Significance of increased proliferation of immature plasma cells in the appendix of patients with ulcerative colitis

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Abstract. The etiology of ulcerative colitis (UC) is not known. Recent studies support a primary role of the appendix in the pathogenesis of UC, however phenotypical studies of proliferating cells in the appendix have not been reported. We report phenotypical studies of lymphocytes and of proliferating subpopulations in the appendix of patients with inflammatory bowel disease and of controls. Surgical samples of the appendix were obtained from 5 patients with colon cancer, 5 with acute appendicitis, 12 with UC and 7 with Crohn's disease (CD). Frozen sections were cut from fixed samples, and immunostained with lymphocyte markers and anti-Ki-67 antibodies. The number of Ki-67$^+$ proliferating cells, CD19, and CD138 cells was significantly higher in the appendix of patients with UC than in controls, patients with acute appendicitis, and patients with CD. Immunohistological double staining revealed significant proliferation of CD3, CD19, and CD138 cells in the appendix of patients with UC. The proportions of Ki-67$^+$ cells in CD3, CD19, and CD138 cells were significantly higher in both total UC patients and patients in remission-stage UC, than in controls, patients with acute appendicitis, and patients with CD. Lamina propria cells in the appendix of patients with UC showed augmented proliferation with increased numbers of CD19 and CD138 cells. The number of CD3 cells was not significantly increased, but the proportion of proliferating CD3 cells was increased. An increased proportion of Ki-67$^+$ cells in CD19 and CD138 cells represents proliferation of immature plasma cells in the appendix of patients with UC, and proliferation of such immature plasma cells was seen in both active- and remission-stage UC. Proliferation of immature plasma cells in the appendix of patients with UC suggests a primary role of humoral immune responses in the pathogenesis of UC.

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Introduction

The appendix has long been considered a redundant organ (1), because its precise function is not known, but an immunological role of the appendix is suspected. Although the pathogenesis of ulcerative colitis (UC) remains unknown (2), two recent observations have focused attention on the role of the appendix, as it contains gut-associated lymphoid tissue (GALT), including Peyer's patches. First, appendiceal inflammation as a discontinuous lesion has been reported by endoscopic study in patients with UC (3-5). Second, recent epidemiological investigation has revealed that appendectomy reduces the risk of UC (6-8). In an animal model of young T cell receptor (TCR)-deficient mice, removal of the appendix either protected against or improved experimental colitis (9). In addition, recent case-control studies suggest that previous appendectomy is rare in patients with UC and is associated with a less severe course of the disease (10-12). Moreover, several case reports suggest that appendectomy after the onset of UC could be a possible therapeutic option (13-15).

We have conducted phenotypical studies of lymphocytes using a panel of antibodies, and evaluated the proliferating activity of infiltrating cells in the lamina propria of the appendix from patients with UC, Crohn's disease (CD), or acute appendicitis, and normal appendix from patients with colon cancer, using antibodies against Ki-67, one of the representative cell proliferation-associated nuclear antigens (16). We have further analyzed the proliferative activity of lymphocyte subsets by immunohistological double staining with various lymphoid cell markers and anti-Ki-67 antibodies. This morphometric analysis based on an immunohistological method was introduced by Saiki *et al* in a study on immune responses in Epstein-Barr virus-associated gastric cancer (17). In inflammatory bowel disease (IBD), Ki-67 has been used to assess the proliferative activity of epithelial cells in dysplasia (18,19). Fell *et al* applied immunohistological double staining of Ki-67

Table I. Clinical backgrounds.

	Control	Acute appendicitis	Ulcerative colitis	Crohn's disease
Age (years)				
Median (range)	59 (40-71)	33 (24-52)	34 (18-62)	31 (28-38)
Sex				
Male/female	2/3	3/2	7/5	6/1
Location of the disease				
Ileitis				2
Colitis				1
Ileocolitis				4
Emergency/elective	0/5	5/0	4/8	0/7
Total	5	5	12	7

