

A Study of Fine Yellowish-White Plaques in Mucosa of Ulcerative Colitis Using Immunoperoxidase Staining: A Comparison with Reddish Lesions

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Fine yellowish-white plaques (YWP), which are seen in the bowel mucosa of patients with ulcerative colitis (UC), are minute erosive lesions where infiltration of inflammatory cells are histologically observed. These lesions are thought to constitute the minimum unit of inflammation. To clarify the factors that determine the activity level of UC, we biopsied YWP, which persisted in the inactive stage of the disease, as well as reddish inflamed lesions (RL) in the active stage of the disease, and performed immunoperoxidase staining of intraepithelial cells and lamina propria mononuclear cells by using monoclonal antibodies against various cell markers.

Both RL, and YWP showed activation of T cells, B cells and macrophages, compared with the normal mucosa. No decrease in TCR $\gamma\delta$ type T cells was seen in YWP or RL. An increase in CD8 positive T cells was noted in YWP. The cell densities of HLA-DR positive cells was higher in RL than in YWP. As HLA-DR positive cells can act as antigen-presenting cells, this finding may be relevant in determining the activity level of UC.

Key words : Fine Yellowish-White Plaques in Mucosa, Ulcerative Colitis, Immunoperoxidase Staining, Reddish Lesions

Introduction

Using a combination of magnifying endoscopy and dye-staining techniques, we have found that fine yellowish-white plaques (YWP) are often detected in the border between the slightly inflamed bowel mucosa and the intact mucosa of patients with ulcerative colitis (UC). YWP are minute erosive lesions and are histologically found to contain invading inflammatory cells. We speculated that YWP constitute the minimum unit of inflammation in cases of UC¹⁾. YWP continue to be seen even when UC has entered an inactive phase. Further assessment of YWP may provide insight into the etiology of UC.

Although the etiology of UC remains unknown, abnormal immune responses are thought to have an important role. Assessment of different aspects of immune function

in UC is the focus of much current research. In UC, it is possible that abnormal immune responses only occur within localized areas of the colon. To clarify the etiology of this disease, it may be therefore necessary to analyze inflammatory cells which can invade the mucosa of the large intestine affected by UC.

In our previous studies, we isolated lamina propria mononuclear cells (LPMC) from the large intestinal mucosa of untreated patients, and analyzed cell subsets by two color flow cytometry^{2),3)}. A limitation of enzymatic digestion as a method of isolating LPMC is that this treatment can cause disruption of the mucosal epithelium, and can alter the surface antigens of the isolated cells.

In the present study, in order to avoid the limitations of enzymatic cell isolation, we used a different approach to the study of cell surface antigens. In this study we carried out OCT treatment of the tissue specimens immediately after biopsy. To clarify factors determining the activity level of UC, we biopsied YWP and RL endoscopically, and performed immunoperoxidase staining of intraepithelial cells (IEC) and LPMC by using monoclonal antibodies against various cell markers. We compared the immunohistochemical findings of the lesions in UC with normal bowel mucosa.

Materials and Methods

1. Subjects

Biopsies were taken from 13 patients with untreated or recurrent UC who were admitted to the Nagasaki University Hospital between 1991 and 1992. (7 men and 6 women, a mean age of 40.1 years.) UC was clinically active in 7 cases and inactive in 6 cases. All patients underwent colonoscopy. Mucosal lesions characterized by mucosal erythema and by loss of vascular translucency (reddish lesions) were classed as "active lesions" (Fig. 1-a). In total, 25 lesions were classed as active lesions. Lesions characterized by YWP, usually located at the border of

