

Tropomyosin Isoforms in Intestinal Mucosa: Production of Autoantibodies to Tropomyosin Isoforms in Ulcerative Colitis

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Background & Aims: Autoantibodies against tropomyosins (TMs) have been reported in ulcerative colitis (UC). In this study the hTM isoforms (hTM1–5) present in intestinal epithelial cells and in smooth muscle were investigated, and the immunoreactivity against hTMs by immunoglobulin G (IgG) produced in vitro by colonic mucosal lymphocytes (LPMCs) from patients with UC, Crohn's disease (CD), and controls was examined. **Methods:** TMs were extracted from colonic and jejunal epithelial cells and smooth muscle, and hTM isoforms were identified using isoform-specific monoclonal antibodies by enzyme-linked immunosorbent assay and transblot analysis. The immunoreactivity of IgG produced by colonic LPMCs was analyzed against the recombinant hTM isoforms. **Results:** The major hTM isoforms present in colonic and jejunal epithelial cells are hTM5 and hTM4, whereas intestinal smooth muscle contains the hTM1–3 isoforms. The IgG synthesized in vitro by LPMCs from UC ($n = 19$) recognized hTM5 and hTM1, more significantly ($P < 0.04$ to < 0.001) when compared with CD ($n = 12$) and controls ($n = 17$). However, IgG produced by LPMCs from CD did not show such anti-hTM reactivity. Mucosal anti-hTM IgG mainly belonged to the IgG1 subclass. **Conclusions:** Intestinal epithelial cells and smooth muscle have distinct hTM isoforms. Patients with UC, and not CD, show mucosal autoantibody response against hTM isoforms, particularly hTM5 and hTM1.

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) of unknown cause, although autoimmunity has been emphasized in the pathogenesis of the disease.¹ A marked increase in the mucosal immunoglobulin G (IgG) immunocytes² and IgG antibodies against colonic antigens³ and neutrophil antigen⁴ have been reported in the colon in UC. However, the specific antigen(s) involved in the IgG-immune recognition has

not been clarified. The mucosal IgG overproduction could play a pathogenetic role in UC, because the subclass distribution of IgG-producing cells in both active and inactive UC lesions shows a disproportionate local overproduction of IgG1.^{5,6} IgG1 antibodies are more effective in complement activation than IgG2,⁷ and because of this property, IgG1 antibodies can contribute to the perpetuation of tissue damage in UC.

We have previously reported the presence of tissue-bound IgG antibody in the colon in UC⁸ and have shown that this IgG antibody recognized a 40,000–molecular weight colonic protein, p40.⁹ Tissue-bound IgG eluted from Crohn's disease (CD) of the colon and controls did not recognize the p40, suggesting an autoantigenic role of this protein in UC.⁹ By using immunocytochemical methods, other investigators have reported the deposition of IgG1 autoantibody and activated complement products on the colonic epithelium from active UC^{10,11} but not in CD.¹² Recently, p40 was purified from the colon to homogeneity and partially sequenced, and the two peptides derived therefrom showed 93%–100% identity with the cytoskeletal protein tropomyosins (TMs).¹³ TMs are cytoskeletal microfilamental proteins present in all eukaryotic cells with organ-specific isoforms, and multiple isoforms may be present in the same cell.¹⁴ At least eight TM isoforms have been identified from human fibroblast cells,¹⁵ and strong evidence for the distinct functions performed by different TM isoforms has been generated recently.¹⁴ TMs are capable of inducing significant immune responses related to allergy¹⁶ as well as autoimmunity.¹⁷ In a computer-based physicochemical

Abbreviations used in this paper: BSA, bovine serum albumin; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; HBSS, Hank's balanced salt solution; LPMC, lamina propria mononuclear cell; OD, optical density; pANCA, perinuclear antineutrophil cytoplasmic antibody; PMSF, phenylmethylsulfonyl fluoride; PWM, pokeweed mitogen; SDS, sodium dodecyl sulfate; TM, tropomyosin.

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analysis, several sequences of TM residues were considered to be among the most potent autoantigens.¹⁷ We previously reported that blood serum from patients with UC, but not from those with CD or from controls, had IgG antibodies reactive to TMs.¹³

The specific TM isoform(s) present in the human colon epithelium is unknown. In this study, we examined the TM isoforms in freshly isolated normal colonic and small intestinal epithelial cells and in smooth muscle of the intestine from surgical specimens. The immunoreactivity of IgG antibodies produced in vitro by lamina propria mononuclear cells (LPMCs) from subjects with UC and CD and from non-IBD controls against various recombinant hTM isoforms were investigated by using enzyme-linked immunosorbent assays (ELISAs).

Materials and Methods

Extraction of TMs From the Intestinal Tissue

Colonic and small intestinal mucosal extracts enriched in TMs. Colonic mucosa (including epithelium and stromal tissue and muscularis mucosa) was stripped from surgical specimens of colon from patients with colon cancer (normal segments; $n = 4$). Jejunal mucosa was obtained from normal subjects ($n = 4$) who underwent gastric bypass surgery for morbid obesity. Mucosa was minced, washed with phosphate-buffered saline (PBS), resuspended in the buffer containing 1 mol/L NaCl, 50 mmol/L Tris, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 5 mmol/L dithiothreitol (DTT), and 2 mmol/L phenylmethylsulfonyl fluoride (PMSF), and ultrasonicated for 3 minutes on ice. The sample was centrifuged at 1000g for 5 minutes, and the supernatant was boiled for 10 minutes, centrifuged at 7000 rpm for 30 minutes, and further purified by ammonium sulfate precipitation (initially 40% and then 60%)¹⁵ and stored until use.

Extraction of TMs from colonic and jejunal epithelial cells. Stripped mucosa from the surgical specimens was washed with Hank's balanced salt solution (HBSS) containing 50 U/mL gentamicin, 100 U penicillin, and 100 mg streptomycin. To remove the mucus, the tissue was treated with 1 mmol/L DTT for 15 minutes at 37°C followed by three washings in HBSS. The tissue was subjected to dispase treatment (3 mg/mL in RPMI 1640 for 30 minutes at 37°C). Cells were collected from the supernatant, and epithelial cells were further purified by using Percoll (Pharmacia, Piscataway, NJ) gradient (44% and 66%) and centrifugation at 1000g for 30 minutes. The top layer was transferred and washed with PBS, and the cells were counted and stored at -80°C until further extraction. The purity of epithelial cells was found to be at least 90%. TMs were extracted by homogenization of the cell pellet in the buffer containing 0.35 mol/L NaCl, 50 mmol/L Tris, 1 mmol/L EDTA, 5 mmol/L DTT, and 2 mmol/L PMSF and centrifuged at 1000g for 5 minutes, and the supernatant

was boiled for 10 minutes, followed by centrifugation at 7000 rpm for 30 minutes. The supernatant was further purified by sequential ammonium sulfate precipitation.