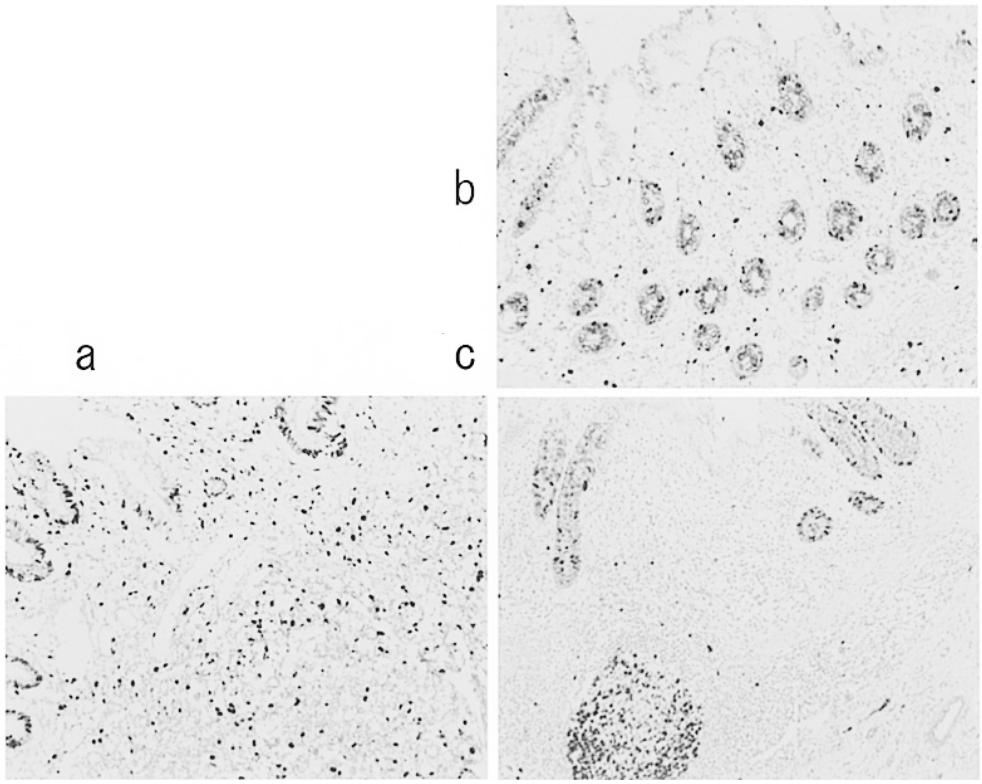

Figure 1. Immunohistological staining with anti-Ki-67 antibody in a patient with UC (a), CD (b), and control (c). Ki-67-positive cells are shown in brown products, x100.

and lymphoid cell markers to the analysis of lamina propria lymphocytes (LPL) in IBD, without referring to B cells (20). Jo *et al* reported histological and immunological characteristics of the appendix of patients with UC with T cell markers (21).

Here, we investigated histological and immunological characteristics of the appendix of patients with UC, and revealed that Ki-67 labeling in lymphoid cells is a predominant feature of UC, particularly in a subset of plasma cells in the lamina propria, both in clinically active and inactive stages of UC.

Materials amd methods

Tissue. Appendiceal tissue specimens were obtained during surgical operations performed on 12 patients with UC, 7 with CD, 5 with acute appendicitis, and 5 with colon cancer who underwent ileocecal resection. All UC patients had total colitis and were treated with corticosteroids. The average cumulative prednisolone dose at the time of surgery was 13,237 mg (420-30,000 mg), and average recent prednisolone dose was 36 mg per day (5-80) for patients with UC. Four patients with UC

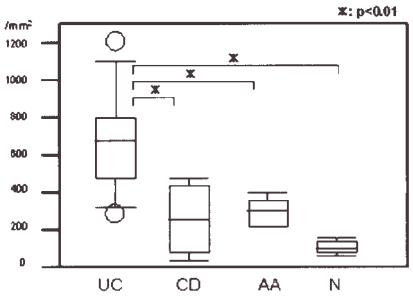

Figure 2. The number of Ki-67-positive cells in lamina proplia of appendix. The number of Ki-67-positive cells is higher in patients with UC than patients with CD, acute appendicitis and normal controls. Boxes show the median with the 25th and 75th percentiles (lines at ends). Bars show the 10th and 90th percentiles. Circles show data points below the 10th percentile and above the 90th percentile.

underwent emergency surgery and the remaining 8 underwent elective surgery. Patients with CD included 2 with ileitis, one with colitis, and 4 with ileocolitis. For preoperative treatment, all patients with CD received total parenteral nutrition (22), with only one patient receiving corticosteroid therapy. All patients with acute appendicitis were taking antibiotics before appendectomy. For control tissue, normal appendixes were obtained from five patients with colon cancer (Table I).

