 30 min. All antibodies were diluted with 0.1M PBST containing 1% normal donkey serum, 0.2% bovine serum albumin, and 0.1% NaN_3. The $F(ab')_2$ fragment of immunoglobulins was used as a secondary antibody to avoid background staining caused by nonspecific binding between intrinsic mouse immunoglobulins and the Fc fragment of the secondary antibodies (Lu and Partridge, 1998). No background activity in lymphocytes was observed in a control study in which the primary antibodies were omitted. The specificity of the VIPR1 and VIPR2 antibodies was checked with Western blotting by Shibata et al. (2008). The preparations were washed with PBS, mounted in antifade reagent (Bio-Rad Laboratories, Hercules, CA), and examined with either a confocal laser microscope (LSM 510; Carl Zeiss, Oberkochen, Germany) or with a fluorescence microscope (Eclipse 600).

Cell counting

IgA^+ lymphocytes and IgG^+ lymphocytes in the colon lamina propria were measured, and classified based on whether they were close to the basement membrane (BM^+) and the VIP^+ nerve fibers (N^+). The numbers of cells were counted in randomly selected fields i.e., BM^+, to examine the migration of the cells involved directly in the immunoglobulin secretion to the basement membrane; N^+, to examine the role of the VIP^+ nerve fibers in the cells; BM^+N^+, to examine the contact with the basement membrane and with the VIP^+ nerve fibers; and BM^-N^-, to examine the behavior of cells not close to either the basement membrane or the VIP^+ nerve fibers. The cell counting was performed in

Table 1. Histological colitis scoring.

Feature	Score	Description
Epithelium	0	Normal morphology
	1	Loss of goblet cells
	2	Loss of crypt, epithelium present
	3	Loss of crypt and epithelium
	4	Loss of crypt and epithelium (large area)
Infiltration	0	No infiltration
	1	Crypt basis
	2	Mucosa
	3	Submucosa
	4	Submucosa(extensive)
Ulcer	0	None
	2	Positive
	4	Positive(extensive)

Histological criteria were used to evaluate inflammation severity. The scores range from 0 to 12 (total score), which represents the sum of scores from 0 to 4 for epithelium, infiltration, and ulcer. All evaluations were performed by observers unaware of the treatment groups.

30 fields (x40/field; size, 1024 pixel) from three intact mice, and from three DSS-treated mice. The 8-μm thick sections were separated into 0.75-μm tomogram images using a confocal laser microscope (LSM 510). Lymphocytes close to the basement membrane or the VIP^+ nerve fibers were determined as such when they clearly contacted the basement membrane or the VIP^+ nerve fibers in extended 0.75-μm images. The percentage of BM^+, N^+, and BM^+N^+ to total IgA^+ and IgG^+ lymphocytes in the colon lamina propria was calculated.

Statistical analysis

The difference in the number or percentage of IgA^+ lymphocytes, IgG^+ lymphocytes close to the epithelial basement membrane, and the VIP^+ nerve fibers in the colon lamina propria was analyzed using an unpaired t test between the two groups, or by Student-Newman-Keuls test among three groups.

The parametric correlation between HCS and the number or percentage of IgA^+ lymphocytes close to the epithelial basement membrane and the VIP^+ nerve fibers was analyzed in 90 fields from 9 mice, 3 with DSS0 and 6 with acute colitis. These were calculated as means per field. A *P*-value of less than 0.05 was considered statistically significant. The data were analyzed by a two-tailed test.

Results

Histological analysis

In the proximal colon and distal colon mucosa, acute-DSS4 and acute-DSS7 induced goblet cell loss, crypt loss, intestinal epithelium deformation and inflammatory cell infiltration (Fig. 1). The same findings were observed in the colonic mucosa of chronic-DSS7x2 mice.

In the proximal colon, the HCS of the acute-DSS7 was significantly higher than that of DSS0 and acute-DSS4 (Fig. 2A). In the distal colon, the HCS of both acute-DSS4 and acute-DSS7 mice was significantly higher compared with DSS0 (Fig. 2B). On the other hand, the HCS of chronic-DSS7x2 mice was significantly higher in both the proximal colon and distal colon compared with the DSS0 mice (Fig. 2C,D).

Contact between IgA^+ lymphocytes expressing VIP receptor and VIP^+ nerve fibers in the colon lamina propria

Some IgA^+ lymphocytes in the lamina propria were close to the VIP^+ nerve fibers (Fig. 3). Varicosities protruding into the cytoplasm of lymphocytes were also observed (Fig. 3). Most IgA^+ lymphocytes in the lamina propria were positive for VIPR1 or VIPR2. Lymphocytes positive for IgA^+ and $VIPR^+$ were occasionally observed close to the basement membrane (Fig. 4).