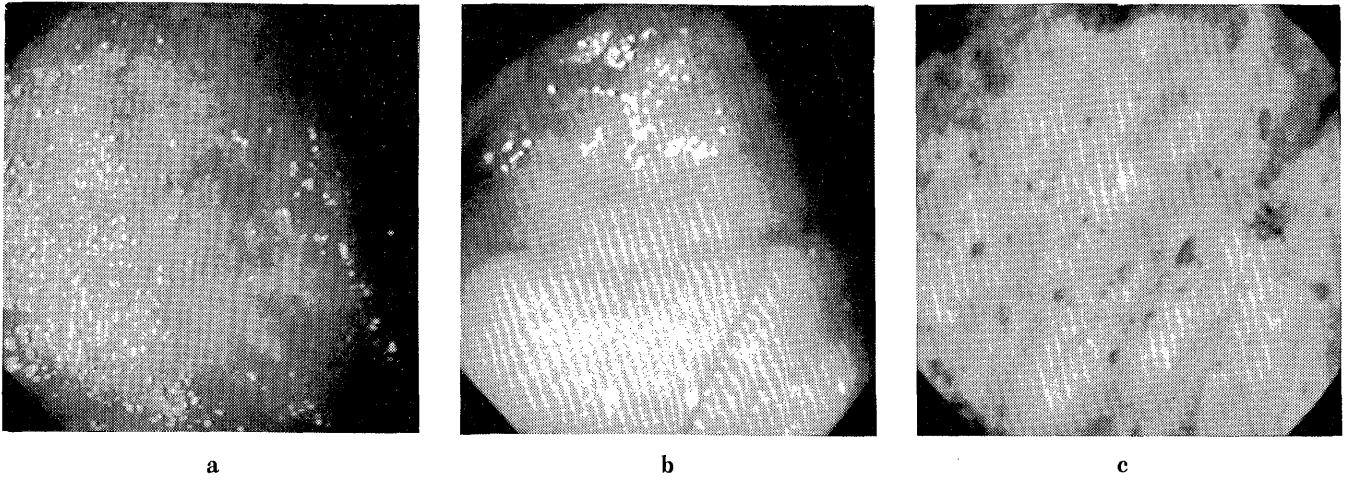


Fig. 1. Endoscopic appearance of UC. a; RL, b; YWP, c; YWP (after methylene blue staining)

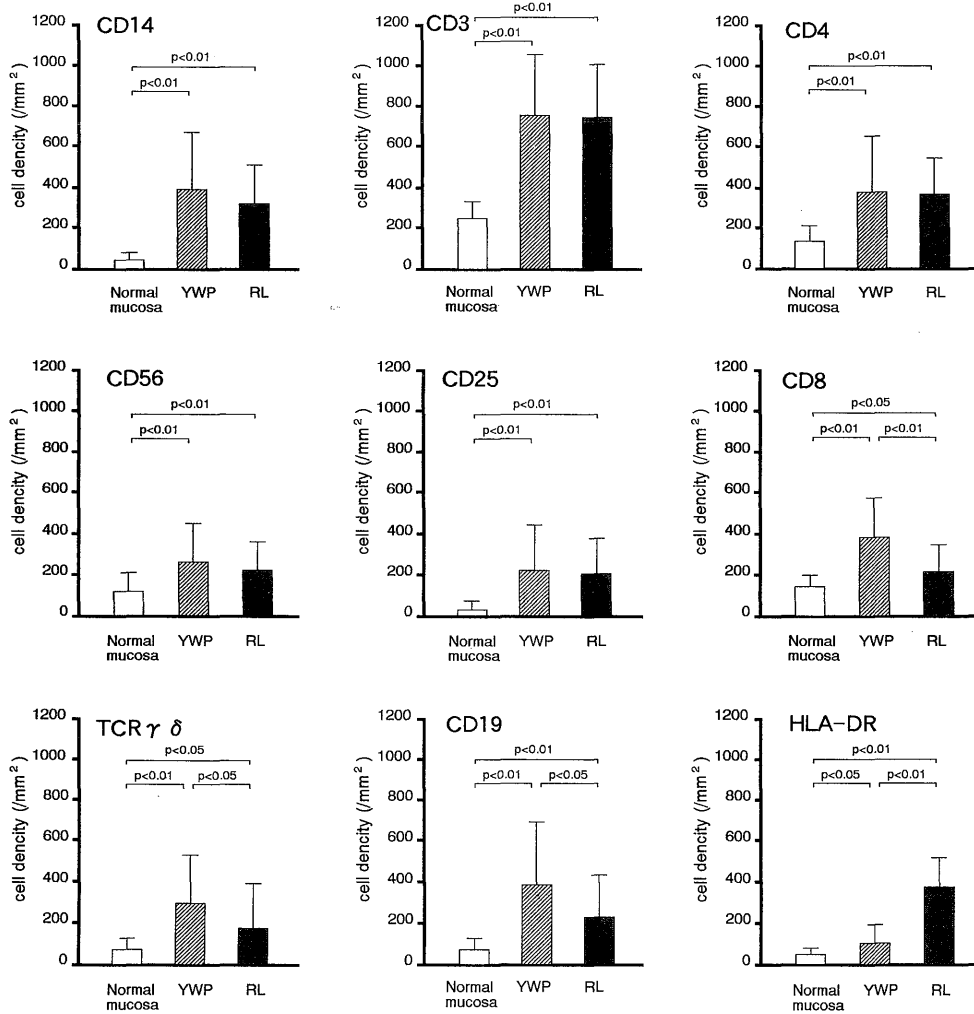


Fig. 2. Densities of immunoreactive cells in mucosa of UC.

normal and inflamed mucosa were classed as "inactive lesions" (Fig. 1-b, c). The classification of a mucosal lesion as "inactive" or "active" was made irrespective of the clinical status of the patient. In total, 60 inactive lesions were biopsied. Twenty mucosal biopsies, collected from 10 patients who had no macroscopic abnormalities, on colorectal endoscopy, served as normal controls.