Immunohistochemistry. Immediately after surgical resection, fresh tissue samples were cut into small pieces and fixed in periodate-lysine-2% paraformaldehyde (PLP) at 4˚C for 6 h, followed by sequential washing in phosphate-buffered saline (PBS) containing 10, 15, and 20% sucrose for 4 h in each step. The specimens were embedded in O.C.T. compound (Miles, Elkhart, Indiana), rapidly frozen in dry-ice-acetone and stored at -80˚C until use. Cryostat sections, 5 μm thick, were mounted on glass slides and air-dried. Sections were pretreated with 2% non-immune goat serum to inhibit non-specific protein binding, and incubated with the primary antibodies for 24 h at 4˚C in a moist chamber. Endogenous peroxidase activity was inactivated by treatment with PBS containing 0.3% hydrogen peroxide and 0.1% sodium azide for 15 min. After washing with PBS, sections were conjugated to peroxidase-labeled dextran polymer (Envision staining kit; Dako Japan, Kyoto, Japan). Peroxidase-substrate reaction was developed with 0.03% 3',3-diaminobenzidine tetrahydrochloride (DAB) (Dojin, Kumamoto, Japan) containing 0.006% hydrogen peroxide and 0.065% sodium azide Tris-buffered saline. Primary antibodies used were mouse monoclonal antibodies against CD3 (clone UCTH1, applied at 1:200, Immunotech, Marseille, France), CD19 (clone J4.119, 1:200, Immunotech), CD20 (clone B-Ly1, 1:200, Dako, CA, USA), CD138 (clone MI15, 1:200, Dako) and Ki-67 (clone MIB1, 1:50, Dako). For Ki-67 staining, the sections were heat-treated in 0.1 M citrate buffer (pH 6.0) at 97˚C for 15 min for antigen retrieval. The specificity of immunoreactivity was confirmed by replacing the primary antibodies with isotype-matched control antibodies at the same concentration (Dako), and sections were counterstained with methyl green.

Morphometric analysis of Ki-67⁺ cells. We counted the number of Ki-67⁺ mononuclear cells in a unit area of 0.0625 mm² that corresponded to one microscopic grid area in a x400 field. Cells were counted for 5 fields in each specimen and expressed as number per mm². Ki-67 expression in germinal centers and in epithelial cells was carefully excluded. The areas measured were the appendiceal mucosa of the appendix of patients with UC (n=12), CD (n=7), acute appendicitis (n=5), and of normal appendix (n=5).

Immunohistological double staining of Ki-67 and lymphocyte subpopulations (enzyme-linked method). We performed immunohistochemical double staining for CD3, 19, 20, or 138, and Ki-67 with minor modification of Saiki *et al* (17). First, sections were processed for CD3, 19, 20, or 138 with DAB as a chromogen, using EnVision kit (Dako). Slides were incubated with 0.1 N hydrochloric acid for 2 h and immersed in citrate buffer in plastic jars and heat-treated as above. After the heat-mediated antigen retrieval of Ki-67 antigen, sections were stained with anti-Ki-67, with True Blue (Kirkegard & Perry Laboratories, Gaithersburg, MD, USA) as a chromogen. The labeling index was defined as the percentage of double-positive cells for Ki67⁺ and each lymphocyte marker among the total numbers of corresponding lymphocytes in a unit area. The unit area was the same as the number of Ki67-positive mononuclear cells, and the areas we measured were the same as above. Areas with germinal centers were excluded in these analyses.

The clinical activity of UC. The activity index of UC was calculated according to a modified disease activity index. The parameters involved were stool frequency, rectal bleeding, mucosal appearance and disease activity, rated by the physician (23).

Statistical analysis. Results are expressed as medians (25th, 75th percentiles), and the differences in the number of Ki-67⁺ mononuclear cells among these groups were tested by the Kruskal-Wallis test and Mann-Whitney U test. The correlation between the labeling index and the proliferative activity of lymphocytes was tested by the Spearman's rank test. P-values <0.05 were considered significant.

This study was approved by the University Ethics Committee, and informed consent was obtained from patients and controls.

