Changes in enteroendocrine and immune cells following colitis induction by TNBS in rats

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Abstract. Approximately 3.6 million individuals suffer from inflammatory bowel disease (IBD) in the western world, with an annual global incidence rate of 3-20 cases/100,000 individuals. The etiology of IBD is unknown, and the currently available treatment options are not satifactory for long-term treatment. Patients with inflammatory bowel disease present with abnormalities in multiple intestinal endocrine cell types, and a number of studies have suggested that interactions between gut hormones and immune cells may serve a pivotal role in the pathophysiology of IBD. The aim of the present study was to investigate alterations in colonic endocrine cells in a rat model of IBD. A total of 30 male Wistar rats were divided into control and trinitrobenzene sulfonic acid (TNBS)-induced colitis groups. Colonoscopies were performed in the control and TNBS groups at day 3 following the induction of colitis, and colonic tissues were collected from all animals. Colonic endocrine and immune cells in the obtained tissue samples were immunostained and their densities were quantified. The densities of chromogranin A, peptide YY, and pancreatic polypeptide-producing cells were significantly lower in the TNBS group compared with the control group, whereas the densities of serotonin, oxyntomodulin, and somatostatin-producing cells were significantly higher in the TNBS group. The densities of mucosal leukocytes, B/T-lymphocytes, T-lymphocytes, B-lymphocytes, macrophages/monocytes and mast cells were significantly higher in the TNBS group compared with the controls, and these differences were strongly correlated with alterations in all endocrine cell types. In conclusion, the results suggest the presence of interactions between intestinal hormones and immune cells.

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Introduction

Abnormalities in several intestinal endocrine cell types have been reported in patients with inflammatory bowel disease (IBD) and in animal models of human IBD (1-20). The association between the neuroendocrine peptides/amines in the gut and the immune system has been previously investigated, and it was suggested that interactions between gut hormones and immune cells may serve a pivotal role in the pathophysiology of IBD (8,10,11,21-29).

The etiology of IBD is unknown and the currently available treatments are not completely satisfactory (2,30). Treatment with 5-aminosalicylates and corticosteroids are not effective for the long-term treatment of the majority of patients with IBD. In addition, thiopurine analogues, mercaptopurine and azathioprine, as well as methotrexate, have been used. Short and long-term side effects limit the use of these agents. Biological agents, such as antibodies against tumor necrosis factor α (TNFα), have been used for two decades. However, only ~65% of patients with ulcerative colitis and Crohn's disease respond to treatment with anti-TNFα, and surgery remains the only option for many IBD patients (2,30). Understanding the role of the gut neuroendocrine peptides/amines in the pathophysiology of IBD may provide an insight into its etiology and lead to the use of agonists or antagonists to these peptides and amines as a treatment for IBD (26).

Using a model of human ulcerative colitis (UC) in dextran sulfate sodium (DSS)-induced rats, a recent study demonstrated that abnormalities in the large intestine endocrine cells were strongly correlated with the alterations in immune cells (31). The present study investigated the large intestine endocrine cells in an animal model of Crohn's disease (CD), which involved the induction of colitis in rats using trinitrobenzene sulfonic acid (TNBS). The aim of the current study was to determine whether a change in immune cell number is correlated with abnormalities in the endocrine cells.

Materials and methods

Animal model. A total of 30 male Wistar rats (6 weeks of age; Wistar Hannover GALAS; Taconic Biosciences, Inc.,

Lille Skensved, Denmark), with a mean body weight of 276 g (range, 235-380 g), were housed in Makrolon III cages with water and food available *ad libitum*. They were fed a standard diet (B&K Universal AS, Nittedal, Norway) and were maintained at a temperature of 20-22˚C, a relative humidity of 50-60% and under 12 h light/dark cycles. Rats were acclimated to these animal house conditions for a minimum of 7 days prior to the start of the experiments. They were then divided equally into the following 2 groups: Control and TNBS-induced colitis.