Extraction of TMs from colonic and small intestinal smooth muscle. Smooth muscle was dissected from the intestinal specimens after stripping off the mucosa, and TMs were extracted, as described above.

Preparation of recombinant TM isoforms. The full-length complementary DNAs (cDNAs) encoding various TM isoforms (hTM1, hTM2, hTM3, and hTM5) were prepared from human fibroblast, as reported previously.¹⁵ An hTM4 clone was not available. We have subcloned these four cDNA clones into prokaryotic expression vector pET8c. The resulting plasmids were transformed into the *Escherichia coli* BL21 (D3) LysS strain. Recombinant human TM isoforms were purified from bacterial lysates by ammonium sulfate fractionation, DE-52 ion-exchange chromatography, and hydroxyapatite column chromatography. The purity of the hTM isoforms was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the immunoreactivity was analyzed by ELISA and transblot analysis using isoform-specific monoclonal antibodies. Any contamination of *E. coli* protein was investigated using an anti-*E. coli* polyclonal antibody (a gift from Dr. David Alcid, Associate Professor of Medicine, Infectious Disease Division, Robert Wood Johnson Medical School) by an ELISA and by transblot analysis against each of the purified hTM isoforms. The anti-*E. coli* antibody reacted with the sonicated extract of the *E. coli* BL21 (D3) LysS strain, which was used in parallel as a control.

Monoclonal Antibodies Against Various TM Isoforms

The isoform-specific murine monoclonal antibodies CG1 (IgG1) against hTM1, CG β 6 (IgM) against hTM2/hTM3, and CG3 (IgM) against hTM5 have been generated and characterized previously.^{14,15} CG β 6 reacts with both hTM2 and hTM3 but not against hTM1, hTM4, or hTM5. The isoform-specific monoclonal antibodies LC24 (IgG1) against hTM4 and LC1 (IgG1) against hTM5 were generated by immunizing mice with the recombinant hTM isoform.¹⁸

Determination of Specific TM Isoform(s) in the Colon and Small Intestine by Immunotransblot Analysis and ELISA

Extracted TMs from mucosa, isolated epithelial cells, and smooth muscle from the small and large intestine were examined by SDS-PAGE followed by immunotransblot analysis.⁹ Five micrograms of enriched TM extracts were separated by 12% SDS-PAGE and stained with Coomassie brilliant blue. Separated proteins were also transferred to a nitrocellulose sheet blocked with 1% bovine serum albumin (BSA) and probed with isoform-specific murine anti-TM monoclonal antibodies (CG1, 1:1000; CG3, 1:500; CG β 6, 1:1000; LC1, 1:500; LC24, 1:500), followed by horseradish peroxidase-conjugated appropriate anti-mouse IgM or IgG antibodies. The reactivity was

detected by chemiluminescence using the Renaissance Kit (Dupont, Boston, MA), and the respective recombinant hTM isoform (2 μ g) was run in parallel as a positive control. Unrelated isotype-specific murine monoclonal antibodies such as MOPC-IgM or MOPC-IgG were also used to examine any nonspecific reactivity.

TM extracts from jejunal and colonic mucosa, smooth muscle, and small intestinal enterocytes and colonocytes were also analyzed by ELISA. The extracts were plated (0.3 μ g/well) in carbonate buffer (pH 9.6) on an ELISA plate and left overnight. The TM isoform-specific monoclonal antibodies, as described above, were incubated at 37°C for 1 hour after the plate was blocked with 1% normal goat serum followed by alkaline phosphatase-conjugated appropriate (anti-mouse IgM or anti-mouse IgG raised in goat) second antibody. The optical density (OD) values were measured at 405 nm within 2 hours.

Examination for Antibodies Synthesized In Vitro by LPMCs

Patients. Forty-eight patients undergoing colonic resection ($n = 30$) or colonoscopy ($n = 18$) at the Division of Gastroenterology, Universita La Sapienza, Rome, Italy, were included for these studies. There were 19 patients with UC (12 men and 7 women; mean age $\pm$ SEM, 51 ± 2 years; range, 21–65 years), 10 symptomatic and 9 asymptomatic at the time of the study. Of these 19 patients with UC, 13 underwent routine colonoscopy and 6 underwent colonic resection. Indications for surgery in these 6 patients were steroid dependence ($n = 4$), diffuse polyposis ($n = 1$), and no response to medical treatment ($n = 1$). The colitis was left-sided in 14 patients and extensive in 5 patients. At the time of the study, 10 of the 19 patients with UC were taking oral steroids; 7 patients were on mesalamine therapy (orally in 2 patients, rectally in 5), and 2 patients were not undergoing any treatment. Twelve of the 19 patients were clinically and endoscopically active, and 7 were in remission. The mean ($\pm$ SEM) duration of the disease in patients with UC was 11 ± 2 years (range, 1–31 years). Twelve patients had colonic CD (7 men and 5 women; mean age, 42 ± 3 years; range, 22–62 years). In the CD group, there were 6 symptomatic and 6 asymptomatic patients. Four of these 12 patients with CD underwent routine colonoscopy, and 8 underwent surgery. Indications for surgery were steroid dependence ($n = 4$), no response to medical treatment ($n = 2$), and abdominal abscess ($n = 2$). Five patients were taking steroids, and 7 were being treated with oral mesalamine. The mean duration of the disease was 9 ± 2 years (range, 1–17 years). The non-IBD group included 1 patient with diverticular disease who underwent colonoscopy and 16 patients with colonic carcinoma who underwent colonic resection. Among them, 11 were men and 6 were women; the mean age was 58 ± 3 years (range, 34–72 years). In all subjects, the diagnosis was made according to the usual clinical, endoscopic, radiological, and histological criteria. Patients with indeterminate colitis were not included in this study. Disease activity was assessed in patients with UC through clinical history and endoscopic findings¹⁹ and in patients with CD through Crohn's Disease Activity Index.²⁰

Tissue samples. Colonic mucosal specimens for isolation of LPMCs were taken from each disease group immediately after colonic resection (total $n = 30$; UC, $n = 6$; CD, $n = 8$; non-IBD, $n = 16$). In case of colonoscopy, 4–5 biopsy specimens were obtained during the procedure from 18 subjects with UC ($n = 13$), CD ($n = 4$), and non-IBD ($n = 1$). Surgical and colonoscopic biopsy specimens were obtained from the distal 20 cm of the colon (UC, $n = 12$; CD, $n = 7$; non-IBD, $n = 10$) and from the area proximal to the sigmoid colon (UC, $n = 7$; CD, $n = 5$; non-IBD, $n = 9$).