Results

Single immunohistological staining revealed that Ki-67⁺ mononuclear cells were abundant in the lamina propria of the appendix of patients with UC (Fig. 1a), and did not usually form a follicular pattern. Ki-67⁺ mononuclear cells were scattered in the appendix of patients with CD (Fig. 1b), as well as in those with acute appendicitis. In normal controls, the Ki-67⁺ mononuclear cells were mostly confined to germinal centers, which are typical areas of B cell proliferation (Fig. 1c). The number of Ki-67⁺ cells per mm² in the lamina propria of appendixes was 670 (508, 812) in patients with UC, 243 (72, 424) in patients with CD, 288 (224, 328) in patients with acute appendicitis, and 115 (88, 140) in normal controls (Fig. 2). The number of Ki-67⁺ cells was significantly higher in patients with UC than in controls (p=0.002), patients with

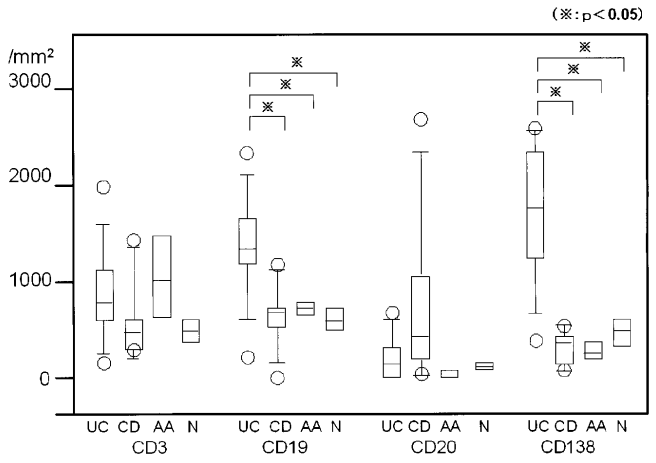

Figure 3. The number of CD3, CD19, CD20 and CD138 positive cells in lamina propria of appendix. The number of CD19 or CD138 positive cells is higher in patients with UC than patients with CD, acute appendicitis and normal controls.

CD (p=0.008), and patients with acute appendicitis (p=0.013). There was no positive correlation between the number of Ki-67$^+$ cells in the appendix of patients with UC and the clinical activity of UC at the time of the operation.

Quantitative studies of LPL showed that the number of CD19 and CD138 cells was significantly higher in the lamina propria of the appendixes of patients with UC than in those of controls (p=0.011 and p=0.004, respectively), patients with acute appendicitis (p=0.043 and p=0.004, respectively), and patients with CD (p=0.005 and p=0.038, respectively; Fig. 3). The number of CD3 and CD20 cells in the lamina propria was not significantly different in the appendixes of controls, patients with acute appendicitis, UC, and CD.

Immunohistological double staining showed that some CD3 positive cells expressed Ki-67, while CD20 positive cells hardly expressed Ki-67, and a number of CD19 positive and CD138 positive cells expressed Ki-67 antigen in the appendix of a patient with UC (Fig. 4a-d). Ki-67 labeling indices in CD19$^+$ and CD138$^+$ cells were significantly higher in patients with UC than in controls (p=0.013 and p=0.004, respectively), patients with acute appendicitis (p=0.025 and p=0.004, respectively), and patients with CD (p=0.006 and p=0.001, respectively). The Ki-67 labeling index in CD3$^+$ cells was higher in patients with UC than in controls (p=0.007) and patients with acute appendicitis (p=0.029). Ki-67 labeling indices of CD20$^+$ cells were very low in all groups (Fig. 5).

We next searched for possible clinical implications of our findings. There was significant positive correlation between the labeling index of Ki-67 among CD138$^+$ cells in the appendix of patients with UC and the clinical activity of UC at the time of the operation (Fig. 6).