Induction of colitis with TNBS. Rats were fasted for 24 h prior to TNBS administration. A single dose of TNBS (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) was administered to the colon of each rat (25 mg/animal in a 50% ethanol solution; 0.5 ml/rat) followed by 2 ml air, at 8 cm from the anal margin using an 8.5 cm-long, 2.5-mm-wide round-tipped Teflon feeding tube (AgnTho's AB, Lidingö, Sweden) under isoflurane (Schering-Plough Pharmaceuticals, North Wales, USA) anesthesia. The animals were kept in a prone position with their hind legs raised for at least 2 min following the administration of the TNBS. They were supervised until recovery and then monitored several times daily. The control group received the same treatment as the TNBS group, except that 0.9% saline instead of TNBS was introduced into the colon. Any rats that exhibited signs of pain were injected subcutaneously with 1 ml Temgesic (0.3 mg Temgesic/ml; Merck Sharpe & Dohme, Hoddesdon, UK).

Colonoscopy. Colonoscopies were performed in the control and TNBS rats at 3 days following the administration of 0.9% saline and TNBS, respectively. The bowels were prepared as described previously (32). Briefly, prior to the colonoscopy, the rats were deprived of food for 24 h and received gastric doses of 1 and 2 ml Picoprep (Ferring Holding SA, Saint Prex, Switzerland) followed by 2 ml water at 24 and 12 h, respectively. Picoprep was administered using an 8.5 cm-long, 2.5 mm wide round-tipped Teflon feeding tube (AgnTho's AB). Picoprep (150 ml) contains 10 mg sodium sulfates, 3.5 g magnesium oxide, and 12 g citric acid.

Rats were anesthetized by inhalation of isoflurane (Merck Sharpe & Dohme) prior to and during the colonoscopy. They were placed in a supine position and secured to an acrylic surgical table (World Precision Instruments, Sarasota, FL, USA), and a warming pad (T/Pad; Gaymar Industries, Inc., Orchard Park, NY, USA) with a heat therapy pump (Gaymar TP500 T/Pump; Gaymar Industries, Inc.) was used to maintain normothermia during the procedure. The top of a video gastroscope (GIF-N180; Olympus Corporation, Tokyo, Japan) was lubricated with 2% lidocaine (Xylocaine; AstraZeneca, Södertälje, Sweden) and introduced gently into the anus.

Endoscopic inflammation was scored according to the same grading scale as described by Vermeulen *et al* (33). This scale comprises the following five subscales (total score, 0-19 points): Degree of inflammation (0-6 points), extent of disease (0-10 points), stenosis (0 or 1 point), edema (0 or 1 point) and active bleeding (0 or 1 point).

Following the procedure, rats were sacrificed by CO_2 inhalation and a postmortem laparotomy was conducted. Tissue samples obtained from the distal colon were examined histopathologically, and with immunostaining techniques as described below.

The local ethical committee for experimental animals at the University of Bergen (Bergen, Norway), which is responsible for implementing the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, approved the protocols employed for the purposes of the current study.

Histopathological and immunohistochemical analysis. Rat colon tissue samples were fixed overnight in 4% buffered paraformaldehyde, embedded in paraffin and sectioned at into 5 μm-sections. The sections were deparaffinized and then stained with hematoxylin-eosin, or immunostained using the ultraView Universal DAB Detection kit (version 1.02.0018; Venata Medical Systems, Inc., Basel, Switzerland) and the BenchMark Ultra IHC/ISH staining module (Venata Medical Systems, Inc.). Tissue sections were incubated with primary antibodies for 32 min at 37˚C. Details of the primary antibodies used are listed in Table I.

Quantification of endocrine and immune cells. The endocrine and immune cells were quantified by manually counting each cell type in 10 randomly selected microscopic fields of view using cellSens imaging software (version 1.7; cellSens; Olympus Corporation). The number of endocrine cells in the lining epithelium, and the number of immune cells in the lamina propria were manually counted in each field using a computer mouse. To achieve this, the epithelial cell area was determined by manually drawing an enclosed region with the computer mouse. A x40 objective was used, which, represented a tissue area of 0.035 mm^2 for each frame (field) on the monitor. The data are presented as the number of endocrine cells/mm^2 of epithelium, and the number of immune cells/field of view. Immunostained sections were coded and mixed, and measurements were determined by the same person (Professor Magdy El-Salhy), who was unaware of which group the slides were derived from.