Blood samples. Venous blood samples were obtained from each patient during the visit for colonoscopy or before surgery; sera were separated and stored at -70°C .

Isolation of LPMCs from surgical specimens. Intestinal mucosa was dissected from surgical specimens within 1 hour of resection. LPMCs were isolated from surgical specimens using the Bull and Bookman enzymatic methods²¹ with minor modifications as described by Pallone et al.²² LPMCs were washed and counted and their viability assessed by 0.1% trypan blue exclusion. Contamination by epithelial cells of the LPMC suspensions ranged between 10% and 16%, as shown by us and others.^{23–25} The LPMC yield ranged from 2.5×10^6 to 11.5×10^6 LPMCs/g wet tissue. The viability of LPMCs ranged from 85% to 90% before cultures and from 50% to 70% on the 10th day of culture.

Isolation of LPMCs from biopsy specimens. LPMCs were isolated using EDTA collagenase as mentioned above with modifications for small samples.^{23,26,27} LPMC preparations obtained from biopsy specimens were comparable with surgical specimens in terms of yield and epithelial cell contamination, ranging from 0.9 to 4×10^6 from 4–5 biopsy specimens. The viability of LPMCs ranged from 85%–95% before culture to 50%–70% on the 10th day.

LPMC cultures. Cells were resuspended in a complete medium (2×10^6 cells/2 mL), placed in 2.8-mL-well tissue culture plates (Falcon Plastic, Franklin Lakes, NJ), and cultured unstimulated at 37°C in 5% CO₂. Previous studies by us²³ and others²⁸ have consistently shown that in vitro pokeweed mitogen (PWM) stimulation does not increase the total and antigen-specific IgG antibody release by LPMCs, suggesting that LPMCs are already maximally stimulated in vivo by luminal antigens permeating the intestinal mucosa. In the present study, we therefore used limited paired cultures of LPMCs with and without PWM (UC, $n = 4$; CD, $n = 6$; non-IBD, $n = 8$). PWM was added at a concentration of 1 μ g/well (GIBCO Laboratories, Grand Island, NY). On the 10th day, cells were harvested, counted, and checked for viability, and supernatants were collected, frozen within 1 hour, and stored at -20°C until testing.

Estimation of IgG and IgG subclass-specific antibodies against hTMs in culture supernatants and sera. Total IgGs in culture supernatants were tested by ELISA as previously described.^{22,23} For anti-hTM IgG reactivities, ELISA plates (Falcon Plastic) were separately coated with the four recombinant human TM isoforms (hTM1, hTM2, hTM3, and hTM5) at 150 ng/well, diluted in the carbonate buffer (pH 9.6; 100 μ L/well). Purified recombinant hTM4 was not available for

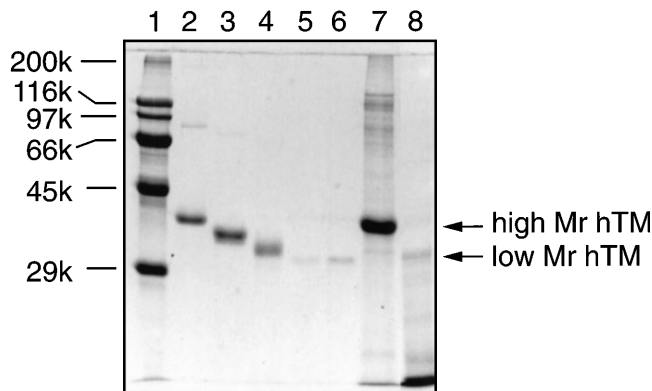


Figure 1. SDS-PAGE followed by Coomassie blue staining of purified recombinant hTM isoforms (hTM1–5) and enriched hTMs from colon epithelial cells and colonic smooth muscle. Lanes 2–6 contain hTM1–5, respectively. The molecular weights of various hTM isoforms vary from 30,000 to 40,000. Lane 7 contains enriched TM preparation from colonic smooth muscle, and lane 8 contains enriched TM preparation from colonic epithelial cells. The colonic epithelial cells contain low-molecular-weight hTMs (30,000–32,000), whereas smooth muscle contains mostly high-molecular-weight hTMs (38,000–40,000).

testing. As blanks, additional wells were coated with BSA (0.5 μ g/100 μ L). All of the hTM isoforms were tested simultaneously with the supernatants. After an overnight incubation at 4°C, plates were washed as above in PBS and 0.05% Tween 20 (200 μ L/well). Nonspecific binding was blocked with 0.25% BSA in PBS (100 μ L/well) for 30 minutes at 37°C. After three washings, diluted supernatants (1:1) or sera (1:100) were added in triplicate (100 μ L/well). A polyclonal anti-TM antibody (1:800 dilution) raised in rabbit that reacts with all of the isoforms was included as an internal positive standard. Plates were incubated for 2 hours at 25°C and then overnight at 4°C. After three washings, the alkaline phosphatase-conjugated goat anti-human IgG (100 μ L at 1:6000) and goat anti-rabbit IgG (100 μ L at 1:10,000; Sigma Chemical Co., St. Louis, MO) was added for the measurement of IgG antibodies to TMs. For the IgG subclass analysis, alkaline phosphatase-

labeled anti-human IgG1, IgG2, and IgG3 were used (Zymed, San Francisco, CA) at 1:6000 (100 μ L). After 1 hour at 25°C and three washings, the substrate *p*-nitrophenyl-phosphate-disodium was added (100 μ L; 5 μ g/mL). After 1 hour at 25°C, plates were read at 405 nm. Results for each sample were expressed as the mean OD $\pm$ SD of triplicate values with the blank value against BSA subtracted from each value.

Statistical Analysis

ELISA results from both total and TM-specific IgG antibodies were not normally distributed in all groups. Therefore, the nonparametrical Mann-Whitney *U* test was used for the statistical analysis of the data among groups, and results are expressed as means $\pm$ SEM in both the text and tables.