In the UC group, all 8 patients who underwent elective surgery were in clinical remission at the time of operation, with a clinical activity index of UC of 2.0 (1.0, 3.0). We compared the labeling index of Ki-67 in CD3$^+$, 19$^+$, 20$^+$, and 138$^+$ cells between patients with UC in remission stage to that of the other groups. The labeling indices of CD19$^+$ and CD138$^+$ cells were significantly higher in patients with UC in remission

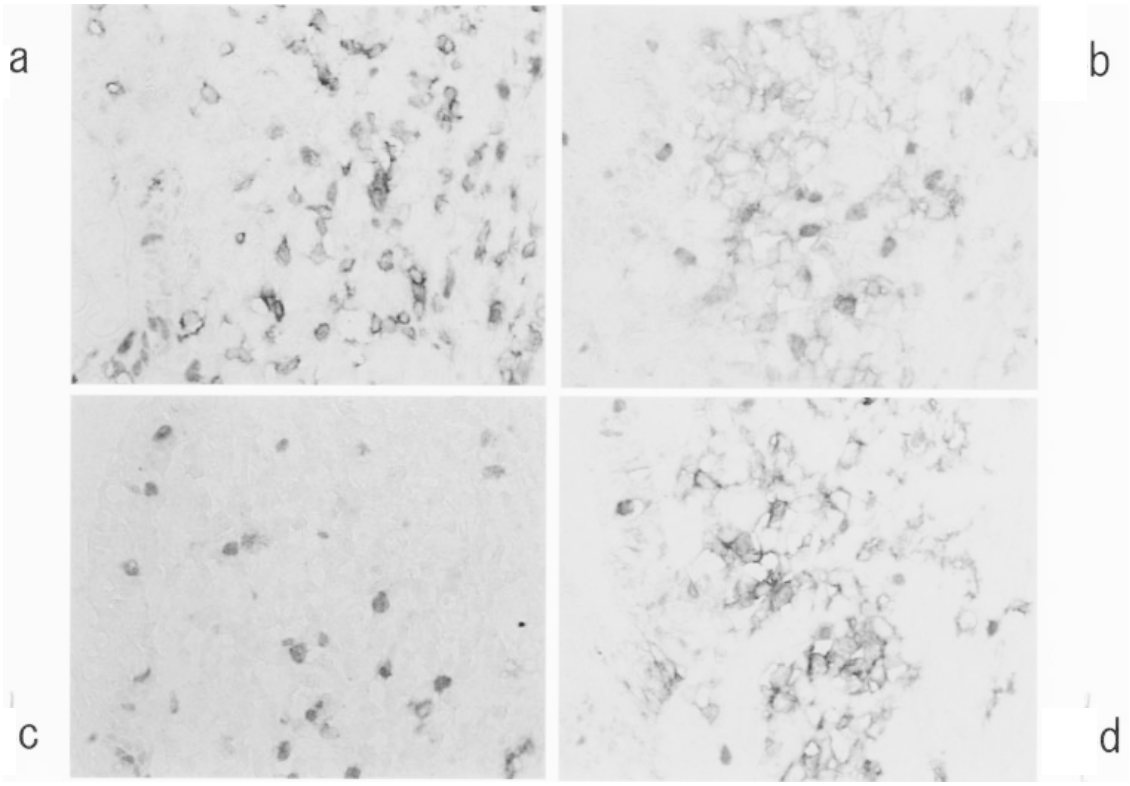

Figure 4. Immunohistological double staining with lymphoid cell markers (brown) and anti-Ki-67 antibody (blue) in a patient with UC. Arrowheads show double positive cells x400. (a) CD3/Ki-67, (b) CD19/Ki-67, (c) CD20/Ki-67, (d) CD138/Ki-67.

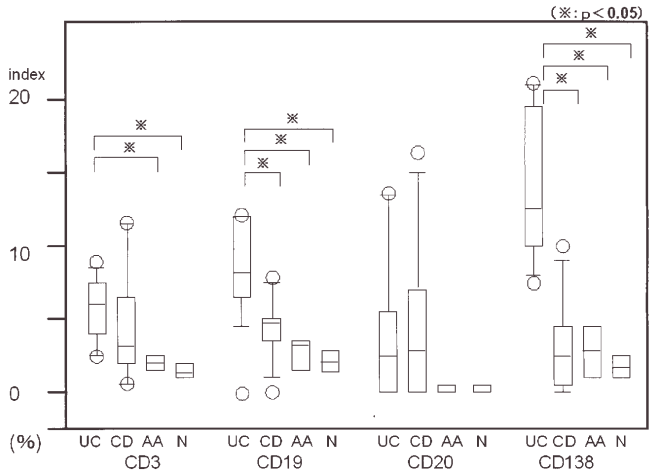

Figure 5. Ki-67 labeling index in CD3, CD19, CD20 and CD138 positive cells.