On average, 2.5 biopsies were taken from each affected area under endoscopic guidance. These specimens were immediately treated with OCT and then stored at -80°C .

2. Immunoperoxidase staining

Frozen specimens were sliced at a thickness of $5\ \mu\text{m}$. The sections were then fixed in acetone at 4°C for 5 min. They were subsequently washed three times in phosphate buffered saline (PBS) for 5 min each time. The specimens were incubated with $40\ \mu\text{l}$ of the primary antibody overnight at 4°C . The specimens were washed in PBS three times (for 5 min each time). Forty μl of secondary antibody (biotinylated anti-mouse IgG, $15\ \mu\text{g}/\text{ml}$; Vector, Burlingame, USA) was then applied. The specimens were then incubated at room temperature for 30 min. The sections were then washed in PBS for one min, and incubated at room temperature for 30 min in the presence of $40\ \mu\text{l}$ of horseradish peroxidase-conjugated avidin D ($5\ \mu\text{g}/\text{ml}$) (Vector, Burlingame, USA). After the specimens were washed twice in PBS (for one min each time), they were immersed in $40\ \mu\text{l}$ of DAB ($0.2\ \text{mg}/\text{ml}$) and 0.5% aqueous solution of hydrogen peroxide for 1-2 min. After this incubation, the specimens were washed twice in PBS (for 5 min each time), and then in distilled water ten times (for 3 min each time). The specimens were then stained with hematoxylin. After discoloration with ethanol, the specimens were cleared with xylene and mounted.

In the present study, we used the following 9 antibodies (products of Becton Dickinson, California, USA) as primary antibodies: Leu4 (anti-CD3), Leu3a (anti-CD4), Leu2a (anti-CD8), Leu12 (anti-CD19), LeuM23 (anti-CD14), Leu19 (anti-CD56), 2A3 (anti-CD25, i.e. anti-IL-2R), L243 (anti-HLA-DR) and 11F2 (anti-TCR $\gamma\delta$). Of these antibodies, Leu 4, Leu19 and 2A3 were diluted with PBS at a ratio of 1 : 12.5, and the other 6 antibodies were diluted with PBS at a ratio of 1 : 25. Tonsillar tissues were also stained as controls. The stained sections were examined at a magnification of $\times 200$, and a 100 square grid at last 5 visual fields were counted⁹. The numbers of positively staining cells were expressed as the number of positively-staining cells per mm^2 . Results were expressed as mean \pm SD. Statistical comparison between groups was made using student's t-test.