Results

Figure 1 shows SDS-PAGE analysis followed by Coomassie blue staining of the recombinant hTM isoforms (hTM1–5 in lanes 2–6, respectively) and enriched TMs from colon smooth muscle (lane 7; mol wt, 38,000–40,000) and colonic epithelial cells (lane 8; mol wt, 30,000–32,000). Recombinant hTM isoforms were synthesized by us from the respective cDNA clones (hTM1, 2, 3, and 5), expressed in the bacterial system, and purified. A small amount of recombinant hTM4 was available, and this was used as a reference sample to examine the specificity of the isoform-specific anti-hTM monoclonal antibodies. The homogeneity of each of the purified hTM isoforms can be seen (Figure 1). The molecular weights of various isoforms varied from 30,000 to 40,000. The anti-*E. coli* polyclonal antibody did not react with any of the purified hTM isoforms, indicating that there is no major contaminating protein from the *E. coli* used for the synthesis of hTMs. As shown in Figure 2, using isoform-specific anti-TM murine monoclonal anti-

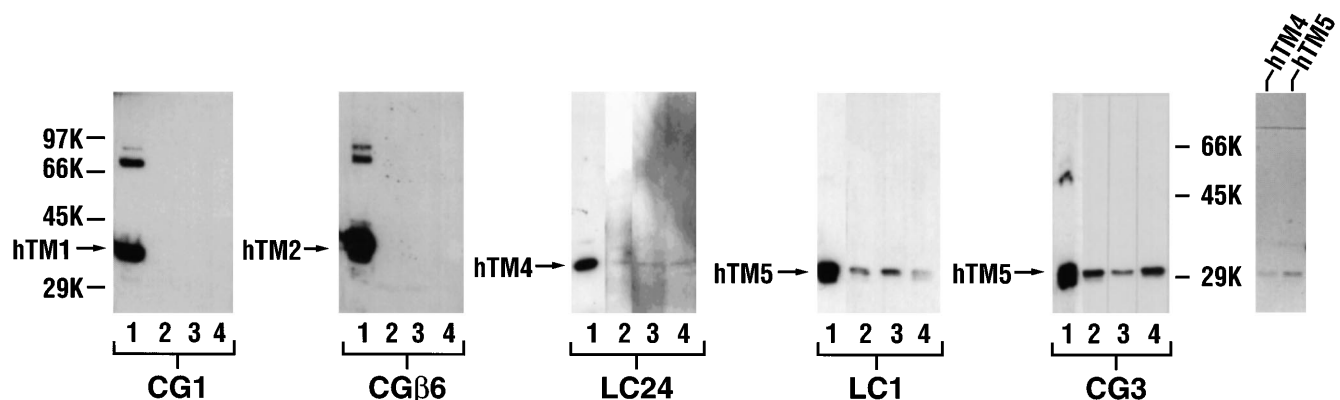


Figure 2. Immunotransblot analysis of the enriched TM extracts from colonic and jejunal epithelial cells using isoform-specific monoclonal antibodies CG1 (anti-hTM1), CG β 6 (anti-hTM 2 and 3), LC24 (anti-hTM4), LC1 (anti-hTM5), and CG3 (anti-hTM5). Lane 1 in each panel contains respective recombinant hTM isoforms. For CG β 6, hTM2 was used. Lanes 2 and 3 in each panel contain enriched TM preparations from two different specimens of colonic epithelial cells, and lane 4 in each panel contains enriched TM from jejunal epithelial cells. Both colonic and jejunal epithelial cells contain hTM5 and hTM4, and there was no detectable hTM1, 2, or 3. The far right lanes show recombinant hTM4 and hTM5 (mol wt, $\sim$ 30,000) separated by SDS-PAGE and stained with Coomassie blue.

bodies, the specificity of various hTM isoforms can be shown by transblot analysis. The immunoreactivity was also tested by ELISA. Each of the hTM isoforms was examined using all of the monoclonal antibodies to determine the presence or absence of any cross-reactivity. Isoform specificity of the hTMs and monoclonal antibodies were clearly evident. Whereas CG1 monoclonal antibody (IgG1 subclass) reacted exclusively with hTM1, LC-24 monoclonal antibody (IgG1 subclass) reacted with hTM4 only. LC1 (IgG1 subclass) and CG3 (IgM isotype) monoclonal antibodies both reacted solely with hTM5. These two monoclonal antibodies react with two different epitopes of hTM5 at amino acid residues 1–18 and 29–44, respectively.^{15,18} CG β 6 monoclonal antibody (IgM isotype) reacted with both hTM2 and hTM3. At this time, we do not have a monoclonal antibody to differentiate between hTM2 and hTM3. As also shown in Figure 1, colonic smooth muscle mainly contained high-molecular-weight TM isoforms (lane 7), whereas colonic epithelial cells contained low-molecular-weight TM isoforms (lane 8).

Western blot analysis of TMs extracted from normal colonic and jejunal epithelial cells and intestinal smooth muscle is shown in Figures 2 and 3. The predominant hTM isoforms in normal colonic and jejunal epithelium are hTM5 and hTM4, which are lower-molecular-weight isoforms 30,000–32,000 compared with hTM1–3 (mol wt, 36,000–40,000; Figures 1 and 2). Epithelial extracts did not contain any detectable amount of hTM1, 2, or 3 (Figure 2). In contrast, intestinal smooth muscles from

both colon and jejunum contained predominantly higher hTM isoforms, i.e., hTMs 1, 2, 3 isoforms (Figure 3). In the smooth muscle, there was also a trace of hTM4, but no detectable amount of hTM5. Colonic and jejunal mucosal extracts contributed by epithelial and nonepithelial mucosal tissue, including muscularis mucosa, contained all five isoforms, as can be expected (data not shown).

Total IgG in LPMC Supernatants From UC in Comparison With CD and Non-IBD Controls

Total IgG levels were higher in LPMC supernatants from patients with UC (452 ± 155 ng/mL) and CD (404 ± 147 ng/mL) compared with non-IBD subjects (127 ± 29 ng/mL; $P = 0.04$). Total IgG released by LPMCs from UC and CD did not differ significantly. LPMCs from patients with symptomatic UC spontaneously released higher levels of total IgG (870 ± 243 ng/mL) than LPMCs from UC in remission (93 ± 31 ng/mL; $P = 0.03$), CD in remission ($P = 0.04$), and non-IBD ($P = 0.003$). Levels of total IgG were also higher in LPMC supernatants from symptomatic CD (670 ± 219 ng/mL) than CD in remission (86 ± 33 ng/mL; $P = 0.04$), UC in remission ($P = 0.04$), and non-IBD ($P = 0.002$). There were no significant differences in terms of total IgG release between LPMCs isolated from biopsy specimens and surgical specimens from the patients with IBD and the non-IBD controls.

In patients with IBD, total IgG levels in LPMC supernatants did not significantly differ also in relation to steroid treatment, disease duration, or age. As expected, PWM stimulation did not significantly increase the release of total IgG by LPMCs from all groups (data not shown).