Number and percentage of IgA^+ lymphocytes in the colon lamina propria

In the proximal colon, the mean number of total IgA^+ lymphocytes in acute-DSS7 mice, but not in acute-DSS4 mice, was significantly increased, compared to that in DSS0 mice (Fig. 5A). The mean numbers of BM^+, N^+, and BM^+N^+ in acute-DSS4 and acute-DSS7 were significantly increased compared to those in DSS0

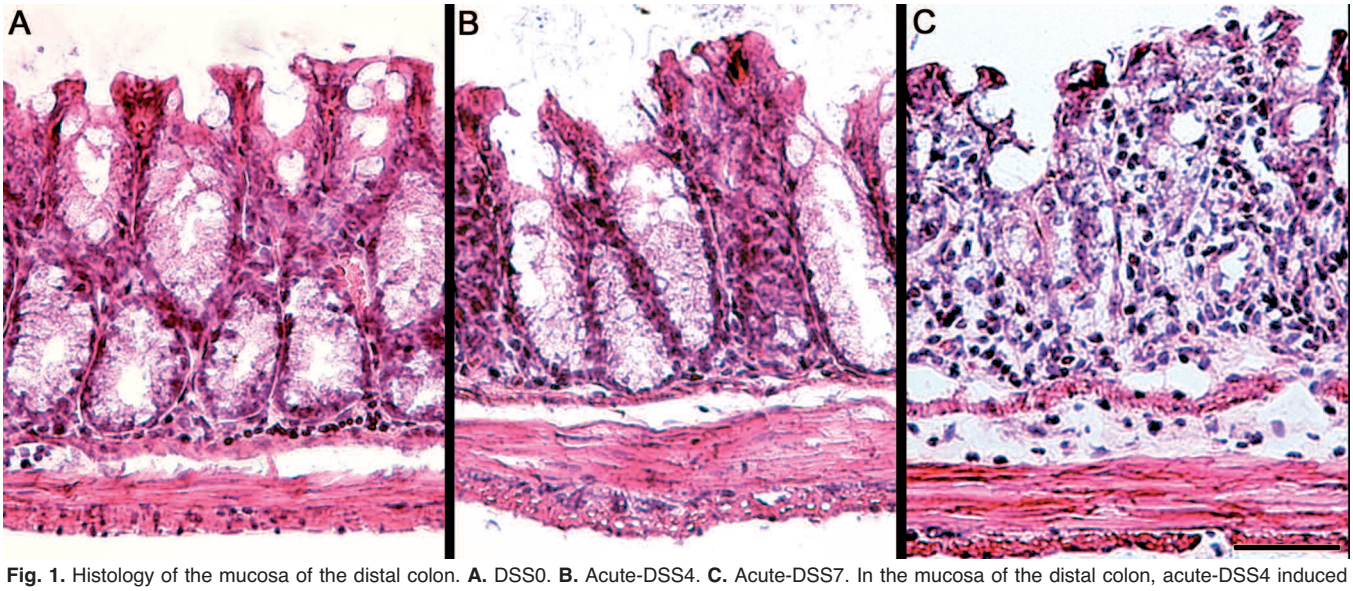

Fig. 1. Histology of the mucosa of the distal colon. **A.** DSS0. **B.** Acute-DSS4. **C.** Acute-DSS7. In the mucosa of the distal colon, acute-DSS4 induced crypt loss in addition to goblet cell loss. Acute-DSS7 induced deformation of the intestinal epithelium and infiltration of inflammatory cells in addition to crypt loss and goblet cell loss. Scale bar: 10 μm.