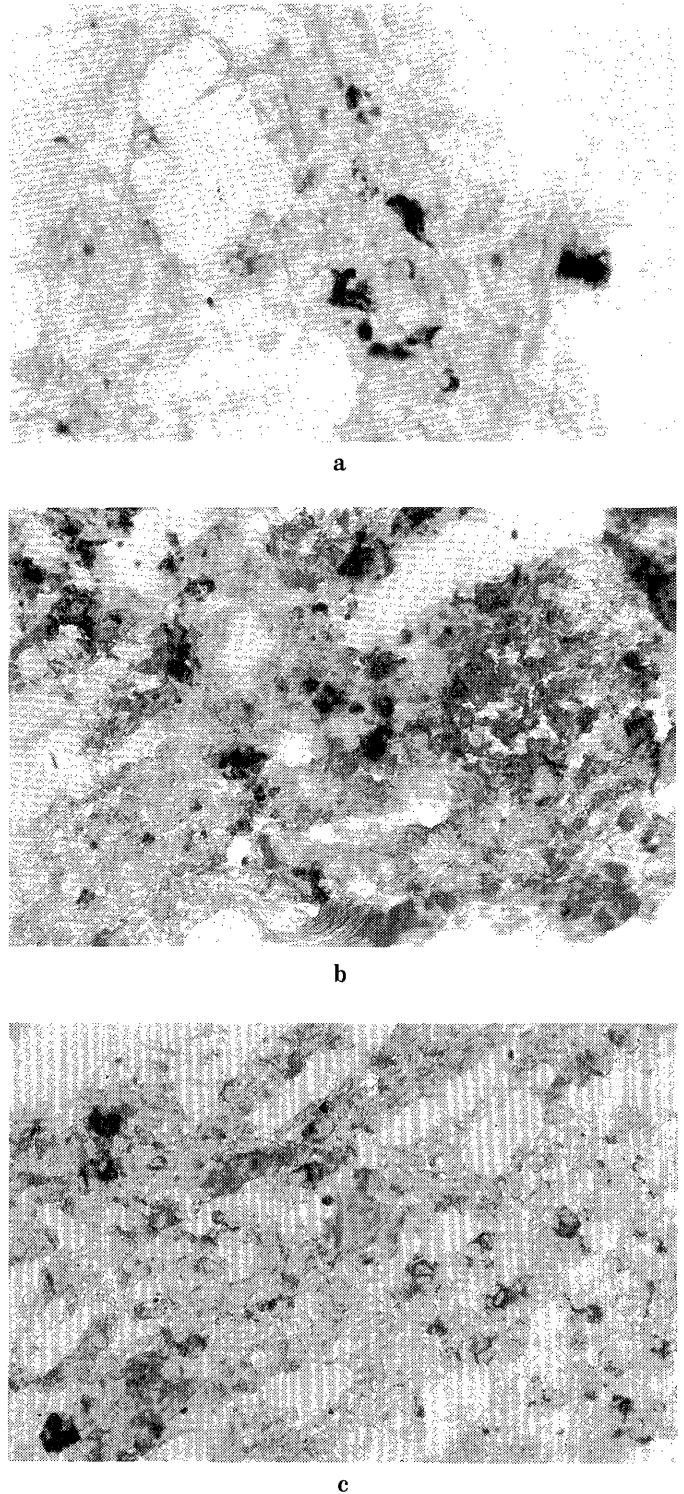


Fig. 3. Immunoperoxidase staining of CD8 positive cells in mucosa. ($\times 200$) a; normal, b; YWP, c; RL

Results

The results were summarized and shown in Fig. 2. The cell density of CD14 positive cells (a marker of monocytes/macrophages) was significantly higher in YWP and RL compared to normal mucosa. The cell density of CD14 positive cells did not differ significantly between YWP and RL.

The cell densities of CD3 positive cells (a marker of pan T cells), CD4 positive cells (a marker of helper T cells), CD56 positive cells (a marker of killer T cells) and CD25 positive cells also were higher in YWP and RL than in the normal mucosa. The cell densities of these cell types did not differ significantly between YWP and RL.

The cell densities of CD8 positive cells (a marker of killer/suppressor T cells) (Fig. 3), $\gamma\delta$ T cells, and CD19 positive cells (a marker of B cells) were higher in both YWP and RL than in the normal mucosa. The cell density of those cells was higher in YWP than in RL.

The cell density of HLA-DR positive cells in RL ($373 \pm 144/\text{mm}^2$) and in YWP ($100 \pm 95/\text{mm}^2$) was significantly higher than that in the normal mucosa ($50 \pm 28/\text{mm}^2$). The cell density of HLA-DR positive cells in YWP ($100 \pm 95/\text{mm}^2$) was significantly lower than in RL ($373 \pm 144/\text{mm}^2$) ($p < 0.01$).

Discussion

Abnormal immune response to intestinal commensal bacteria may be important in the etiology of UC⁵. Monocytes/macrophages recognize microorganism and act as antigen presenting cells to CD4 positive cells. CD4 cells can be divided into T helper 1 cells (Th1 cells) and T helper 2 cells (Th2 cells). Th1 cells produce IL-2 and IFN γ , and activate cell-mediated immunity through activating monocytes/macrophages and inducing CD8 positive T cells. Th2 cells produce IL-4 and IL-10 and activate B cells, resulting in enhanced antibody production. The IL-4 and IL-10, produced by Th2 cells, suppress monocytes/macrophages production of IL-1, TNF α and IL-12 (a negative feedback mechanism). Thus, Th1 and Th2 cells are involved in a complex interaction.

The epithelium of the intestine is characterized by the presence of $\gamma\delta$ T cells which recognize antigens of microbial origin. Mediated by IFN γ , $\gamma\delta$ T cells activate macrophages and thus protect the host from infection. It is thought that the normal epithelial cells of the intestine express MHC class II antigens and induce suppressor T cells that are not specific to particular antigens.

The mucosa of the large intestine of humans with UC has been reported to contain activated T and B cells, and to express excessive class II antigens in its epithelial cells. Elevated blood immunoglobulin levels and the presence of

anti-colon antibodies have also been reported in such patients⁵. Fukushima et al found a decrease in $\gamma\delta$ T cells⁶ and Kusugami et al reported a decrease in IL-2 producing CD4 positive cells in such patients⁷.