IgG Antibodies Against Different hTM Isoforms Present in LPMC Supernatants From UC in Comparison With CD and Non-IBD Controls

In the ELISA, using the supernatants from cultured LPMCs against the four hTM isoforms (hTM1, 2, 3, and 5), the OD values are shown for each of the 48 patients in the scatterogram (Figure 4). The thick horizontal bar indicates the mean value for each patient's group against the hTM isoforms. The immunoreactivity of LPMC supernatants from UC against hTM1 and hTM5 was significantly higher compared with LPMC supernatants from both CD and non-IBD (hTM1, $P = 0.006$ and $P = 0.001$; hTM5, $P = 0.04$ and $P = 0.01$, respectively). However, the reactivity against hTM1 and hTM5

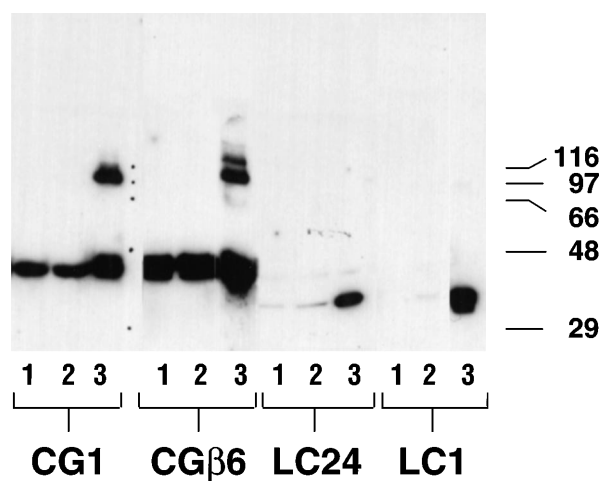


Figure 3. Immunotransblot analysis of enriched TM preparations from the smooth muscle of intestine probed with the hTM isoform-specific monoclonal antibodies against hTM1–5. Lane 3 in each panel contains respective recombinant hTM isoforms. Lane 1 contains enriched TM extract from colonic smooth muscle, and lane 2 contains a similar preparation from jejunal smooth muscle. Both colonic and jejunal smooth muscle contain mainly hTM1, hTM2, and/or hTM3 and a trace of hTM4. There was no detectable hTM5.

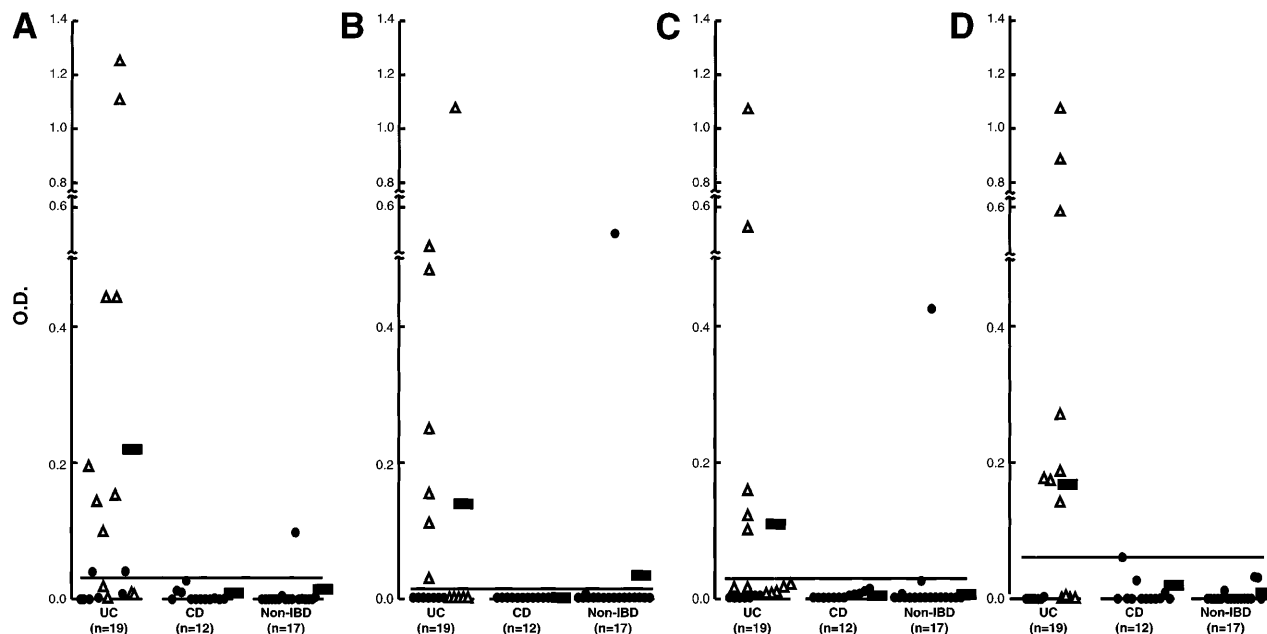


Figure 4. Scattergram showing the immunoreactivity of spontaneously produced IgG by LPMCs against (A) hTM1, (B) hTM2, (C) hTM3, and (D) hTM5. The individual OD value for each of the LPMC supernatants from the 48 subjects with UC ($n = 19$), CD ($n = 12$), and non-IBD controls ($n = 17$) is shown. In the UC group, there were 12 symptomatic patients (Δ), and 7 patients were in remission ($\bullet$). Of the 12 patients with CD with colonic disease, 6 were active and 6 were in remission. —, Mean value plus 3 SD for the CD group against the respective hTM isoforms; ■, mean value for the group.

did not differ between CD and non-IBD controls. Unlike values for hTM1 and hTM5, the mean OD values for IgG antibodies against hTM2 and hTM3 in LPMC supernatants did not significantly differ among the total group of UC, CD, and non-IBD controls.

The supernatants from cultured LPMCs from 12 of 19 patients with UC (63%) had clearly higher anti-TM reactivity against one or multiple hTM isoforms compared with the mean + 3 SD value for CD (shown in Figure 4 by the long horizontal line for each of the four hTM isoforms). These 12 patients included all 10 patients who were symptomatic both clinically and endoscopically, and 2 patients who were in clinical remission. Six of the 10 symptomatic patients had reactivity against both the hTM1 and the hTM5 isoform. Four additional patients (2 symptomatic and 2 in remission) showed high immunoreactivity against hTM1 but not hTM5, and 2 other patients (both symptomatic) showed reactivity against hTM5 and not against hTM1. Five of these 6 patients' sera (3 of 4 in the hTM1 group and 2 of 2 in the hTM5 group) were also tested against hTM1 and hTM5. Four of the 5 sera (2 in each group) showed parallel anti-hTM reactivity, whereas 1 serum did not react with either of the two hTM isoforms. The 2 sera in the hTM1 group reacted with both hTM1 and hTM5.