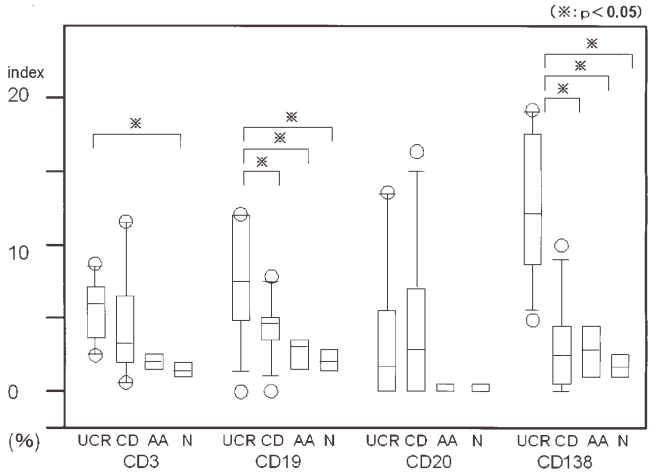

Figure 7. Ki-67 labeling index in CD3, CD19, CD20 and CD138 positive cells in patients with UC in remission stage (UCR) and the other.

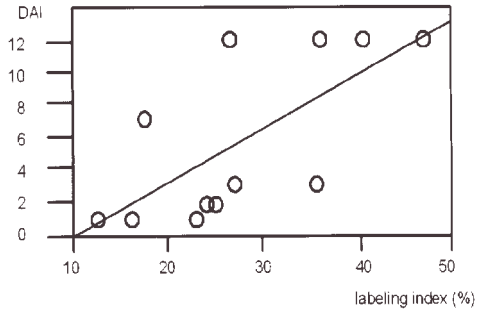

Figure 6. The relation between the labeling index of Ki-67 among CD138$^+$ cells in the appendix of UC and the clinical activity of UC at the time of operation. There was a significant positive correlation. (y=0.324x - 3.219, r^2=0.473, p=0.012).

stage than in patients with CD (p=0.024 and p=0.004, respectively), acute appendicitis (p=0.034 and p=0.007, respectively) or normal controls (p=0.034 and p=0.007, respectively) (Fig. 7). In addition, the labeling index of Ki-67 in CD3$^+$ cells was higher in patients with UC in remission stage than normal controls (p=0.017).

Discussion

This is the first report to show that the appendix of patients with UC is characterized by abundant Ki-67$^+$ mononuclear cells, irrespective of the clinical UC activity. Quantitative study showed significantly higher numbers of CD19 and CD138 cells in the appendix of patients with UC, than in the appendixes of controls, patients with acute appendicitis, and patients with CD, while the number of CD3 and CD20 cells did not significantly differ.

Immunohistological double staining showed increased numbers of CD3$^+$Ki-67$^+$, CD19$^+$Ki-67$^+$, CD20$^+$Ki-67$^+$, and CD138$^+$Ki-67$^+$ cells in the appendix of patients with UC compared with controls, patients with acute appendicitis, and patients with CD, which indicates involvement of both T and B cell lineage proliferation in the appendix of patients with UC. Differences were seen between the quantitative study and the study of proliferating subpopulations for CD3 cells, as the number of CD3 cells did not differ between the groups, but the proportion of proliferating cells was significantly higher in the appendix of patients with UC than in controls, patients with acute appendicitis, and patients with CD. Such discrepancy may reflect rapid turnover of CD3 cells, rather than B cells, in the appendix of patients with UC. Furthermore, the labeling indices of Ki-67 in CD19$^+$ and CD138$^+$ cells in patients with UC, even in remission stage, were significantly higher than those in the other groups examined. Memory B cells expressing CD19 in peripheral lymphoid organs are activated by the antigen and differentiate into short-lived proliferating plasma blasts that express CD19$^+$, CD20^{low}, CD138$^+$ phenotype. Then plasma blasts migrate into bone marrow or into the lamina propria of the mucosa where they become plasma cells and secrete abundant immunoglobulins (24-26). Such recruitment of plasma cells in the lamina propria is highly consistent with the classical pathological finding of basal plasmacytosis seen in the mucosa of patients with UC. Proliferation of CD19$^+$ and CD138$^+$ cells may suggest proliferation of memory B cells into plasma blasts, which occurred in the appendix of patients with UC in remission stage. Proliferation of plasma blasts in the appendix of patients with UC, irrespective of the activity of colitis, suggests an essential role of humoral immunity in the pathogenesis of UC.