Statistical analysis. Differences between the control and TNBS groups were analyzed using the nonparametric Mann-Whitney U test. The existence of a correlation between abnormalities/alterations in the densities of endocrine cells and immune cells was determined using the nonparametric Spearman's rank correlation test. The data are presented as the mean ± standard error of the mean. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Histopathological examination. Histopathological examination of the colonic tissues demonstrated that those derived from control rats displayed a normal histology, whereas those from the TNBS group exhibited an abnormal mucosal architecture, the presence of crypt abscesses, edema, bleeding and infiltration of immune cells into the mucosa and submucosa (Fig. 1).

Colonoscopy. Rat colons in the control group displayed a normal appearance with undamaged mucosa and clear branching of blood vessels, whereas the colonic mucosa in the

Table I. Details of primary antibodies used.

Target protein	Species raised in	Target species	Dilution	Source	Catalogue number
Chromogranin A	Mouse[a]	N-terminal of purified chromograninA	1:1,000	Dako (Glostrup, Denmark)	M869
Serotonin	Mouse[a]	Serotonin	1:1,500	Dako	5HT-209
Peptide YY	Rabbit[b]	Peptide YY	1:1,600	Alpha-Diagnostics International (San Antonio, TX, USA)	PYY 11A
Oxyntomodulin	Rabbit[b]	Porcine glucagon	1:200	Acris Antibodies GmbH (Herford, Germany)	BP508
Pancreatic polypeptide	Rabbit[b]	Synthetic human pancreatic polypeptide	1:500	Diagnostic Bio-Systems (Pleasanton, CA, USA)	114
Somatostatin	Rabbit[b]	Synthetic human somatostatin	1:800	Dako	A566
Leukocytes	Mouse[a]	Human CD45	1:600	Dako	M0701
B/T lymphocytes	Mouse[a]	Human CD5	1:500	Dako	IS082
T lymphocytes	Mouse[a]	Human CD57	1:200	Dako	IS647
B lymphocytes	Mouse[a]	Human CD23	1:400	Dako	IS781
Monocytes and macrophages	Mouse[a]	Human CD68	1:100	Dako	M0814
Mast cells	Mouse[a]	Human mast cell tryptase	1:800	Dako	M7052

[a] and [b] denote monoclonal and polyclonal primary antibodies, respectively.

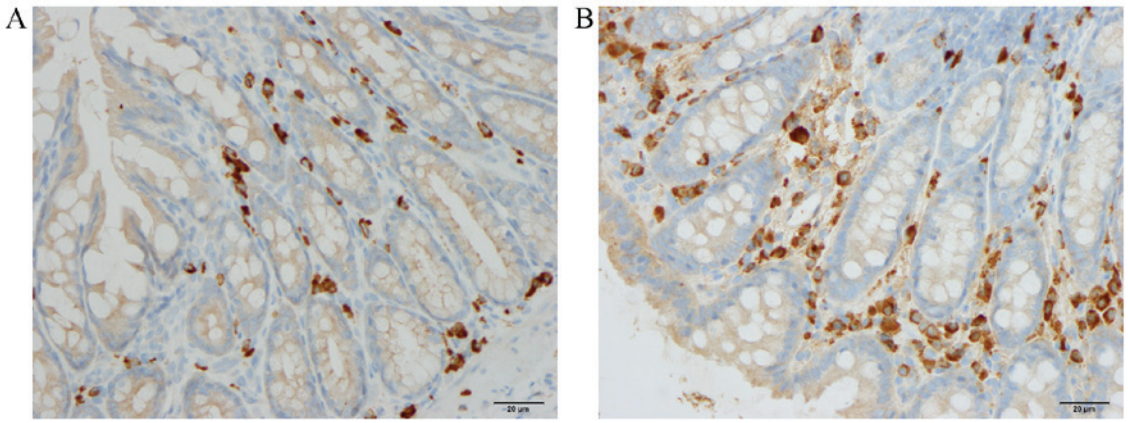

Figure 1. Identification of macrophages/monocytes in the lamina propria of the colon derived from (A) a control rat (B) a rat with trinitrobenzene sulfonic acid-induced colitis.