The specificity of the IgG reactivity in the LPMC supernatant against the hTM5 isoform was examined by an inhibition ELISA²⁹ using anti-hTM5 monoclonal

antibody, LC1 (IgG1 subtype). The supernatants from 5 of the 8 patients with UC reactive to hTM5 were available for the additional inhibition assay. LC1 antibody blocked the binding of each of the five LPMC supernatants with hTM5. The mean percentage of inhibition of the binding was $87\% \pm 6\%$, with a range of 74%–97%. The control antibody did not show any significant inhibition ($8\% \pm 6\%$). The change in the reactivity, if any, of these five supernatants was also examined by inhibition ELISA using the extract prepared from *E. coli* BL21 (D3) LysS strain. There was no significant inhibition of the binding of supernatants to the hTM5 by the addition of the *E. coli* extract. The mean percentage of inhibition by the addition of the *E. coli* extract was only $6\% \pm 5\%$, which was similar to that in the control.

The specific reactivity of the anti-hTM5 autoantibody was further examined by immunoaffinity chromatography.⁹ Because the amount of IgG present in the LPMC supernatants is very low and the volume of supernatant is very limited, affinity chromatography using the supernatants could not be performed, and hence we have used purified serum IgG. Recombinantly purified hTM5 (total, 8 mg) was covalently coupled with CnBr-activated Sepharose 4B (bed volume, 4 mL). IgG was purified from serum from 2 patients with UC and 2 non-IBD controls using protein G. These four sera were selected on the basis of their relatively higher OD values in the respective

group against hTM5 in the ELISA. Three milligrams of purified serum IgG from each subject was subjected to the hTM5 immunoaffinity column chromatography. Because the efficiency of the affinity column may be reduced by repeated use, the two non-IBD serum IgG preparations were sequentially subjected to the affinity column first and then followed by the two UC serum IgG. The amounts of serum IgG bound to the column were 45 and 66 μ g in the 2 patients with UC, whereas there was no binding of IgG to the hTM5 column in 1 of the 2 non-IBD controls. The bound IgG with the second non-IBD subject was 10 μ g. Using immunotransblot analysis with a chemiluminescence detection system, the immunoreactivity of the affinity column-eluted IgG (1 μ g/mL) against the hTM5 isoform (2 μ g/lane) is shown in Figure 5. Affinity column-eluted IgG from both the UC serum IgG clearly reacted with hTM5 (Figure 5, lanes 1 and 3). The IgG eluted from the column in 1 of the 2 non-IBD subjects did not show any reactivity against the hTM5 isoform (Figure 5, lane 2). The small amount of IgG that bound to the column from this non-IBD subject was probably nonspecific binding. Furthermore, the unbound IgGs (flow-through fraction from the column) from both of the patients with UC as well as from the non-IBD controls did not show any reactivity in the immunotransblot analysis against hTM5, even with 5 μ g/mL concentration (data not shown).

None of the 12 patients with CD (6 active and 6 in remission) had values beyond the cutoff line of mean + 3 SD for CD. Only 1 of the 17 patients in the non-IBD control group had high OD value against hTM1, 2, and

3, but not against hTM5 (Figure 4). In general, LPMCs from the symptomatic group with UC produced higher IgG antibodies to hTM1 and hTM5 (mean OD $\pm$ SE: 0.386 ± 0.162 and 0.337 ± 0.140 , respectively) than LPMCs from UC in remission (mean OD $\pm$ SE: 0.049 ± 0.019 and 0.051 ± 0.025 , respectively; $P = 0.04$), symptomatic CD (mean OD $\pm$ SE: 0.007 ± 0.005 and 0.016 ± 0.009 , respectively; $P = 0.03$), and non-IBD (mean OD $\pm$ SE: 0.006 ± 0.002 and 0.003 ± 0.002 , respectively; $P = 0.002$). Although not statistically significant as a whole group of UC, the LPMC supernatants from 7 of the 12 UC patients who were reactive against hTM1 and/or hTM5 also showed higher reactivity against hTM2 and 5 of these 7 against hTM3 as well (Figure 4), and all of them were symptomatic. However, the OD values for the IgG antibodies against any of the four hTM isoforms did not differ between patients with CD, symptomatic or in remission, and the non-IBD controls.

The higher IgG immunoreactivity against hTM1 and hTM5 by LPMC supernatants in UC was not related to higher levels of total IgG levels in the same samples. Indeed, the total IgG for UC-positive vs. UC-negative for hTM1 was 351 ± 249 vs. 538 ± 206 ng/mL, respectively, and for hTM5, the total IgG was 466 ± 329 vs. 445 ± 184 ng/mL ($P =$ not significant for both). PWM stimulation did not significantly increase the release of hTM-specific IgG antibodies from LPMCs (data not shown). Within each disease group, levels of total and hTM-specific IgG did not significantly differ in supernatants from LPMCs isolated from biopsy or surgical specimens, or between LPMCs isolated from the distal 20 cm of the colon or from the descending colon proximal to 20 cm. Levels of total and hTM-specific IgG antibodies produced by LPMCs from UC also did not differ in relation to clinical variables such as age, sex, duration of disease, or therapy.

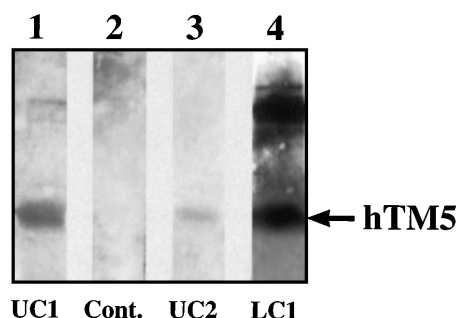


Figure 5. Immunotransblot analysis of recombinantly synthesized hTM5 isoform probed with serum IgG eluted from the affinity column prepared with hTM5 covalently coupled with CnBr-activated Sepharose 4B. Each of the four lanes contained 2 μ g of purified hTM5. Lanes 1 and 3 were probed with affinity column-eluted IgG (1 μ g/mL, total 5 mL used for each lane) from the 2 patients with UC, UC1, and UC2. Lane 2 was probed with an equal amount of IgG from a non-IBD control (Cont.) subject. Lane 4 was probed with anti-hTM5 monoclonal antibody (LC1, 1 μ g/mL) as a positive control. A clear reactivity against hTM5 with both UC serum IgGs is evident. The non-IBD control did not react. The band at $\sim 64,000$ molecular weight is most likely hTM5 dimer, which reacted with the anti-hTM5 monoclonal antibody and with one of the two UC IgGs.

