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Circulating autoantibodies against purified colonic mucin in ulcerative colitis

Hiromasa Takaishi[1], Susumu Ohara[2], Kyoko Hotta[2], Tomoharu Yajima[1], Takanori Kanai[3], Nagamu Inoue[1], Yasushi Iwao[1], Mamoru Watanabe[3], Hiromasa Ishii[1], and Toshifumi Hibi[1,3]

[1] Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
[2] Department of Biochemistry, School of Medicine, Kitasato University, Kanagawa, Japan
[3] Keio Cancer Center, Tokyo, Japan

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Abstract: To clarify the role of colonic mucin in the autoimmune process of ulcerative colitis, circulating antibodies against human colonic mucin were investigated. Purified colonic mucin, obtained from human colonic mucosa by gel filtration, using a Bio-Gel A-1.5-m column and CsCl equilibrium density gradient, was divided into soluble mucin (S-mucin) secreted extracellularly and membranous mucin (M-mucin) binding to cell membrane. Sodium dodecylsulfate polyacrylamide gel electrophoresis and Western blotting analysis showed that antibodies in the serum samples of some patients with ulcerative colitis recognized purified S- and M-mucin of >180-kD. By enzyme-linked immunosorbent assay (ELISA), anti-mucin antibodies were detected in 11 of 60 patients with ulcerative colitis (18%). In contrast, the antibodies were not detected in 22 patients with Crohn's disease. The titers of anti-mucin antibodies against S-mucin and M-mucin were not different in each patient. By ELISA using mucin in which the sugar chains were destroyed by neuraminidase or $NaIO_4$ treatment, it was demonstrated that anti-mucin antibodies recognized the epitopes of either the sugar chain or the core protein exposed through destruction of the sugar chains. We then investigated the relationship between anti-mucin antibodies and the patients' clinical features. Anti-mucin antibodies were detected in 6 of 15 patients with chronic continuous type ulcerative colitis (40%) and in 5 of 26 patients with relapsing-remitting type (19%), but there was no anti-mucin antibody-positive serum in patients who had had only one attack without any relapse. These results suggest that anti-mucin antibodies could be a disease marker for ulcerative colitis and that immunological abnormalities in colonic mucin contribute to the persistence of colonic mucosal inflammation.

Key words: ulcerative colitis, Crohn's disease, mucin, anti-mucin antibodies, ELISA

Introduction

Ulcerative colitis is a chronic inflammatory bowel disease of unknown etiology. Autoimmune mechanisms may play an important role in the pathogenesis of this disease. Various kinds of autoantibodies, such as anti-neutrophil cytoplasmic antibodies[1–4] and anti-tropomyosin antibodies,[5–7] have been demonstrated in serum samples of patients with ulcerative colitis.

Using an immunohistochemical method and flow cytometry analysis, we have demonstrated that antibody to colonic epithelial cells (anti-colon antibody) was frequently seen in the serum of patients with ulcerative colitis. Moreover, we and other investigators have indicated that this antibody may contribute to the epithelial cell injury of colonic mucosa through antibody-dependent cell-mediated cytotoxicity (ADCC).[8–12] In addition, we have established anti-colon antibody-producing B-cell lines from peripheral blood lymphocytes (PBL) and lamina propria lymphocytes (LPL), using Epstein-Barr virus (EBV) transformation and the limiting dilution method.[13] Through the analysis of monoclonal antibodies produced by these cell lines, we have revealed that anti-colon antibodies include two kinds of autoantibodies, an anti-colonic epithelial cell antibody which reacts with colonic epithelial cells and an anti-goblet cell antibody which specifically reacts with goblet cells. Ulcerative colitis is histologically characterized by goblet cell depletion. It is well established that goblet cells produce mucin, which consists of glycoprotein, and that the secreted mucin forms a mucous

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Reprint requests to: T. Hibi

layer in the lumen to protect the mucosa from the invasion of intestinal microbes and various antigens. Therefore, abnormalities in the colonic mucin may contribute to injury of the colonic mucosa. In the present study, we focused on colonic mucin in goblet cells and investigated autoantibodies against purified colonic mucin in patients with ulcerative colitis.