TNBS group exhibited patchy and discontinuous erythema, edema and occasional hemorrhage (Fig. 2). In addition, aphthoid ulcers abruptly surrounded by normal mucosa were observed in the colons of TNBS rats (Fig. 2B). The deep ulcerations coalesced, which led to mucosal detachment and the presence of few mucosal islands. Ulcerated stenosis was also observed in TNBS rat colons (Fig. 2B). The endoscopic inflammation scores were 0 and 6.4±0.8 in the control and TNBS groups, respectively.

Endocrine cells. The densities of various endocrine cells are presented in Figs. 3, 4 and 5. The density of chromogranin A (CgA), peptide YY (PYY) and pancreatic polypeptide (PP) staining was reduced in the colon tissues from rats in

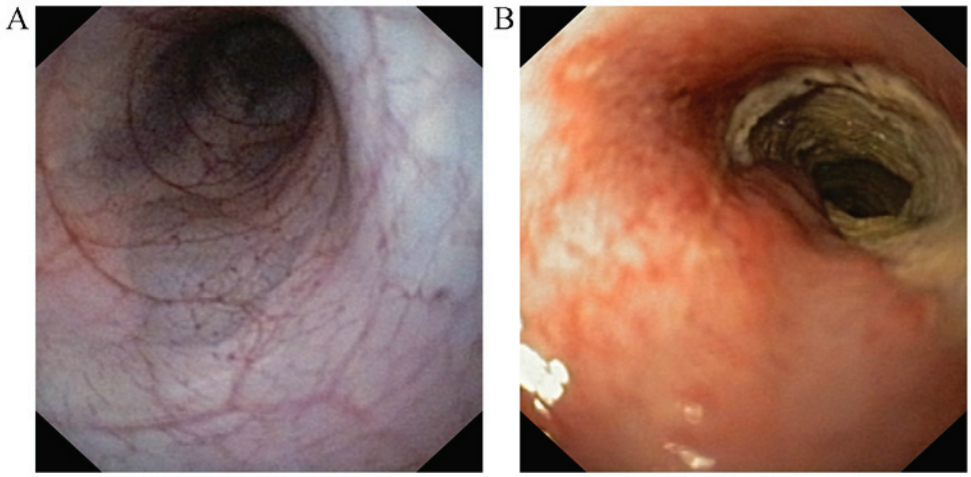

Figure 2. Endoscopic appearance of the colon in (A) a normal control rat and (B) a rat with trinitrobenzene sulfonic acid-induced colitis, demonstrating mucosal erythema, edema and ulcerated stenosis.