IgG Subclass Antibodies Against hTM1 and hTM5 Isoforms in Culture Supernatants and Sera From UC in Comparison With CD and Non-IBD Controls

As shown in Table 1, the major IgG reactivities against hTM1 and hTM5 of LPMC supernatants from patients with UC belong to the IgG1 subclass. Antibodies against hTM1 and hTM5 belonging to IgG2 and IgG3 subclasses were almost undetectable in the LPMC supernatants. The IgG1 reactivities against hTM1 and hTM5 of the LPMC supernatants from patients with UC were significantly ($P = 0.02$ – 0.04) higher compared with those from patients with CD and from non-IBD controls (Table 1). The IgG1 reactivities against hTM1 and hTM5 for the UC sera were also significantly

Table 1. Levels of IgG Subclass Antibodies (IgG1, IgG2, and IgG3) Against hTM1 and hTM5 Isoforms in Supernatants From Cultured Mucosal Lymphocytes (LPMCs) and Sera From Patients With UC and CD and Non-IBD Controls

Patients	Antibodies against hTM1 and hTM5 isoforms (mean OD $\pm$ SE)			
	hTM1		hTM5	
	LPMC	Sera	LPMC	Sera
UC				
IgG1	0.146 $\pm$ 0.010 ^a	0.248 $\pm$ 0.021 ^b	0.208 $\pm$ 0.026 ^c	0.691 $\pm$ 0.077 ^d
IgG2	0.003 $\pm$ 0.001	0.108 $\pm$ 0.013 ^e	0.014 $\pm$ 0.009	0.218 $\pm$ 0.042 ^f
IgG3	0.010 $\pm$ 0.005	0.007 $\pm$ 0.004	0.012 $\pm$ 0.008	0.004 $\pm$ 0.002
CD				
IgG1	0.116 $\pm$ 0.007	0.152 $\pm$ 0.032	0.133 $\pm$ 0.010	0.420 $\pm$ 0.111
IgG2	0.001 $\pm$ 0.002	0.010 $\pm$ 0.002	0.003 $\pm$ 0.006	0.058 $\pm$ 0.028
IgG3	0.005 $\pm$ 0.002	0.002 $\pm$ 0.003	0.003 $\pm$ 0.003	0.002 $\pm$ 0.003
Non-IBD				
IgG1	0.113 $\pm$ 0.007	0.102 $\pm$ 0.008	0.135 $\pm$ 0.007	0.363 $\pm$ 0.080
IgG2	0.003 $\pm$ 0.003	0.003 $\pm$ 0.002	0.001 $\pm$ 0.003	0.063 $\pm$ 0.018
IgG3	0.003 $\pm$ 0.002	0.004 $\pm$ 0.002	0.002 $\pm$ 0.006	0.002 $\pm$ 0.003

^a*P* = 0.02 UC vs. CD and *P* = 0.03 UC vs. non-IBD.

^b*P* = 0.01 UC vs. CD and *P* = 0.03 UC vs. non-IBD.

^c*P* = 0.04 UC vs. CD and non-IBD.

^d*P* = 0.04 UC vs. CD and non-IBD.

^e*P* = 0.03 UC vs. CD and *P* = 0.01 UC vs. non-IBD.

^f*P* = 0.04 UC vs. CD and non-IBD.

(*P* = 0.01 to 0.04) higher compared with sera from patients with CD and controls. In addition, unlike mucosal IgG subclass antibodies against hTM1 and hTM5, UC sera also contained IgG2 subclass antibodies against hTM1 and hTM5. This reactivity was significantly (*P* = 0.01–0.04) higher in UC compared with CD and non-IBD controls (Table 1). Such a difference was not observed in UC sera for the IgG3 reactivity against hTM1 or hTM5. There was no difference in the anti-hTM1 and anti-hTM5 reactivities in CD sera compared with non-IBD controls (Table 1).

Discussion

The results from this study show that normal human intestine contains several isoforms of TMs, and there are clear differences between epithelium and smooth muscle. Epithelial cells from both colon and jejunum contain mainly hTM5 followed by hTM4, whereas cells from smooth muscle contain hTMs1, 2, and 3. Because the monoclonal antibody CG β 6 does not differentiate between hTM2 and hTM3, at present we do not know whether intestinal smooth muscle contains either or both of these two hTM isoforms. Because the mucosa contains both epithelium and muscularis propria and other cells, total mucosal TM preparation included all of the five isoforms. Indeed, these results explain our earlier findings of multiple TM-related bands in colonic mucosal extracts.²³

TMs can induce significant autoimmune responses.¹⁷ Physicochemical and biochemical analyses of 109 human

autoantigens showed that sequences longer than 27 residues with coiled-coil α helices are the forerunner autoantigens for various human autoimmune diseases, and several sequences of TM residues were considered to be the most potent autoantigens.¹⁷ TM has also been found to be the major allergen related to seafood and house dust mite allergies,^{16,30} and ~80% of shrimp-allergic subjects contain IgE and IgG serum antibody reactive with a 36-kilodalton TM extracted from shrimp.¹⁶

Multicellular organisms show a multiplicity of TM isoforms. For example, at least 12 isoforms have been identified in the rat on the basis of primary sequence differences with molecular weights ranging from 30,000 to 40,000.¹⁴ At least eight distinct isoforms of TMs have been isolated from human fibroblast,¹⁵ and organ-specific isoforms have been well characterized.¹⁴ Four genes have been identified in humans, and various isoforms are synthesized by an alternate RNA-splicing mechanism restricted to three exon regions.^{14,31–33} The TM molecule is a rod-shaped, coiled-coil dimeric protein associated with the actin filaments, caldesmone, and tropomodulin involved in cell shape, cytokinesis, intracellular granule movement,^{14,33} and other functions such as maintenance of cellular polarity in developing tissue.³⁴ Although by and large TMs are intracellular proteins, they have also been found in the cell membrane, e.g., in erythrocytes,³⁵ including at the root of the brush border of the small intestine.³⁶ Independent protein with partial (35%) homology to TM has also been isolated from the brush border of the small intestine.³⁷

Several independent studies have recently shown IgG autoantibody responses against TMs in UC but not in CD.^{13,38,39} Using a large number of patients with IBD and their first-degree blood relatives, one study from Italy reported that sera from approximately two thirds of these Italian patients with UC contained autoantibody against hTM5, and about half of the patients had autoantibody against hTM1.³⁸ Twenty percent of these patients' healthy first-degree relatives were found to have autoantibody against hTM1 only. In contrast, patients with CD and their healthy relatives did not have such an antibody response ($P < 0.001$).³⁸ Another study from Japan also reported the presence of anti-TM antibody in the circulation of patients with symptomatic UC,³⁹ although hTM isotype was not analyzed. Furthermore, these investigators reported that tryptic peptide(s) of TM bind with specific class II molecule (HLA-DPw9) and is expressed on the surface of L cells transfected with class II genes, and the serum from UC contains autoantibody that recognizes the TM peptide on the surface of transfected L cells.³⁹ In one study from the United Kingdom, anti-TM antibodies were not shown in UC.⁴⁰ In this study, from colonic mucosa, two TM-related proteins of 37 and 39 kilodaltons, which comigrated in SDS-PAGE with porcine TM, were electroeluted and used in an ELISA as antigen. No reactivity was found with any of the human sera, including those from patients with UC and CD and non-IBD controls. This complete nonreactivity can be explained by the loss of antigenicity by SDS treatment that is a common phenomenon with autoantibodies.⁴¹ Using colonic mucosal extract enriched in TMs, we previously reported nonreactivity of autoantibodies in UC by immunotransblot analysis, although the reactivity was evident in the ELISA using the same extract prepared without SDS treatment.²³