Patients and methods

Patients

The subjects of the present study included 60 patients with ulcerative colitis (33 males and 27 females), 22 patients with Crohn's disease (12 males and 10 females), 7 patients with infectious colitis, 6 patients with chronic nephritis, and 7 patients with chronic hepatitis. Diagnosis of ulcerative colitis and Crohn's disease was based on clinical, radiographic, endoscopic, and pathological features. The patients with ulcerative colitis were divided into three groups on the basis of their clinical course as followed-up over a 1- to 7-year observation period: relapsing-remitting type (n = 26), chronic continuous type (n = 15), and one attack only type (n = 19). As the control group, 32 healthy volunteers (16 males and 16 females) of the same age group were used.

Serum samples were stored at −80°C after filtration through a 0.45-μm millipore filter. Prior to use, serum samples were inactivated at 56°C for 30 min and centrifuged for 60 min at 105000g.

Purification of colonic mucin

Mucin was purified from the macroscopically normal mucosa of the ascending colon obtained from a patient with ulcerative colitis (left-sided colitis type) who had undergone total colectomy because of rectal stenosis, and from the macroscopically normal mucosa of the descending colon of a patient with sigmoid colon cancer, as previously described.[14] The mucosa was homogenized in 50 mM Tris-HCl (pH 7.2), containing 30 mM dithiothreitol (DTT), 10 mM ethylenediaminetetraacetic acid (EDTA), 2 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 μg/ml leupeptin, and 0.7 μg/ml pepstatin A (buffer A). The homogenate was centrifuged at 100000g for 1 h and the membrane fraction was separated from the supernatant (Sup 1). The membrane fraction was homogenized in buffer A containing 2% Triton X-100 and 6 M urea. The homogenate was centrifuged at 100000g for 1 h and the soluble membrane fraction (Sup 2) was taken. Soluble mucin (S-mucin) and membrane-bound mucin (M-mucin) were purified according to the procedures for Sup 1 and Sup 2, respectively. Sup 1 and Sup 2 were applied to a Bio-Gel A-1.5-m column (BioRad Laboratories, Hercules, CA, USA) and eluted with 0.1% Triton X-100, 50 mM Tris-HCl (pH 7.2), 10 mM EDTA, and 5% 2-mercaptoethanol. The void volume fraction was centrifuged in a CsCl gradient at 150000g for 120 h at 10°C. The starting density of CsCl was 1.4 g/ml. The middle fractions (density, 1.5 g/ml) were collected, dialyzed against distilled water, and lyophilized. Gradient centrifugation in CsCl was conducted one more time. The following analytical procedures were carried out. Hexose was measured by the phenol-sulfonic acid method[15] with galactose as the standard. Protein was determined according to Lowry et al.,[16] using bovine serum albumin (BSA) as the standard. Gas-liquid chromatography was conducted to determine sugar composition, as previously described.[14]

Detection of anti-mucin antibodies by Western blotting

Ten μg of purified mucin, separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with polyacrylamide gel having a 3%–10% gradient, was transferred by electroblotting onto a polyvinylidene difluoride (PVDF) membrane for 2 h at 15 V in transfer buffer. The membrane was blocked with 3% gelatin in Tris buffered saline (TBS). The membrane was then incubated with serum samples diluted 1:100 in 3% gelatin TBS for 1 h, followed by incubation with peroxidase-conjugated goat anti-human IgG antibody diluted 1:1000 in 3% gelatin TBS for 1 h. The membrane was washed extensively with 0.05% Tween-20 in TBS (TTBS) between each incubation step. In the final step, mucin was visualized with enzyme substrate solution of 0.02% H_2O_2, 0.1% diaminobenzidine (DAB) in 0.1 M Tris-HCl (pH 7.2), and 0.8% NiCl. All incubation steps were carried out at room temperature, using an orbital shaker.