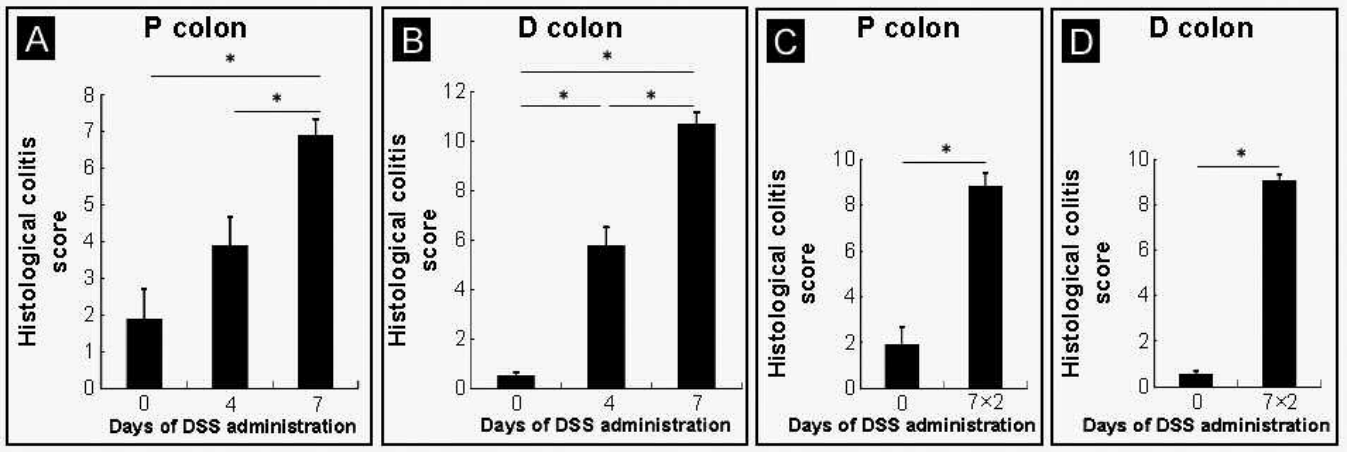

Fig. 2. Histological colitis score in the mucosa of the colon. **A.** Proximal colon of DSS0, acute-DSS4, and acute-DSS7. **B.** Distal colon of DSS0, acute-DSS4, and acute-DSS7. **C.** Proximal colon of DSS0 and chronic-DSS7x2. **D.** Distal colon of DSS0 and chronic-DSS7x2. Histological colitis score (HCS) is expressed as mean ± SE from 4 mice. *P<0.05, **P<0.01.