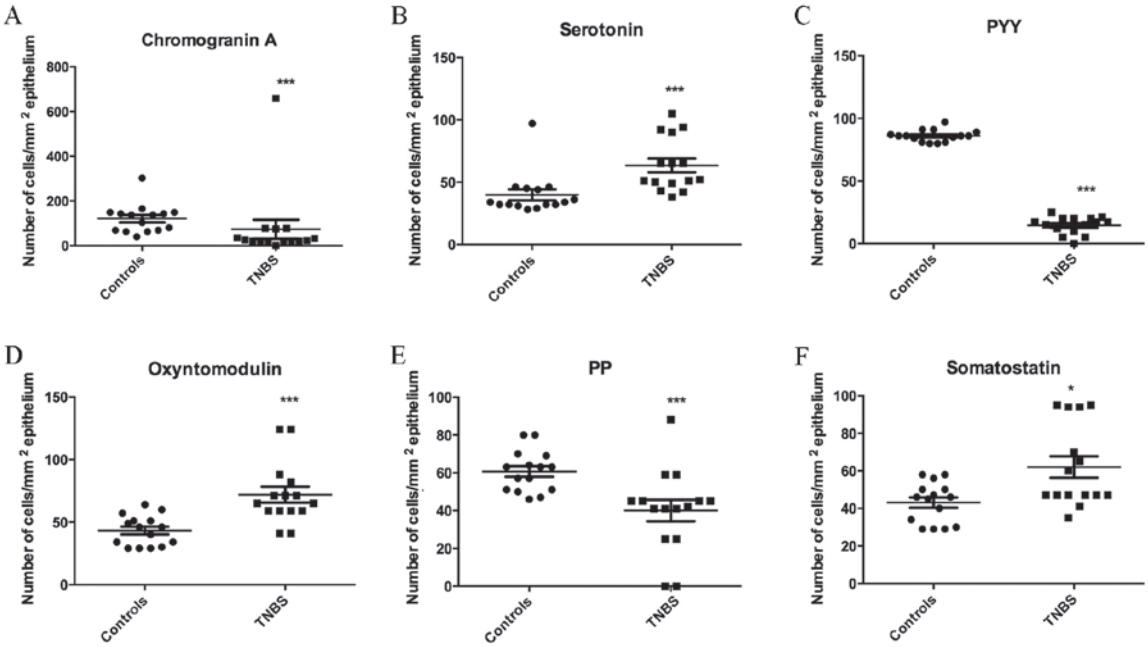

Figure 3. Densities of (A) chromogranin A, (B) serotonin, (C) PYY, (D) oxyntomodulin, (E) PP and (F) somatostatin in the colon tissues derived from normal controls and those from TNBS-induced colitis. Data are presented as the mean ± standard error. *P<0.05 and ***P<0.001 vs. controls. TNBS, trinitrobenzene sulfonic acid; PYY, peptide YY; PP, pancreatic polypeptide.

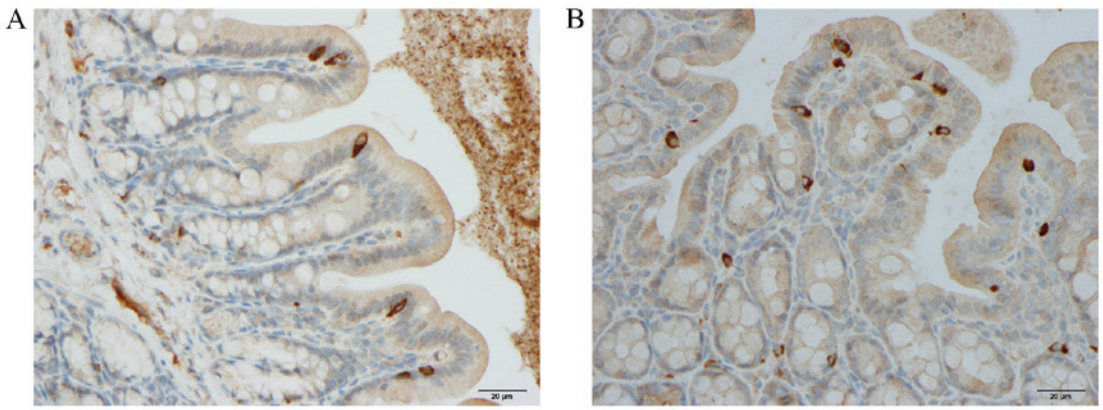

Figure 4. Immunohistochemical staining of serotonin in the colon tissues derived form (A) a control rat and (B) a rat with trinitrobenzene sulfonic acid-induced colitis.

Table II. Spearman's rank correlation coefficients (*r*) and P-values for the association between endocrine and immune cells in rats with TNBS-induced colitis.

	Immune cell type					
Endocrine cell type	Leukocytes	B/T lymphocytes	T lymphocytes	B lymphocytes	Macrophages/ monocytes	Mast cells
Chromogranin A	*r*=–0.7	*r*=–0.3	*r*=–0.6	*r*=–0.6	*r*=–0.7	*r*=–0.5
	P=0.04	P=0.09	P=0.009	P=0.02	P=0.008	P=0.03
Serotonin	*r*=0.7	*r*=0.7	*r*=0.4	*r*=0.6	*r*=0.2	*r*=0.4
	P=0.005	P=0.009	P=0.01	P=0.02	P=0.05	P=0.1
Peptide YY	*r*=–0.5	*r*=–0.7	*r*=0.2	*r*=–0.7	*r*=–0.6	*r*=0.7
	P=0.04	P=0.002	P=0.06	P=0.002	P=0.02	P=0.7
Oxyntomdulin	*r*=0.2	*r*=0.5	*r*=0.6	*r*=0.1	*r*=0.1	*r*=–0.6
	P=0.6	P=0.02	P=0.01	P=0.7	P=0.9	P=0.02
Pancreatic polypeptide	*r*=–0.7	*r*=–0.5	*r*=–0.2	*r*=–0.6	*r*=–0.6	*r*=–0.7
	P=0.004	P=0.8	P=0.5	P=0.008	P=0.01	P=0.002
Somatostatin	*r*=0.6	*r*=0.7	*r*=0.1	*r*=0.2	*r*=–0.7	*r*=–0.6
	P=0.02	P=0.8	P=0.7	P=0.6	P=0.0044	P=0.02