Determination of anti-mucin antibody titer by enzyme-linked immunosorbent assay (ELISA)

Enzyme immunoassay (EIA) plates were coated with purified mucin at 15 ng/per well overnight at 4°C. After being washed, the plates were incubated with 200 μl of blocking buffer (1% BSA in phosphate buffered saline [PBS] at 37°C for 2 h. The plates were washed three times with PBS, and then incubated with 50 μl of serum sample diluted 1:200 in PBS for 1 h at 37°C. Serum was discarded and the plates were washed three times with PBS. The plates were then incubated with 500 μl of peroxidase-conjugated goat anti-human IgG diluted 1:2500 at 37°C for 1 h. After being washed, the plates were incubated with 100 μl of 1 mM 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid; ABTS). The optical density was read at 405 nm. Wells with optical densities greater than two standard deviations above the mean of

the healthy control serum samples were scored as positive for anti-mucin antibody activity.

To determine whether or not the anti-mucin antibodies recognized the sugar chain epitope, EIA plates were coated with purified mucin at 15 ng/per well overnight at 4°C, and then the mucin sugar chain was destroyed with neuraminidase, adjusted to 0.01 U/well and 0.001 U/well with acetate buffer (pH 6.5) or $NaIO_4$ adjusted to 300, 100, 30, 10, and 3 mM with PBS. ELISA was conducted with anti-mucin antibody-positive serum to determine changes in the anti-mucin antibody titer. All data values are expressed as the means of duplicate determinations.

Statistics

All values are expressed as means ± SE. The statistical significance of differences was determined with the Kruskall-Wallis rank test and Wilcoxon rank test. The *P* values computed were two-tailed and *P* values below 0.05 were considered significant.

Results

Composition of purified colonic mucin

After CsCl gradient density ultracentrifugation, it was found that the fractions with a density of about 1.5 g/ml (fractions 8 and 9) in which mucin was present were rich in hexose but low in protein concentration (Fig. 1). Sugar chains of the mucin, extracted and purified by gas-liquid chromatography, were then examined, and were found to show typical patterns for mucin. No significant difference was observed in carbohydrate composition between S-mucin and M-mucin (Table 1).

Detection of anti-mucin antibodies in serum from patients with ulcerative colitis by Western blotting

Western blotting and SDS-PAGE study showed that serum samples from some patients with ulcerative colitis recognized fractions of S- and M-mucin of more than 180-kD molecular weight (Fig. 2). These bands were positive for periodic acid Schiff (PAS) reaction. There was no significant difference in reactivity to the anti-mucin antibody-positive serum samples between the mucin from a patients with ulcerative colitis and that from the patient with colon cancer. These serum samples were proved to react with goblet cells immunohistochemically. Serum samples from the healthy controls did not reveal any bands.

Determination of anti-mucin antibody titer by ELISA

In the 60 patients with ulcerative colitis, the optical density of anti-mucin antibodies was 0.467 ± 0.353 for S-mucin and 0.406 ± 0.400 for M-mucin (mean ± SD). The corresponding optical densities in patients with Crohn's disease were 0.260 ± 0.197 and 0.215 ± 0.165, those in patients with infectious colitis were 0.313 ± 0.029 and 0.314 ± 0.021, those in patients with chronic nephritis were 0.314 ± 0.022 and 0.303 ± 0.024, those in patients with chronic hepatitis were 0.316 ± 0.028 and 0.307 ± 0.026, and those in healthy controls were 0.273 ± 0.161 and 0.304 ± 0.174, respectively. The optical density of anti-S-mucin antibodies was significantly higher in sera of patients with ulcerative colitis ($P < 0.05$, compared with that in sera of normal controls). The optical density of anti-M-mucin antibodies was significantly higher in sera of patients with ulcerative colitis ($P < 0.05$, as compared with that in sera of patients with Crohn's disease). When optical densities greater than two standard deviations above the mean of the healthy controls (S-mucin, $0.273 + 2 \times 0.161$; M-mucin, $0.304 + 2 \times 0.174$) were scored as positive for anti-mucin antibody activity, the frequency of anti-mucin antibodies in ulcerative colitis was 18% (11 of 60 patients) for both S-mucin and M-mucin. Anti-mucin antibodies were not detected in patients with Crohn's disease, infectious colitis, chronic nephritis, or chronic hepatitis (Fig. 3 and Table 2). In the anti-mucin antibody-positive serum samples, the optical densities increased depending on the serum concentration. In contrast, this phenomenon was not observed in the

