

Autoimmunity to Cytoskeletal Protein Tropomyosin

A Clue to the Pathogenetic Mechanism for Ulcerative Colitis¹

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ABSTRACT. Autoimmunity has been emphasized in the pathogenesis of ulcerative colitis and various anticolon antibodies have been described. Nevertheless, the nature of the autoantigens has been elusive. Using ulcerative colitis colon-eluted IgG antibody, we earlier detected an M_r 40,000 protein, P40, in colon extract. In our study, we purified P40 from human colon to apparent homogeneity, sequenced two peptides after proteolytic digestion, and demonstrated that the P40 belongs to the tropomyosin family. Furthermore, we demonstrated that blood serum from 95% of patients with ulcerative colitis contains antibodies that are reactive to tropomyosin. *Journal of Immunology*, 1993, 150: 2487.

UC³, a chronic illness of unknown etiology, is characterized by diarrhea, rectal bleeding and, at times, fever, weight loss, and chronic ill health. The disease is associated with intervals of acute exacerbation and remission. The worldwide incidence of the disease is about 2 to 6 per 10⁵ population. The onset of UC usually occurs between adolescence and the third decade of life. The disease has a high familial incidence.

Autoimmunity has been implicated in the pathogenesis of UC. Serum autoantibodies to colonic epithelial cells have been identified in up to 60% of patients (1–3). In addition, the deposition of IgG antibodies and activated complement products on colonic epithelium in patients

with UC has been described (4). However, the cellular Ag recognized by these antibodies is unknown. In an earlier study, we observed that tissue-bound IgG antibody, when extracted from colons, affected by UC, recognized an P40 present in all colon extracts. IgG similarly extracted from colons with other diseases did not show such reactivity (5). The structure and function of this protein has been unknown.

Our study was undertaken to purify P40 from human colon to homogeneity, sequence the protein, and demonstrate its immunoreactivity against UC sera.

Materials and Methods

Extraction of P40

Twelve normal specimens of colon were obtained from patients undergoing colectomy for colon cancer. After the serosal fat had been removed from it, the tissue was minced in 50 ml of buffer A, containing 50 mM Tris HCl, pH 8.0, 0.15 M NaCl, 2 mM EDTA, 2 mM PMSF, 0.3 μ M aprotinin, 1 μ M pepstatin, and 1 μ M leupeptin. The tissue was washed in the same buffer at least seven times until the supernatant was clear. The final pellet was homogenized in buffer A with 10 mM EDTA. The homogenate was centrifuged at 10,000 $\times g$ for 30 min. The resulting supernatant was ultracentrifuged at 100,000 $\times g$ for 90 min. The supernatant was frozen and thawed three times, then centrifuged for 10

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³ Abbreviations used in this paper: UC, ulcerative colitis; P40, M_r 40,000 protein; FPLC, fast phase liquid chromatography; HIC, hydrophobic interaction chromatography; CD, Crohn's disease; PBS, pH 7.4, containing 0.1% Tween-20.

min at $10,000 \times g$ to remove precipitates. The supernatant was dialyzed against 20 mM bis-tris propane, pH 6.5, (buffer B) at 4°C. To remove lipids, the supernatant was mixed vigorously with an equal volume of 1,1,2-trichlorotrifluoroethane, and centrifuged. The aqueous phase was filtered through 0.20- μ cellulose acetate membrane filter. The filtrate was then subjected to anion exchange chromatography using a Mono Q HR 5/5 column (Pharmacia Fine Chemicals, Piscataway, NJ) in FPLC. Five mg of the colon extract were loaded in the column, which was then washed with buffer B. Proteins were eluted with a step gradient of 0.2, 0.35, and 0.48 M NaCl in buffer B. The 0.35 M and 0.48 M NaCl eluates were pooled and subjected to HIC. For this purpose, a 1×10 -cm Econocolumn (Bio-Rad, Richmond, CA) was packed with a 5-ml bed volume of phenyl Sepharose 4B equilibrated with 20 mM phosphate and 0.15 M NaCl, pH 7.4, containing 0.8 M ammonium sulfate. After extensive washing, the column was eluted stepwise with 20 mM phosphate containing 0.15 M NaCl, pH 7.4, then with distilled water.

Analysis of P40 and sequencing of tryptic peptides

Eluted proteins were analyzed by 10% SDS-PAGE. Immunoreactivity was determined by immunotransblot (5, 6) and ELISA techniques (5, 7). The amino acid composition of the P40 was then analyzed by means of a Beckman Model 6300 amino acid analyzer (Beckman Instruments, Inc., Fullerton, CA) using ninhydrin as the detection method. Analysis of the N-terminal of the P40 revealed that it was blocked; hence, the protein was subjected to tryptic digestion in situ (8), followed by microsequencing (8). Briefly, after the protein had been electroblotted, the nitrocellulose strips containing the P40 were transferred to a microcentrifuge tube and incubated with the appropriate concentration of enzyme for 30 min at 37°C. The enzyme-to-substrate ratio was kept at 1:20 (w/w). After digestion, the resulting peptide fragments were separated by narrow-bore reverse-phase HPLC, then sequenced in a gas-phase sequencer (9).