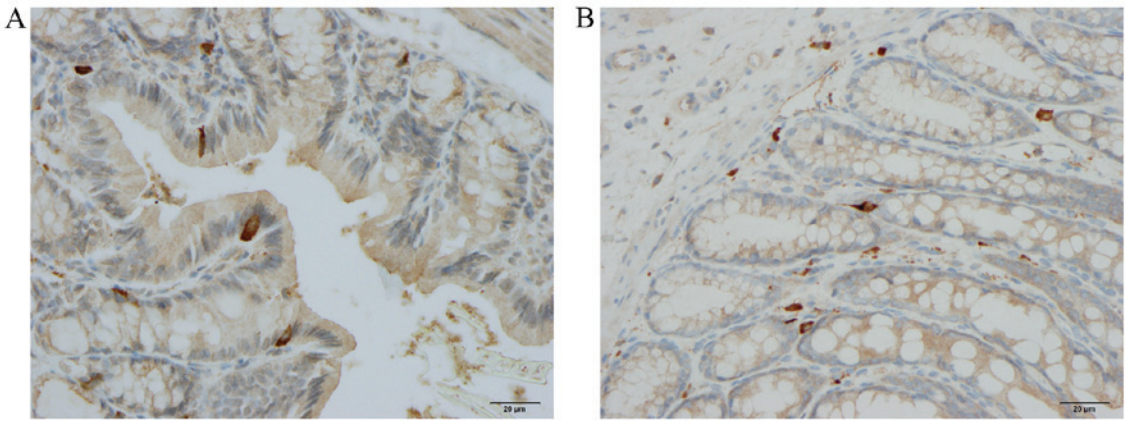

Figure 5. Immunohistochemical staining of peptide YY in the colon tissues derived from (A) a control rat and (B) a rat with trinitrobenzene sulfonic acid-induced colitis.

the TNBS group compared with those of the control group (P<0.0001, P<0.0001 and P=0.0002, respectively; Fig. 3). In contrast, serotonin oxyntomodulin and somatostatin densities were increased in the colon tissues from the TNBS group compared with those of the controls (P<0.0001, P<0.0001 and P=0.01, respectively; Fig. 3).

Immune cells. As presented in Figs. 1 and 6, the densities of all types of immune cells were significantly greater in the TNBS group compared with the control group [leukocytes, 5.9±0.4 vs. 23.3±2.2 cells/field (P<0.0001); B/T lymphocytes, 9.0±0.7 vs. 35.8±2.3 cells/field (P<0.0001); T lymphocytes, 10.5±0.6 vs. 26.6±2.9 cells/field (P<0.0001); B lymphocytes, 9.7±0.4 vs. 27.7±2.6 cells/field (P<0.0001); macrophages/monocytes, 7.6±0.7 vs. 909.0±46.3 cells/field (P<0.0001); and mast cells 5.5±0.5 vs. 27.3±2.9 cells/field (P<0.0001)].

Correlation between endocrine and immune cells. The Spearman correlation coefficients and P-values for the correlations between different endocrine cell types and various immune cells are presented in Table II. The number of CgA, PYY, and PP-producing immune cells was observed to be negatively correlated with the number of specific types of immune cells, whilst positive correlations were observed for serotonin, oxyntomodulin, and somatostatin cells.