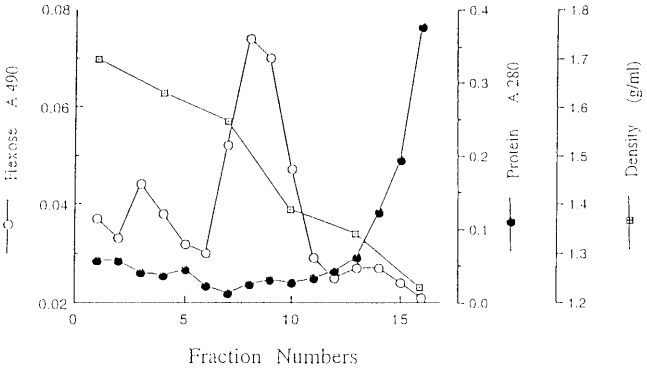

Fig. 1. Mucin was purified by CsCl density gradient ultracentrifugation. The purified mucin contained a high amount of hexose and low amount of protein, and its density was 1.5 g/ml

Table 1. Carbohydrate composition of human colonic mucin

	GalNAc	GlcNAc	Gal	Fuc	NANA
S-mucin[a]	1.00	1.00	0.66	0.53	0.67
M-mucin[a]	1.00	1.13	0.60	0.62	0.60