Etiological studies raised the possibility of a protective effect of appendectomy in the development of UC (7,8,10). Discontinuous inflammation in the appendix (skip lesion) has been reported in patients with UC (3-5), while UC is typically characterized by continuous and diffuse inflammation from the rectum. Radford-Smith *et al* (12) reported that patients with UC who had undergone appendectomy had clinically milder disease with a reduced requirement for immunosuppressants and colectomy. Furthermore, Naganuma *et al* (15) revealed that appendectomy reduces the extent and recurrence of established UC. However, the effect of appendectomy on established UC may be transient (21). Therefore, the role of the appendix in the pathogenesis of UC remains rather complicated.

Despite the many clinical and pathological reports on the appendix of patients with IBD, immunological events in such appendixes are not well documented. Only Jo *et al* (21) reported histological and immunological characteristics of the appendix of patients with UC, with T cell markers, CD25 as an activation marker and CD45RO as a memory marker in T-lymphocytes within the appendiceal mucosa, and showed that the LPL of the appendix of patients with UC contains significantly higher numbers of activated memory T cells, both in helper/inducer and suppressor/cytotoxic subsets, than the appendix of patients with acute appendicitis and of controls. This is the first study to show proliferation of $CD19^+$ and $CD138^+$ cells in the lamina propria of the appendix of patients with UC, with this proliferation characteristically found in such patients. Together with analyses of CD138, we have previously found abundant infiltration of the ulcer base and inflamed mucosa of UC by $CD19^+$, $CD20^-$, $CD138^\pm$ plasma cells that expressed a higher labeling of Ki-67 (data not shown). This change is considered specific for UC, since most $CD138^+$ plasma cells in patients with CD lacked CD19 expression, retaining the pattern of mature plasma cells. Since CD19 is still positive in early plasma cells (plasma blasts) (24-27), our findings herein suggest that the $CD19^+$, $CD20^-$, $CD138^\pm$ plasma cells in UC include a considerable number of immature plasma cells. These data indicate that LPLs of the appendix of patients with UC are characterized by proliferating immature plasma cells.

Th1/Th2 imbalance causes various immunological diseases. Increased numbers of IFN-*r*-producing $CD4^+$ T cells and IL-12 producing macrophages in an inflamed intestine (28,29) have been reported in Th1-dominant Crohn's disease (30). In contrast, dramatic B cell response (31,32), comprehensive messenger RNA analysis of cytokines (33), and increased production of IL5 by CD4 T cells in the diseased intestine (34), strongly suggest Th2 predominance in the pathogenesis of UC. Generation of autoantibodies against tropomyosin (35) and perinuclear anti-neutrophil antibodies (p-ANCA) (36) is a characteristic humoral immune response in patients with UC. B cell clones spontaneously producing p-ANCA were isolated from colonic LPL of patients with UC, but peripheral blood and mesenteric lymph node B cells lacked spontaneous p-ANCA production (36). The number of total IgG-producing B cells was significantly higher in UC and CD colonic mucosa than controls. However, the number of LPL B cells producing anti-tropomyosin isoform 5 antibodies was significantly higher in UC than in CD and controls (35). Differences in antibody production in colonic LPL in patients with UC suggest the existence of abnormal humoral immune responses in mucosal B cells of UC. The existence of a specific antigen that induces memory B cell differentiation and plasma blast proliferation in the appendix of patients with UC is doubtful. Our data from intestinal HLA-DR-bound antigenic peptides, suggest stimulation of B cells by various antigens rather than a specific antigen (37). It is very interesting that increased proliferation of CD3, CD19 and CD138 positive cells was observed in the appendix of patients with UC, especially in patients with inactive colitis. Possibly those T and B cells that arise from the appendix of patients with UC are recruited to colonic LPL where they begin to produce various cytokines and antibodies causing pathological reactions in the UC mucosa.

In conclusion, proliferation of T cells and immature plasma cells in the appendix of patients with UC, not only in clinically active but also in remission stage, strongly suggests that the appendix has a primary role in the pathogenesis of UC.

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