Discussion

TNBS-induced colitis in rats closely mimics human CD (34-39). Although this model exhibits clinical and morphological features similar to human CD (39-41), it lacks the chronicity observed in human CD (39). The present study observed that the frequency of all types of colonic endocrine cells was affected in rats with TNBS-induced colitis. In addition, abnormalities in the colonic endocrine cells were strongly correlated with the alterations in the number of different types of immune cells following the induction of colitis. These observations support the previously suggested

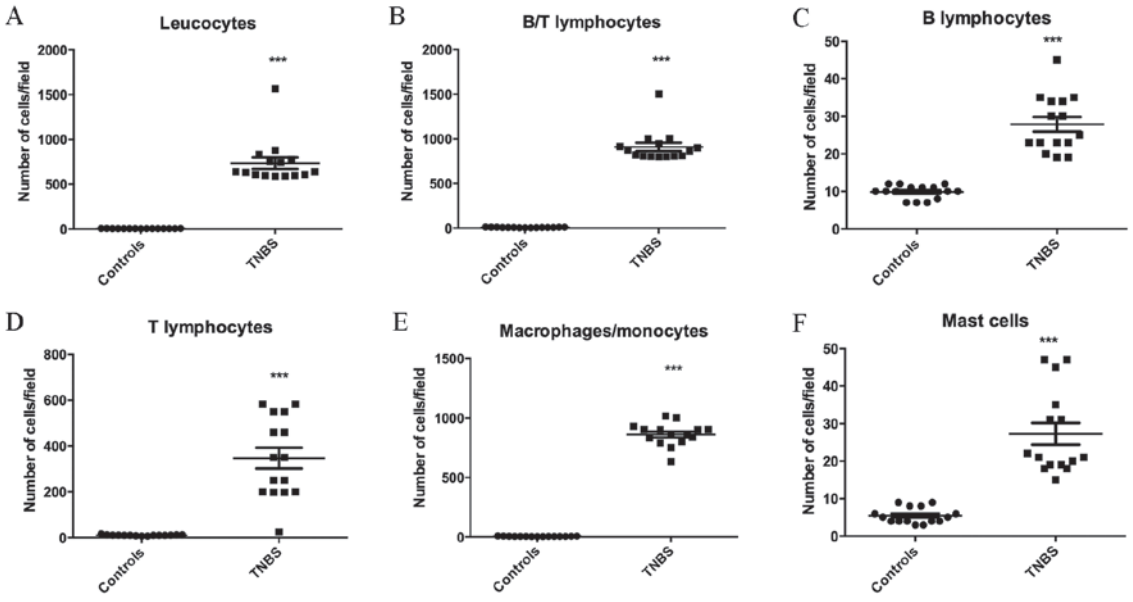

Figure 6. Densities of (A) leucocytes, (B) B/T lymphocytes, (C) B lymphocytes, (D) T lymphocytes, (E) macrophages/monocytes and (F) mast cells in the lamina propria of the colon derived from normal control rats and rats with TNBS-induced colitis. Data are presented as the mean ± standard error. ***P<0.001 vs. controls. TNBS, trinitrobenzene sulfonic acid.

role of gut hormones in immune activation and inflammation (21,22,42).

In the present study, the alterations in the number of colonic endocrine cells observed in rats with TNBS-induced colitis differ from those observed in rats with DSS-induced colitis in a previous study (31). In TNBS and DSS-induced colitis, the densities of serotonin and oxyntomodulin were increased, while the density of PP was reduced compared with normal controls. However, the CgA and PYY-producing immune cell densities were increased in DSS-induced colitis, whereas they were reduced in TNBS-induced colitis compared with normal controls. In addition, the density of somatostatin was reduced in DSS-induced colitis (31), however was increased in TNBS-induced colitis in the present study. Differences in the alterations of the number of colonic endocrine cells between CD and UC have been reported previously by El Salhy *et al* (1). This study demonstrated that the densities of CgA and serotonin were increased in CD and UC, while the densities of PYY and PP were reduced, and oxyntomodulin was decreased in CD only.