[a] Results are expressed as ratios of each carbohydrate concentration/GalNAc

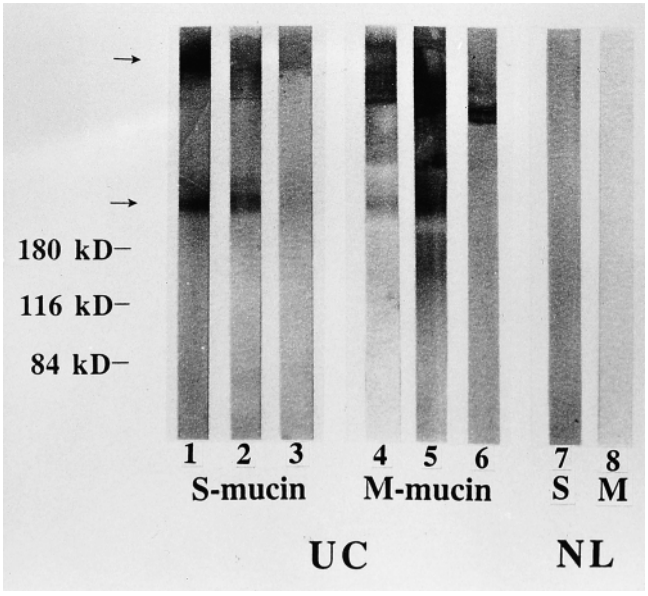

Fig. 2. Western blot analysis demonstrated that anti-mucin antibodies in serum from patients with ulcerative colitis (*UC*) recognized fractions of S- and M-mucin of more than 180 kD purified from the colonic mucosa of a patient with ulcerative colitis (*arrows*). These bands were stained with periodic acid schiff (PAS; data not shown). *Lane 1*, reactivity of ulcerative colitis serum sample 1 to purified S-mucin; *lane 2*, reactivity of ulcerative colitis serum sample 2 to purified S-mucin; *lane 3*, reactivity of ulcerative colitis serum sample 3 to purified S-mucin; *lane 4*, reactivity of ulcerative colitis serum sample 1 to purified M-mucin; *lane 5*, reactivity of ulcerative colitis serum sample 2 to purified M-mucin; *lane 6*, reactivity of ulcerative colitis serum sample 3 to purified M-mucin; *lane 7*, reactivity of a healthy control serum sample to purified S-mucin; *lane 8*, reactivity of a healthy control serum sample to purified M-mucin. *NL*, normal control

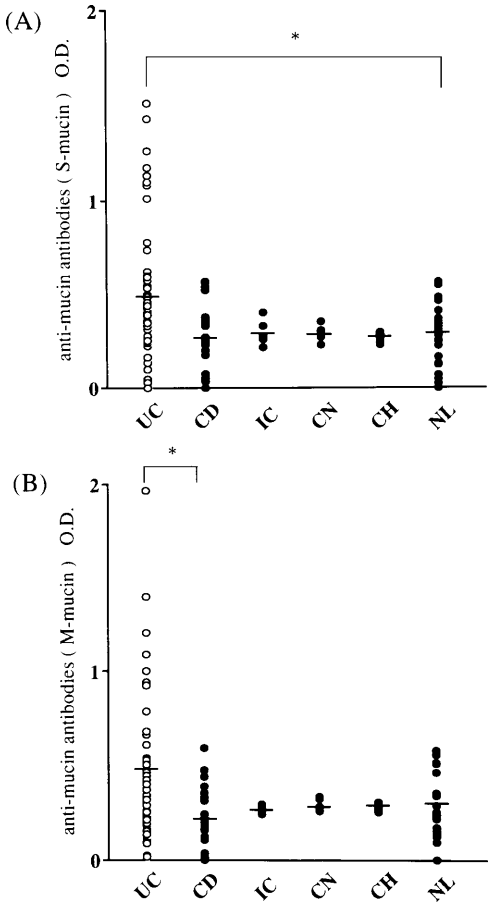

Fig. 3A,B. Anti-mucin antibodies against **A** S-mucin and **B** M-mucin were detected by enzyme-linked immunosorbent assay (ELISA) in various patient populations. Both anti-mucin antibodies were present in 18% of ulcerative colitis (*UC*) samples. No anti-mucin antibody-positive serum samples were found in other patient populations. *O.D.*, optical density; *CD*, Crohn's disease; *IC*, infections colitis; *CN*, chronic nephritis; *CH*, chronic hepatitis. *NL*, normal control

serum samples from healthy controls. Thus, this ELISA system was found to detect an antigen-specific reaction. There was a significant correlation between the S-mucin and M-mucin anti-mucin antibody titers.

To determine the corresponding epitope of these anti-mucin antibodies, we conducted ELISA after the mucin sugar chains had been destroyed by neuraminidase and $NaIO_4$. The antibody titer was elevated with an increase of neuraminidase and $NaIO_4$ concentrations in some serum samples, while it was reduced in other serum samples (Fig. 4). From these results, it is considered that some anti-mucin antibodies recognize sugar chains, while others may recognize protein which is exposed after the destruction of the sugar chains.

These findings in mucin purified from macroscopically normal colonic mucosa from a patient with ulcerative colitis were the same in mucin purified from the patient with sigmoid colon cancer.

Anti-mucin antibody as a clinical marker

Positive rates of anti-mucin antibodies were examined in the three clinical types of ulcerative colitis (Table 3). Anti-mucin antibodies were detected in 40% of patients with the chronic continuous type and in 19% with the relapsing-remitting type. Anti-mucin antibodies were not detected in any patients with the one-attack only type.