Recently, IgG antibodies against TM have been shown in an animal model of TCR^{-/-} mice that develop spontaneous colitis. Interestingly, there was a positive correlation of anti-TM antibody titer with the severity of colitis.⁴² However, it is unknown whether the autoantibody response in this mouse model of colitis is directed against any of the known hTM isoforms. These investigators further showed that autoantibody-producing B cells directed against TM were increased in the appendix lymphoid follicle compared with in Peyer's patches, suggesting selectivity of the mucosal immune response against TM.⁴³

In this study, we have purified recombinant hTM isoforms, and using the four hTM isoforms (hTM1–3 and hTM5), we have shown that unstimulated LPMCs from the colon of 12 of 19 patients (63%) with UC synthesize IgG autoantibody in vitro that preferentially reacts

against certain hTM isoforms, particularly hTM5 and hTM1, as shown by ELISA. The specificity of the autoantibody reactivity against hTM5 was also shown by inhibition of the binding of autoantibody with the hTM5 monoclonal antibody. In addition, hTM5 affinity chromatography showed immunoreactivity of UC serum IgG against hTM5, and the binding of the affinity column eluted IgG to hTM5 using immunotransblot analysis. As mentioned previously, in our earlier study,²³ we could not show the immunoreactivity against colonic TMs by immunotransblot analysis. This is probably a result of the loss of antigenicity by SDS treatment and a low amount of the autoantibody in the LPMC supernatants. However, a significant enrichment of the autoantibody by the immunoaffinity column facilitated the clear reactivity of the autoantibody against the recombinant hTM5 in the transblot analysis, as shown in this study. The autoantibody response against the purified hTM5 isoform was not influenced by the *E. coli* extract, as shown by the inhibition ELISA, and in the transblot analysis there was no other immunoreactive band other than the hTM5 protein. Ten of the 12 patients with anti-TM autoantibody reactivity were symptomatic. After antigenic stimulation by a TM peptide(s), the autoreactive B cells may be polyclonal and may produce antibodies to different parts of the TM isoform, which will show cross-reactivity to other hTM isoforms because of the shared areas among the isoforms. Indeed, 5 of the 12 symptomatic patients with UC showed anti-TM activities to all four hTM isoforms, whereas the others had more restricted antibody activity against hTM5 and/or hTM1. However, none of the patients with CD showed any reactivity against any of the four hTM isoforms, although each of the 12 patients with CD had colonic involvement, and 6 of these patients had active disease. This suggests that the autoantibody response is not a secondary effect of colonic inflammation. The absence of anti-TM autoantibody response in the remaining 7 of 19 patients with UC who were mostly in remission may be due to insufficient in vitro synthesis of IgG by LPMCs for detection by the ELISA. The nonreactive group may also suggest a subgroup. Although we did not examine the purified hTM5 extracted from patient's own colonocytes and instead used recombinant hTM5, it is expected that local autoantibody should react with the autologous hTM5. This is supported by our previous observation that colon tissue-bound IgG from patients with UC recognized the TM-related 40,000-molecular weight protein isolated from the autologous colon.⁹ In this study, we did not examine the perinuclear antineutrophil cytoplasmic antibody (pANCA) status of the patients. However, the study from Italy³⁸ reported a significant ($P < 0.04$) correlation of positive pANCA

status with the presence of anti-hTM5 antibody in sera from patients with UC. The predominant hTM isoform present in the colonic epithelium, as shown here, is hTM5, and autoantibody response against hTM5 is evident. hTM4 is also present in smaller amounts in the epithelial cells. Recombinant hTM4 was not available, and antibody response against hTM4 was not studied. It is interesting that a significant autoantibody response in UC was shown against hTM1. This may suggest that the major autoantigenic epitope(s) may be located in the common region(s) between hTM5 and hTM1. The autoantibody response against hTM1 may also be the result of an ectopic expression of an hTM1-related epitope in UC colonic epithelium that is currently unknown.

To understand a possible role of hTM5 or related protein in the pathogenesis of UC, one needs to explain that because hTM5 is present in both colonocytes and small intestinal enterocytes, why is there no small bowel involvement in patients with UC? It is intriguing that we recently observed a selective expression of hTM5 on the surface of colon epithelial cells and not on the surface of small intestinal enterocytes, although both cells have intracellular hTM5.⁴⁴ Of all the five hTM isoforms (hTM1–5), only the hTM5 epitope was identified on the surface of normal colonic epithelial cells by fluorescence-activated cell sorter analysis and immunoelectronmicroscopy.⁴⁴ Indeed, none of the monoclonal antibodies against hTM1–4 reacted with the freshly isolated normal colon epithelial cells, whereas CG3 monoclonal antibody (anti-hTM5) consistently reacted on colonocytes in the fluorescence-activated cell sorter analysis. In contrast to colonocytes, small intestinal (both jejunal and ileal) epithelial cells, however, did not react with any of the monoclonal antibodies, including CG3. This raises the possibility that the hTM5-related epitope expressed on the surface of colon epithelial cells may be involved in stimulating the effector immune system as well as a target for immune attack restricted to the colon. Such an organ-specific antigenic display may play an important role for a local trigger of immune response and perpetuation of the disease in the colon and not in the small intestine. Indeed, antigenic display of myosin has recently been found to be involved in the pathogenesis of autoimmune myocarditis,⁴⁵ which may also be genetically determined.⁴⁶ Molecular mimicry, related to a specific peptide in streptococcal M protein and tropomyosin, has been found to be an important pathogenetic factor in autoimmune myocarditis.⁴⁷ Such a molecular mimicry against bacterial product⁴⁸ may influence the mucosal immune functions and be responsible for perpetuation and flare-up of the disease.

Whereas IgG autoantibody response against TM in patients with UC has been shown by three groups of investigators,^{13,38,39} T-cell response to TMs in UC is unknown. Physicochemically, TM peptide(s) could be strong potential autoantigen(s).¹⁷ The knowledge of normal colonic epithelium-specific hTM isoforms and the autoantibody response against hTMs in UC will facilitate future studies in focusing on the identification of the hTM peptide(s) capable of cellular and humoral immune responses in patients with UC. Such investigation should provide important biochemical information to explain the autoimmune mechanism in UC and may help in the development of strategies for possible immune therapy.

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