Although the abnormalities in the colonic endocrine cells in DSS-induced colitis were strongly correlated with leukocytes, B lymphocytes, T lymphocytes, macrophages/monocytes and mast cells (31), the results for TNBS-induced colitis in the present study demonstrated that specific endocrine cell types were correlated with particular immune cell types. CgA is a member of the granin family (43,44), which is localized to gut endocrine cells (45-48), and is considered to be a common marker for gastrointestinal endocrine cells (49,50). The reduction in the density of CgA-producing cells observed in the present study may reflect reductions in the densities of all colonic endocrine cells following the induction of colitis by TNBS. The density of CgA-producing cells was negatively correlated with increases in all immune cell types except for B/T lymphocytes. CgA suppresses the release of interleukin (IL)-16 and IL-5, and consequently reduces the number of lymphocytes at sites of inflammation, and reduces the pro-inflammatory actions of lymphocytes and monocytes (51-53). In addition, CgA, inhibits the vascular leakage caused by tumor necrosis factor-α (54). CgA is generally considered to exert an anti-inflammatory effect (54). It can therefore be speculated that the reduced density of CgA results from a direct action exerted by immune cells.

In the present study, the observed increase in the density of colonic serotonin-producing cells in TNBS-induced colitis relative to controls is consistent with previous observations in patients with UC, CD and microscopic colitis, as well as animal models of colitis (1,3,55-57). The increased density of serotonin-producing cells in the present study was correlated with increases in the number of all types of immune cells examined, apart from macrophages/monocytes and mast cells. Lymphocytes, macrophages, and dendritic cells express serotonin receptors (58), and IL-13 receptors have been localized on serotonin cells (59). In addition, serotonin inhibits the apoptosis of immune cells, promotes the recruitment of T cells, affects the proliferation of lymphocytes and protects natural killer cells (60-63). Furthermore, a previous study demonstrated that there are fewer serotonin-producing cells in mice lacking T-lymphocyte receptors (51). Serotonin stimulates gastric and intestinal motility, and intestinal secretion (64,65). Therefore, the increase in serotonin may accelerate gastrointestinal motility and increase intestinal secretion thus resulting in diarrhea, which is the primary symptom in TNBS-induced colitis.

PYY is colocalized with oxyntomodulin in endocrine L cells (66,67). In the present study, the density of PYY reduced while oxyntomodulin increased, which indicates that L cells downregulate the expression of PYY, but upregulate the expression of oxyntomodulin in TNBS-induced colitis in rats. PYY stimulates the adhesion of macrophages, chemotaxis, phagocytosis and the production of superoxide anions (68).

PYY mRNA has been detected in mouse macrophages (69). The precise interaction between oxyntomodulin and immune cells has not yet been determined. In the present study, the density of PYY cells was negatively correlated with increases in the number of B/T lymphocytes, B lymphocytes and macrophages/monocytes, whereas oxyntomodulin density was positively correlated with B/T lymphocytes, T lymphocytes, and mast cells. These results indicate the presence different interactions of PYY and oxyntomodulin with immune cells. PYY delays gastric emptying, is a pivotal mediator of the ileal brake, and stimulates the absorption of water and electrolytes (70). The reduction in the number of PYY-producing cells observed in the current study may have contributed to the acceleration of gastrointestinal motility and increased intestinal secretion observed in TNBS-induced colitis.

The reduction in the number of PP-producing cells observed in the current study is consistent with previous reports for UC and CD (1). However, the interaction between PP and immune cells remains to be fully elucidated. In the present study, PP density was negatively correlated with the number of B lymphocytes, macrophages/monocytes and mast cells. PP stimulates gastric acid secretion and the motility of the stomach and small intestine in addition to relaxing the gallbladder (64). The increased density of somatostatin cells in TNBS-induced colitis observed in the present study contradicts previous observations of UC, CD and DSS-induced colitis, where somatostatin cell density was reported to reduce (1,19,20). Somatostatin inhibits lymphocyte proliferation, immunoglobulin synthesis and the release of neutrophil elastase (71-75). In the present study, the density of somatostatin-producing cells was observed to be positively correlated with the number of macrophages/monocytes and mast cells. The strong correlation observed between alterations in PP and somatostatin-producing cell densities and specific immune cell types, indicate that they may be involved in the inflammatory process.

The present observations, demonstrating that alterations in the number of immune cells are strongly correlated with alterations in large intestinal cells in an animal model of human of Crohn's disease, support the debated suggestion of an interaction between intestinal hormones and the gut immune system. Understanding this interaction may improve our understanding of the pathophysiological mechanisms involved in IBD, and may provide us with novel therapeutic approaches to treat this condition.

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