Discussion

It is generally accepted that immunological abnormalities are involved in the pathogenesis of ulcerative colitis, although the precise mechanism remains unclear. Cumulative evidence suggests that ulcerative colitis is one of the autoimmune diseases, in which various au-

Table 2. Anti-mucin antibodies in serum of ulcerative colitis by ELISA

	Number of subjects	Anti-mucin antibodies (S-mucin) Percentage of positive cases	Anti-mucin antibodies (M-mucin) Percentage of positive cases
Ulcerative colitis	60	0.467 ± 0.353 18%	0.406 ± 0.400 18%
Crohn's disease	22	0.260 ± 0.197 0%	0.215 ± 0.165 0%
Infectious colitis	7	0.286 ± 0.065 0%	0.270 ± 0.020 0%
Chronic nephritis	6	0.290 ± 0.042 0%	0.290 ± 0.033 0%
Chronic hepatitis	7	0.268 ± 0.025 0%	0.278 ± 0.019 0%
Healthy controls	32	0.273 ± 0.161 0%	0.304 ± 0.174 0%

ELISA, enzyme-linked immunosorbent assay

Table 3. Relationship between anti-mucin antibodies and clinical type of ulcerative colitis

	Clinical type		
	One attack only	Relapsing-remitting type	Chronic continuous type
Number of patients	19	26	15
Anti-mucin antibodies (S-mucin)[a]	0.344 ± 0.193	0.476 ± 0.380	0.625 ± 0.421
Anti-mucin antibodies (M-mucin)[a]	0.217 ± 0.124	0.433 ± 0.478	0.620 ± 0.416
Percentage of positive cases	0%	19.2%	40.0%

[a] Optical density

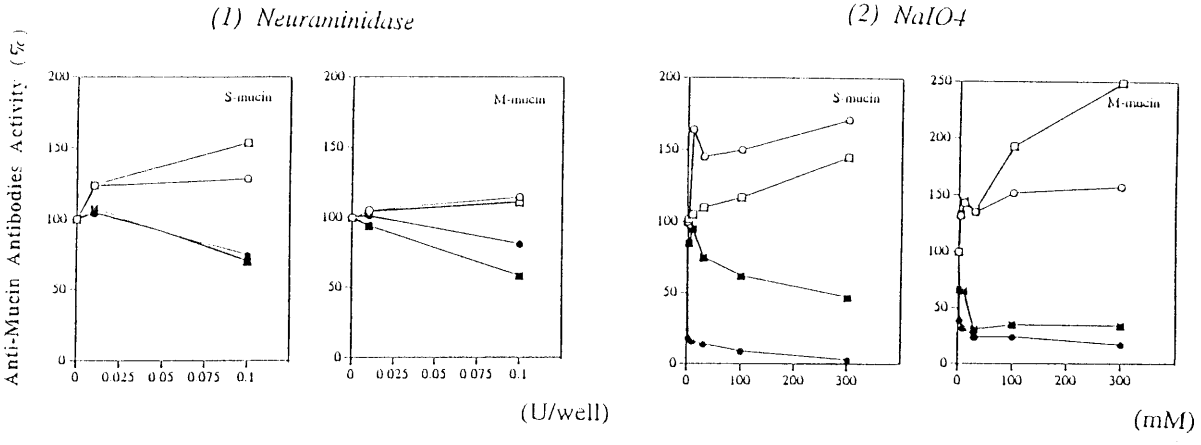

Fig. 4. Anti-mucin antibody activity detected by ELISA before and after treatment of mucin with neuraminidase or $NaIO_4$. Serum anti-mucin antibody activity was decreased after treatment in some patients (*closed circles* and *closed squares*), but was increased in other patients (*open circles* and *open squares*). *Closed circles*, closed squares; *open circles*, or *open squares* means each serum from independent patient

toantibodies are often found. As goblet cell depletion is frequently seen in the colonic mucosa of ulcerative colitis, we investigated mucin produced from goblet cells as the target of immunological abnormalities in the present study.