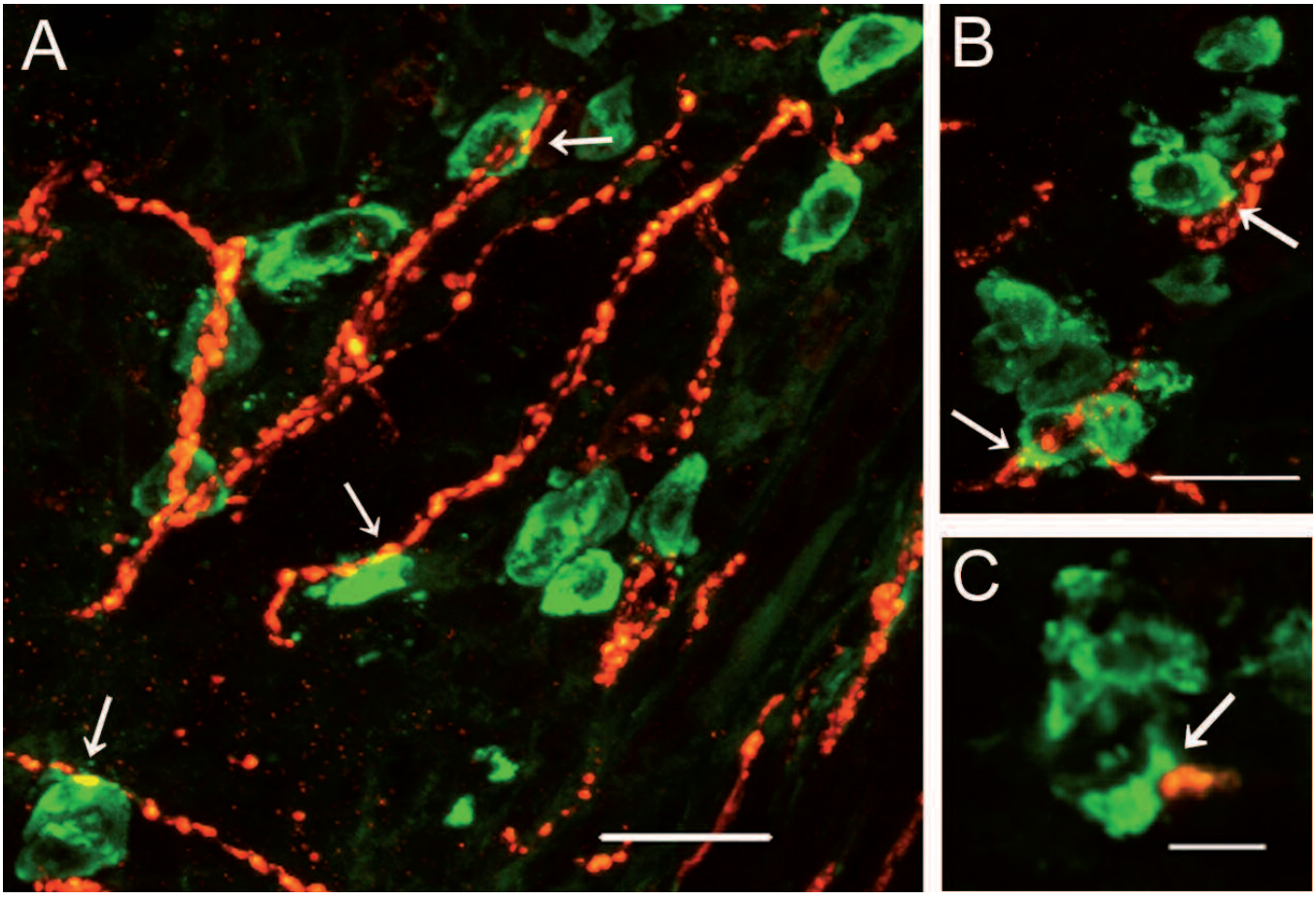

Fig. 3. Contact between IgA$^+$ lymphocytes and VIP$^+$ nerve fibers in the proximal colon lamina propria. **(A and B)** Lymphocytes immunoreactive for IgA (green) were close to nerve fibers (arrows) immunoreactive for VIP (red), as assessed by confocal microscopy. Tomograms of A and B: 8-μm thick. **C.** A varicosity immunoreactive for VIP (red) was in close apposition (arrow) to a lymphocyte immunoreactive for IgA (green). Tomogram of C: 0.75-μm thick. Scale bars: A, B, 20 μm; C, 5 μm.

mice. The mean percentage of BM^+, N^+, and BM^+N^+ per total IgA^+ lymphocytes was also significantly increased compared to that in DSS0 mice. On the other hand, in acute-DSS4 and acute-DSS7, the mean number and percentage of BM^-N^- were significantly decreased, compared to those in DSS0 mice (Fig. 5A,B).

In the distal colon, the mean numbers of total lymphocytes and BM^+, N^+, and BM^+N^+ in acute-DSS4 and acute-DSS7 mice were significantly increased, compared to those in DSS0 mice (Fig. 5C). The mean

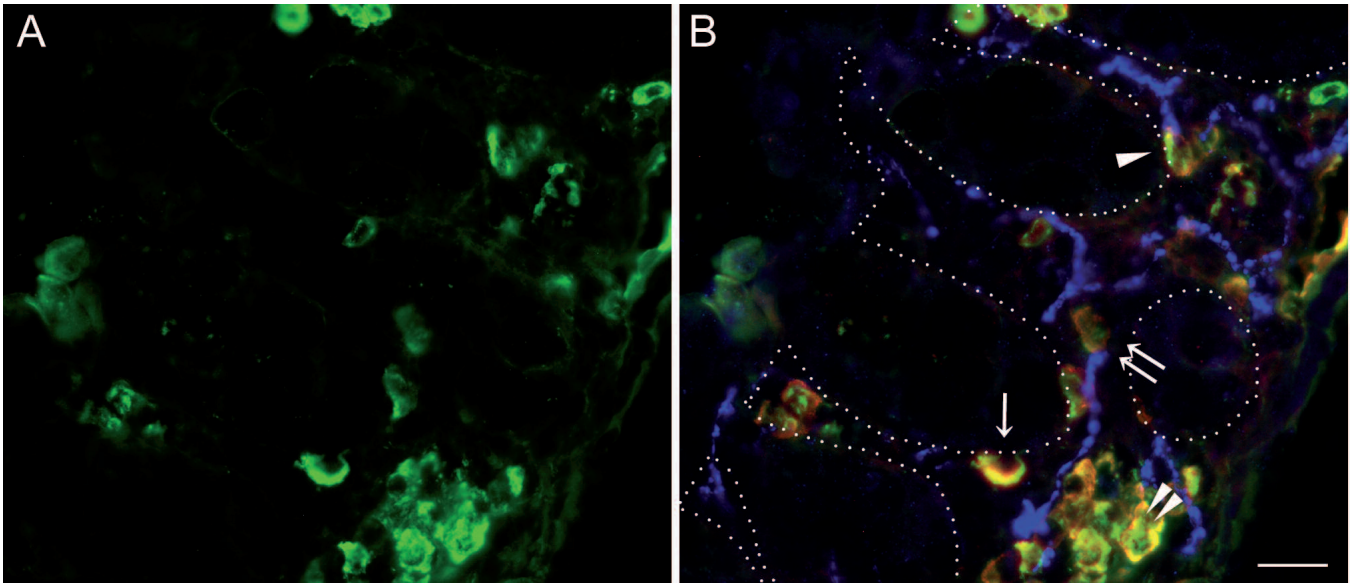

Fig. 4. Labeling of IgA^+ lymphocytes, $VIPR^+$ lymphocytes and VIP^+ nerve fibers in the proximal colon lamina propria of acute-DSS4. **A.** IgA^+ lymphocytes. **B.** Lymphocytes immunoreactive for IgA (green), and VIPR1 (red) were sometimes close to the nerve fibers immunoreactive for VIP (blue). Dotted line: basement membrane. Arrow: BM^+; double arrow: N^+; arrowhead: BM^+N^+; double arrowhead: BM^-N^-. Scale bars: 20 μm in B (applies also to A).