In the present study, activation of T cells, B cells, and macrophages was seen in both RL and YWP. In contrast to the findings of Fukushima et al.⁶, the numbers of $\gamma\delta$ T cells in RL and YWP were not decreased in our study. This discrepancy may be attributed to two factors: (1) Fukushima et al. found a decrease in CD4⁻CD8⁻ $\gamma\delta$ T cells, while we only examined CD3⁺ $\gamma\delta$ T cells; and (2) The cellular fractions we examined were contaminated by small amounts of IEL. In fact, even in the study by Fukushima et al.⁶ the number of CD3⁺ $\gamma\delta$ T cells in LPMC did not differ between the normal mucosa and the lesions, similar to our finding.

The cell density of HLA-DR antigen positive cells was higher in RL than in YWP. This suggest that HLA-DR positive cells have a role in determining the activity level of UC⁸. The increase of CD8 positive suppressor T cells in YWP also supports the classification of YWP as inactive lesions.

A limitation of the design of the current study is that it is difficult to assess whether UC is caused by persistence of abnormal stimuli, or by abnormal inflammatory reactions. New experimental approaches are needed. IL-2 is a cytokine which plays an important role in immune responses and inflammatory reactions. It promotes T cell proliferation, causes differentiation of B cells, and activation of macrophages, NK cells and LAK cells. Sadlack et al. recently reported that mice with an abnormal IL-2 gene product (IL-2 deficient mice) developed a condition resembling human UC⁹. These "knock out" mice have been attracting attention as an animal model of UC. This model closely resembles human UC in terms histopathological features. Immunological assessment of these mice as shown activated T and B cells, elevated immunoglobulin levels, the presence of anti-colon antibodies and excessive expression of class II antigens, similar immune abnormalities are found in human UC. When IL-2 deficient mice were placed in germfree state, none of them developed UC. Based on these findings, Sadlack et al. speculated that abnormal immune responses to normal nonpathogenic microbial flora trigger inflammatory changes. They also suggest that human UC is a disease of multigenic etiology, because mice with an abnormal TCR gene also developed an inflammatory bowel disease (IBD)- like disease⁹. These new animal models also pose new questions about lymphocyte function in UC — for example, what stimulates T cell activation in IL-2 deficient mice? The pathophysiology of chronic IBD such as UC will be further clarified through additional studies in the future.

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References

- 1) K. Makiyama : Magnifying endoscopy and inflammation of the large bowel. *Gastroenterol Endosc* 3 : 451-460 (1991) (In Japanese)
- 2) M. Senju, K. C. Wu, Y. R. Mahida, D. P. Jewell : Two-color immunofluorescence and flow cytometric analysis of lamina propria lymphocyte subsets in ulcerative colitis and Crohn's disease. *Dig. Dis. Sci.* 36 : 1453-1458 (1990)
- 3) M. Senju, K. C. Mahida, D. P. Jewell : Coexpression of CD4 and CD8 on peripheral blood T cells and lamina propria T cells in inflammatory bowel disease by two colour immunofluorescence and flow cytometric analysis. *Gut*, 32 : 918-922 (1991)
- 4) M. Senju : Reaginic hypersensitivity in ulcerative colitis. *Jpn J Gastroenterol* 85 : 2168-2177 (1988)
- 5) D. K. Podolsky : Inflammatory bowel disease. *N. Engl. J. Med.* 325 : 928-937 (1991)
- 6) K. Fukushima, T. Masuda, H. Ohtani, I. Sasaki, Y. Fukushima, S. Matsuno and H. Nagura. Immunohistochemical characterization, distribution, and ultrastructure of lymphocytes bearing T-cell receptor $\gamma \delta$ in inflammatory bowel disease. *Gastroenterology*, 101 : 670-678 (1991)
- 7) K. Kusugami, T. Matsuura, G. A. West, K. R. Youngman, D. Rach-milewitz and C. Focchi : Loss of interleukin-2-producing intestinal CD4⁺ T cells in inflammatory bowel disease. *Gastroenterology*, 101 : 1594-1605 (1991)
- 8) L. Mayer, D. Eisenhard, P. Salomon, W. Bauer, R. Plous and L. Piccinini. Expression of class II molecules on intestinal epithelial cells in humans : Differences between normal and inflammatory bowel disease. *Gastroenterology*, 100 : 3-12 (1991)
- 9) B. Sadlack, H. Merz, H. Schorle, A. Schipl, A. Feller and I. Horak : Ulcerative colitis-like disease in mice with a disrupted a interleukin-2 gene. *Cell*, 75 : 253-261 (1993)