We first purified human colonic mucin and examined its characteristics. Mucin is a glycoprotein which consists of core proteins and a number of mucin-type sugar chains. The properties of the fractions obtained from human colonic mucosa by gel filtration using the

Bio-Gel A-1.5-m column and CsCl equilibrium density gradient were identical to those of mucin, since fractions with a density of 1.5 g/ml consisted of abundant hexose with a low protein content, and the composition of sugar chains in the fractions was consistent with mucin.

After being produced from goblet cells, mucin is released into the lumen of the gastrointestinal tract, forming the superficial mucous layer to protect the mucosa. It was reported by Podolsky[17] that the sugar chain structure of mucin isolated from human colonic mucosa showed remarkable heterogeneity, which he termed superglycoform. It has also been reported that colonic mucin has a specific sugar chain structure in patients with ulcerative colitis.[18] It is well known that sialomucin is predominant in the right-side colon, and sulfomucin is predominant in the left-side colon. In the present study, we confirmed that the reactivity of anti-mucin antibody against mucin obtained from macroscopically normal mucosa-(right side) of ulcerative colitis and macroscopically normal mucosa (left side) of colon cancer was the same, and this finding suggests that mucin purified from a patient with ulcerative colitis could not be distinguished immunologically from mucin purified from a patient with sigmoid colon cancer. Taking into consideration the fact that colonic mucin is partially destroyed by the intestinal flora and that heterogeneity is also observed in mucin purified from healthy controls, it is difficult to conclude that colonic mucin in ulcerative colitis has abnormalities. The authors who reported a specific sugar chain structure in mucin from patients with ulcerative colitis[18] may have observed continuous changes in the glycoprotein molecules associated with the differentiation and maturation of goblet cells.

It has been reported that the production of antibodies against intestinal antigens or autoantigens is increased in the lamina propria of inflamed colonic mucosa. Various kinds of autoantibodies have been reported in serum from patients with ulcerative colitis, but the role of the autoantibodies has not yet been clarified. We have found that circulating anti-colon antibodies in ulcerative colitis can be classified into those antibodies which react with colonic epithelial cells and those which react with cytoplasm and membrane of goblet cells,[10] and we have demonstrated an ADCC mechanism for the injury of colonic mucosa in ulcerative colitis.[8,9] Here, we investigated circulating anti-mucin antibodies by ELISA, using purified mucin. We previously reported that the frequencies of anti-goblet cell antibody were 38% in ulcerative colitis and 33% in Crohn's disease, by ELISA, using the HT29-18-N2 cancer cell line as the antigen source.[19] Compared with that result, the frequency of anti-mucin antibodies was low in ulcerative colitis (18%). Because the HT29-18-N2 cancer cell line differentiates into goblet cells and produces mucin in confluent cell culture, it is possible that the anti-goblet cell antibodies identified in the previous study may recognize not only mucin but also any other cell components of the cell line. In contrast, the antigen used in this present study was purified mucin, which did not contain other cell components. Thus, when the purified antigen was used, the prevalence of anti-mucin antibodies was low and the specificity was high. Anti-mucin antibodies were not detected in healthy controls, or in patients with Crohn's disease or chronic inflammatory diseases of other organs. These results indicate that colonic mucin is the antigen corresponding to the anti-mucin antibodies and that the antibodies are specific to ulcerative colitis, although the relatively low prevalence of these antibodies may limit their clinical usefulness.