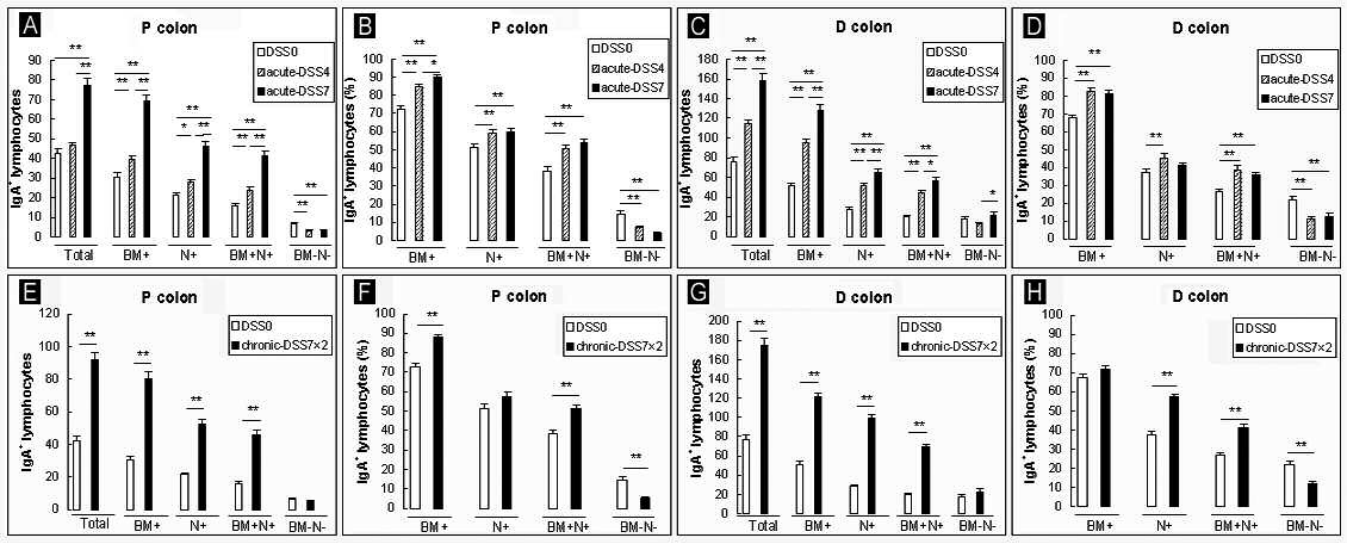

Fig. 5. Number and percentage of IgA^+ lymphocytes in the colon lamina propria. **A, C, E, and G.** Number of IgA^+ lymphocytes. **B, D, F, and H.** Percentage of IgA^+ lymphocytes contacting either the basement membrane or VIP^+ nerve fibers. Total, distributed over the entire lamina propria in the field; BM^+, close to the basement membrane; N^+, close to the VIP^+ nerve fibers; BM^+N^+, close to both the basement membrane and the VIP^+ nerve fibers; BM^-N^-, not close to either the basement membrane or the VIP^+ nerve fibers. The number of cells is expressed as mean ± SE in 30 fields from 3 mice. $^*P < 0.05$, $^{**}P < 0.01$.

percentage of BM^+, N^+, and BM^+N^+ per total lymphocytes in acute-DSS4 mice and the mean percentage of BM^+ and BM^+N^+ per total lymphocytes in acute-DSS7 mice were significantly increased compared to those in DSS0 mice. On the other hand, in acute-DSS4 and acute-DSS7 mice, the mean percentage of BM^-N^- per total lymphocytes was significantly decreased, compared to that in DSS0 mice (Fig. 5D).

In chronic-DSS7x2 mice, the mean numbers of total lymphocytes and of BM^+, N^+, and BM^+N^+ lymphocytes in the proximal colon and the distal colon were significantly increased, compared to those in DSS0 mice (Fig. 5E,G). The mean percentage of BM^+ and BM^+N^+ in the proximal colon, and of N^+ and BM^+N^+ in the distal colon was significantly increased. On the other hand, BM-N- in the proximal colon and the distal colon were significantly decreased, compared to those in DSS0 mice (Fig. 5F,H).

The HCS in the proximal colon and distal colon of acute-DSS4, acute-DSS7, and chronic-DSS7x2 mice significantly correlated with the number and percentage of total, BM^+, N^+, BM^+N^+ of IgA^+ lymphocytes.

Contact between IgG⁺ lymphocytes and VIP⁺ nerve fibers in the colon lamina propria

The lamina propria contained numerous IgG^+ lymphocytes. SomeIgG^+ lymphocytes in the lamina propria were close to the VIP^+ nerve fibers. Many IgG^+ lymphocytes in the lamina propria, however, were

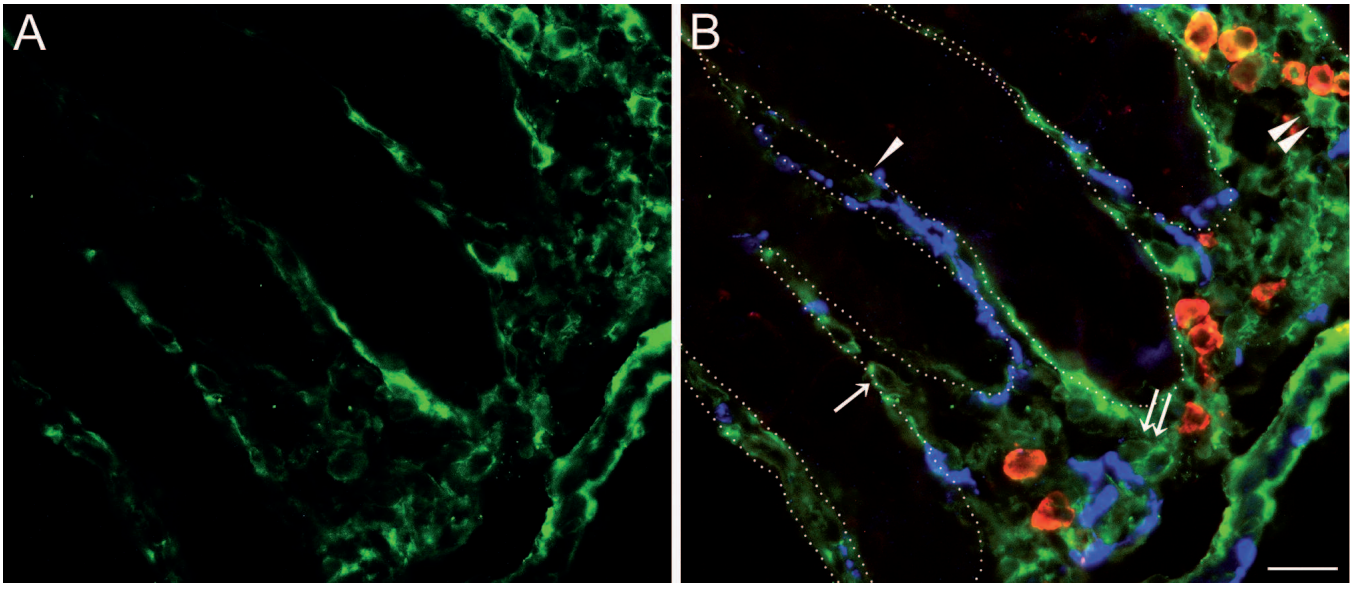

Fig. 6. Labeling of IgG^+ lymphocytes, $VIPR^+$ lymphocytes and VIP^+ nerve fibers in the distal colon lamina propria. **A.** IgG^+ lymphocytes were present. Background reactivity was observed in the submucosal blood vessels. **B.** Lymphocytes immunoreactive for IgG (green), and VIPR1 (red) were sometimes close to the nerve fibers immunoreactive for VIP (blue). Dotted line: basement membrane. Arrow: BM^+; double arrow: N^+; arrowhead: BM^+N^+; double arrowhead: BM^-N^-. Scale bars: 20 μm in B (applies also to A).