Mucin is present either on the epithelial cell surface binding to the cell membrane or in the intestinal lumen released from epithelial cells. As the mechanism for the production of autoantibodies against mucin, it is postulated that mucin comes into contact with blood as a result of the continuous secretion of mucin into the vascular side caused by injury to the epithelial cells. If anti-mucin antibodies injure the colonic epithelium directly, the antibodies may react with membranous mucin (M-mucin) more markedly than with secreted soluble mucin (S-mucin). In the present study, the S-mucin fraction may contain cytosol fractions, in which there may be not only soluble secretory-type but also immature membranous-type mucin synthesized in the endoplasmic reticulum (ER) or Golgi apparatus or transfer vesicles. There is a possibility that anti-mucin antibody against S-mucin may react with immature membranous-type mucin in the S-mucin fraction. As demonstrated in this study, S-mucin and M-mucin were similar in terms of sugar chain composition. Furthermore, antibodies to both S-mucin and M-mucin showed cross reactivity and it was impossible to distinguish these two antibodies immunologically. These results suggest that the inflammation is not caused by a direct effect of anti-mucin antibodies on the colonic mucosa. There may be some other mechanisms, such as a decrease in the function of mucin as a barrier to intestinal substances, through changes in the structure of mucin molecules.[20] Further studies are warranted to elucidate the role of anti-mucin antibodies in the pathogenesis of ulcerative colitis.

In a study of the expression of immunoglobulin heavy chain variable region (VH region) in anti-colon antibody-producing B-cell clones, it has been shown that the VH3 family is expressed specifically (Inoue et al., personal communication). Thus, it is suggested that anti-colon antibody is induced by some specific antigen in the colonic mucosa. To determine the epitopes recognized by anti-mucin antibodies, we examined titers of the antibodies by ELISA before and after the mucin

sugar chains were destroyed by neuraminidase and $NaIO_4$. This investigation demonstrated that there were two types of anti-mucin antibodies: one type which recognizes the sugar chains and another which recognizes core protein that was probably exposed by the treatment of the sugar chains. These findings suggest that the anti-mucin antibodies are heterogeneous.

It is well known that blood group type antigens have mucin-type sugar chains. Therefore, we should be careful of the possibility that antibodies in serum samples in some patients may react with blood group A, B, or H substance but not with mucin antigen. However, the blood group type antigens of individual purified mucins in this study were type B in mucosa from ulcerative colitis and type O in normal mucosa from colon cancer (data not shown). There is little possibility that the antibodies reacted with blood group A, B, or H substance, since we found no differences in the ELISA data in any samples, even when we used the two kinds of mucin fractions, purified from normal mucosa of ulcerative colitis and that of colon cancer.

Although we could not clarify the significance of anti-mucin antibodies in the pathogenesis of ulcerative colitis, there are some reports that autoantibodies may be a disease marker in inflammatory bowel disease.[3,21] We therefore analyzed the relationship between the presence of anti-mucin antibodies and the patients' clinical features. It is considered that ulcerative colitis can be classified according to its causes, such as virus infection, bacterial infection, microcirculation disturbance, and immunological abnormalities. We found that anti-mucin antibodies were frequently detected in patients with the chronic continuous type of ulcerative colitis, but there was no anti-mucin antibody-positive serum in patients who had only one attack without any relapse. These results suggest that there is a subgroup of ulcerative colitis in which circulating anti-mucin antibodies are positive and autoimmune mechanisms have a crucial role in the etiology.

In conclusion, anti-mucin antibodies which react with purified colonic mucin were demonstrated in the serum samples of patients with ulcerative colitis but not in those of patients with infectious colitis or Crohn's disease. A recent study has demonstrated goblet cell autoantibodies not only in patients with ulcerative colitis but also in their first-degree relatives.[22] Ohara et al. have clearly demonstrated the induction of colitis in rats by immunization with colonic mucin purified by the same method as that used in this study (Ohara et al., manuscript submitted for publication). Taken together with the findings in our study that anti-mucin antibodies were frequently shown in the refractory type of ulcerative colitis, it seems that anti-mucin antibodies may reflect underlying immunological abnormalities and may be a serological marker for a group of colitis patients who share the same pathological conditions. The precise autoimmune mechanisms will be clarified by pursuit of the anti-mucin antibodies which react with purified colonic mucin.

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