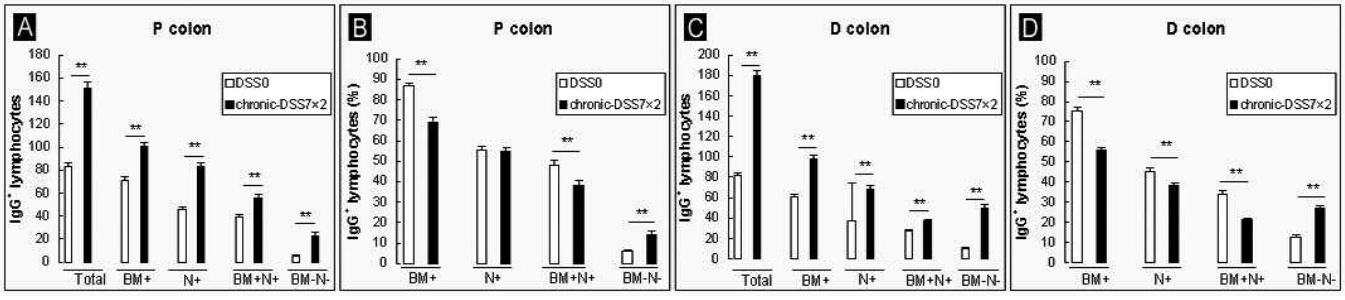

Fig. 7. Number and percentage of IgG^+ lymphocytes in the colon lamina propria. **A and C.** Number of IgG^+ lymphocytes. **B and D.** Percentage of IgG^+ lymphocytes contacting VIP^+ nerve fibers. Total, distributed over the entire lamina propria in the field; BM^+, close to the basement membrane; N^+, close to the VIP^+ nerve fibers; BM^+N^+, close to both the basement membrane and the VIP^+ nerve fibers; BM^-N^-, not close to either the basement membrane or the VIP^+ nerve fibers. The number of cells is expressed as mean ± SE in 30 fields from 3 mice. *P<0.05, **P<0.01.

negative for both VIPR1 and VIPR2. IgG^+ lymphocytes were occasionally observed close to the basement membrane (Fig. 6).

In the proximal and distal colon, the mean numbers of total IgG^+ lymphocytes and of BM^+, N^+, BM^+N^+, and BM^-N^- lymphocytes in chronic-DSS7x2 mice were significantly increased (Fig. 7A,C). On the other hand, the mean percentage of each type of IgG lymphocyte per total IgG^+ lymphocytes was significantly decreased, but the mean percentage of BM^-N^- IgG^+ lymphocytes per total IgG lymphocytes in chronic-DSS7x2 mice was significantly increased, compared to that in DSS0 mice (Fig. 7B,D).

Discussion

Patholgoy of DSS-treated mice

The present study demonstrated that acute-DSS4 and acute-DSS7 induced the pathology of acute colitis in mice. The higher HCS of the distal colon compared with that of the proximal colon in acute-DSS4 and acute-DSS7 suggests that the pathology of the mouse model is similar to that of human inflammatory bowel disease, which originates in the distal colon. The present study also suggested that chronic-DSS7x2 induced chronic inflammation in mice.

IgA^+ lymphocytes contacting the basement membrane or VIP^+ nerve fibers

The present results demonstrated that the total number of IgA^+ lymphocytes in the colon lamina propria, the number of IgA^+ lymphocytes close to the basement membrane or the VIP^+ nerve fibers, and the percentage of IgA^+ lymphocytes close to the basement membrane or the VIP^+ nerve fibers per total IgA^+ lymphocytes were increased in both the acute and chronic DSS-induced colitis models. The HCS scores correlated with the number and percentage of IgA^+ lymphocytes close to either the basement membrane or the VIP^+ nerve fibers, suggesting that the migration of IgA^+ lymphocytes to the basement membrane and the VIP^+ nerve fibers might be promoted with progression of the inflammation-induced pathology. The increased percentage of IgA^+ lymphocytes close to the basement membrane and the VIP^+ nerve fibers per total IgA^+ lymphocytes suggests that the increased number of IgA^+ lymphocytes close to the basement membrane and the VIP^+ nerve fibers does not simply reflect the increased number of IgA^+ lymphocytes migrating to the lamina propria. The inflammation might promote the contact between IgA^+ lymphocytes and the basement membrane, and the VIP^+ fibers.

Although the number and percentage of IgA^+ lymphocytes close to the basement membrane or the VIP^+ nerve fibers increased, some differences were observed between the proximal colon and distal colon, and between different administration regimens. The comparison of each value in the acute colitis model indicates a possible migratory pathway of IgA^+ lymphocytes.

In acute-DSS4, the total number of IgA^+ lymphocytes and the number and percentage of IgA^+ lymphocytes close to the basement membrane or the VIP^+ nerve fibers were significantly increased in the distal colon, which had significantly higher HCS. In the proximal colon, on the other hand, although the total number of IgA^+ lymphocytes was not increased in the proximal colon, the number and percentage of IgA^+ lymphocytes close to the basement membrane or the VIP^+ nerve fibers were increased. These findings suggest that IgA^+ lymphocytes from the lamina propria migrated to the basement membrane or to the VIP^+ nerve fibers before newly migrated lymphocytes increased in the proximal colon. This finding is also supported by the decrease in the number of IgA^+ lymphocytes not close to either the basement membrane or the VIP^+ nerve fibers. In the proximal colon, no significant increase in HCS was observed in acute-DSS4, suggesting that IgA^+ lymphocytes migrate to the basement membrane or the VIP^+ nerve fibers even though inflammation levels are low.

In acute-DSS7, the number of IgA^+ lymphocytes close to the basement membrane or to the VIP^+ nerve fibers was significantly increased in both the proximal colon and distal colon compared to that in acute-DSS4. The percentage of IgA^+ lymphocytes close to the basement membrane or to the VIP^+ nerve fibers per the total IgA lymphocytes, on the other hand, did not change compared to that in acute-DSS4. These results suggest that new IgA^+ lymphocytes migrated from outside of the lamina propria, and were integrated in the IgA secretory mechanisms from 4 days onward. It is possible that the same thing happened in the chronic-DSS7x2 mice.

The functional significance of the increase in IgA^+ lymphocytes close to the VIP^+ nerve fibers in the colon lamina propria is not clear. As in the ileum, the VIP^+ nerve fibers in the colon regulate both fluid secretion from the glands and IgA secretion into the lumen via migration to the basement membrane (Shibata et al., 2008). DSS administration might activate VIP^+ nerves in response to the invasion of intestinal microflora induced by the DSS-induced epithelial damage.

As the acute DSS-colitis is mediated in a T cell-independent manner, migration of IgA^+ lymphocytes to the VIP^+ nerve fibers and the basement membrane in the mucosa might be activated independently of the T cell system. On the other hand, the T cell system might be involved in the recruitment of IgA^+ lymphocytes into the inflamed mucosa, because the number of IgA^+ lymphocytes close to the VIP^+ nerve fibers and the basement membrane further increased in our chronic model, in which T cells might have an important role in the progression of inflammation (Dieleman et al., 1998; Kabashima et al., 2002).

A previous study suggested that some IgA^+ lymphocytes communicate with the nerve fibers prior to

migration to the basement membrane after LPS treatment (Crivellato et al., 1998), suggesting that VIP$^+$ nerve fibers have a regulatory role in the migration of lymphocytes to the basement membrane. The mucosal epithelial cells express chemokines such as CCL28/mucosae-associated epithelial chemokine, which have an important role with receptor CCR10 in the migration and homing in the colon of IgA$^+$ lymphocytes (Hieshima et al., 2004). On the other hand, it is not clear which mediators are involved in the contact between the IgA$^+$ lymphocytes and VIP$^+$ nerve fibers.

IgG$^+$ lymphocytes contacting the basement membrane or VIP$^+$ nerve fibers

In inflammatory bowel disease, IgG antibodies have an important role as a host defense mechanism against microflora invading the mucosa through the dysfunctional epithelial barrier (Brandtzaeg et al., 2006). Previous studies reported that the number of IgG-containing cells (Brandtzaeg et al., 1974; Skinner and Whitehead, 1974; Rosekrans et al., 1980; Scott et al., 1983; van Spreeuwel et al., 1985a,b; Badr-el-Din et al., 1988), and the IgG production (Wu et al., 1989) increase in active and quiescent ulcerative colitis. Our present analysis showed an increase of IgG$^+$ lymphocytes in the chronic-DSS7x2 colon lamina propria. Some IgG$^+$ lymphocytes in the lamina propria were close to the VIP$^+$ nerve fibers in normal mice, and the number of IgG$^+$ lymphocytes close to VIP$^+$ nerve fibers significantly increased with inflammation. Many IgG$^+$ lymphocytes in the lamina propria, however, were negative for VIPR1 or VIPR2, and the percentage of IgG$^+$ lymphocytes close to the VIP$^+$ nerve fibers per total IgG$^+$ lymphocytes, in contrast to IgA$^+$ lymphocytes, which decreased during inflammation. Therefore, the increase in the number of IgG$^+$ lymphocytes close to the VIP$^+$ nerve fibers might simply reflect a significant increase in the total number of lymphocytes in the mucosa. The increased number of IgG$^+$ lymphocytes suggests that a second line of mucosal defense is formed in chronic inflammation (Tokoi et al., 1996; Brandtzaeg et al., 2006), because the IgG immune response is involved in cell damage through activation of the antibody-dependent cell-mediated cytotoxicity system (Tokoi et al., 1996).

Conclusion

Our data suggest that immunoglobulin-producing lymphocytes close to VIP$^+$ enteric nerve fibers in the colon increased during both acute and chronic inflammation. As the percentage of IgA$^+$ lymphocytes, but not IgG$^+$ lymphocytes, close to the basement membrane or the VIP$^+$ nerve fibers was significantly increased, the close relationship to the VIP$^+$ nerve fibers is more important for the activity of IgA$^+$ lymphocytes during inflammation. Therefore, enteric nervous systems inflamed by disorders such as inflammatory bowel disease might have an important role in the activation of the IgA secretory mechanisms of the B cell system. Further studies are required to determine how the IgA secretory mechanisms related to VIP$^+$ enteric nerve fibers are involved in the pathogenic prevention of inflammatory bowel disease.

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