Immunoreactivity of P40 against patients' sera

In preparation for ELISA analysis of the P40, blood serum was obtained from a total of 130 subjects. These included 41 patients with symptomatic UC and 33 patients with active CD, 24 of whom had colonic involvement. UC and CD were diagnosed on the basis of patients' histories, sigmoidoscopic or colonoscopic examinations, and radiographic and histologic studies of intestinal specimens. The patients with UC ranged in age from 12 to 68 yr (mean age 32 yr). The duration of their disease varied from 2 mo to 18 yr (mean 4.8 yr). The patients with CD ranged in age from 15 to 72 years (mean, 37 yr). The duration of CD ranged from

2 mo to 40 yr (mean 6.9 yr). Patients with both UC and CD had active disease when the blood samples were collected. Most of them were not being treated with corticosteroids or other immunosuppressive drugs at that time. Serum samples were also obtained from 32 patients with acute diarrhea caused by specific pathogens, which, as identified by stool examinations, were as follows: *Shigella*, 7; *Salmonella*, 6; *Giardia*, 6; amebiasis, 5; *Cryptosporidia*, 4; *Clostridium difficile*, 3; and *Campylobacter*, 1. Additional serum samples were obtained from 10 patients with symptomatic SLE who were included as an autoimmune-disease control group, and from 14 healthy subjects. All serum samples were decanted by heating at 56°C for 30 min, then delipidated and ultracentrifuged at $30,000 \times g$ for 30 min to remove aggregates.

ELISA

Initial sequencing data from above experiments suggested that P40 belongs to tropomyosin family. Therefore, for the ELISA we have used P40 purified from the colon as described above and commercially available tropomyosin from porcine muscle (T-2400, Sigma Chemical Co., St. Louis, MO) with similar molecular weight. Both proteins reacted with antitropomyosin mAb (clone TM311, Sigma) and antitropomyosin polyclonal antibody (T-3651, Sigma). A 96-well microtitre plate (Immulon II, Dynatech Laboratory, Chantilly, VA) was coated with 0.3 μ g of the protein per well in 100 μ l of carbonate buffer, pH 9.6, overnight at 4°C. Thereafter, wells were washed with PBS-Tween 20. Plates were blocked with 5% BSA and 1% goat serum for 1 h at 37°C. Each serum was diluted 200-fold. Several sera from both UC and non-UC groups were serially diluted up to 3200-fold. One hundred μ l of the diluted serum were added per well in triplicate, incubated for 2 h at 37°C, and washed. A total of 100 μ l of diluted (1/5000) alkaline phosphatase-conjugated goat anti-human IgG (H+L) (Zymed, South San Francisco, CA) was added to each well. The plate was then incubated for 1 h at room temperature followed by addition of substrate, *p*-nitrophenyl phosphate, 100 μ l/well in 1 mM magnesium chloride, and 50 mM sodium carbonate, pH 9.8. Blanks consisted of protein-coated wells that received similar treatment except human serum was not added in these wells. Parallel wells were incubated with anti-actin (A-2668, Sigma), antilaminin (a gift from Dr. Eva Engvall, La Jolla Cancer Research Foundation, La Jolla, CA), and antitropomyosin antibodies in place of human serum as additional controls. The plates were read at 405 nm using a Vmax kinetic microplate reader connected with the computer (Molecular Devices, Palo Alto CA). The mean blank values were automatically deducted by the computer from the experimental values.

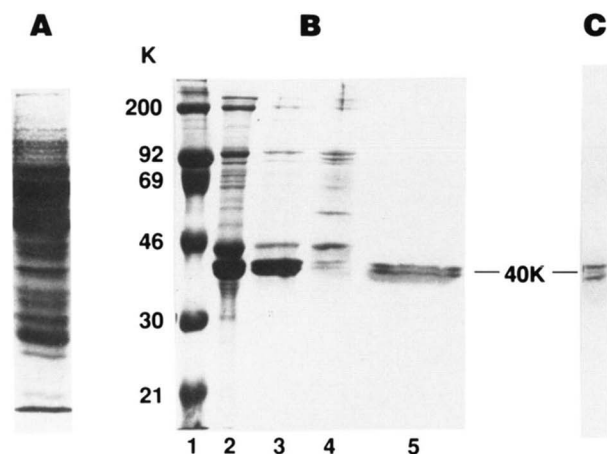


FIGURE 1. Purification of P40 by sequential ultracentrifugation, FPLC using Mono Q column and HIC: SDS-PAGE (10% gel) of different colon extracts stained with Coomassie brilliant blue (A and B). A shows solubilized proteins in a crude colon extract. B, lane 1, Marker proteins. Lane 2, Pre-FPLC colon extract processed by serial ultracentrifugation and freeze-thaw three times. Lane 3, 0.35 M NaCl eluate from Mono-Q column in FPLC. Lane 4, 0.48 M NaCl eluate from Mono-Q column. Lane 5, 25 mM phosphate eluate from HIC column showing only the P40 as doublet. C, Immunoblot analysis using antitropomyosin antibody against the purified P40 (as shown in B, lane 5); both bands reacted with antitropomyosin antibody.

Electroelution of immunoreactive 40K

Both colon extracted P40, and commercial porcine tropomyosin appeared as doublet in SDS-PAGE at P40. The two bands could be separated further from each other by running the gel at 25 V over 16 h. The gel was stained by 0.1% Coomassie brilliant blue (w/v) in 40% methanol and 10% acetic acid and then destained with methanol and acetic acid. The two bands were excised separately and gel slices were transferred to microcentrifuge tubes, minced as finely as possible with a pellet pestle (Kontes Corp., Vine-land, NJ). A total of 200 μ l of elution buffer (50 mM ammonium bicarbonate, pH 7.8, and 0.1% SDS) was added and the mixture was incubated at 37°C for 2 h, and then centrifuged to remove the acrylamide fragments. The supernatant containing the eluted protein was concentrated to dryness in a speed-vac vacuum concentrator (Savant, Farmingdale, NY). A total of 200 μ l of cold (-70°C) 80% acetone was added to the tube, vortexed for 30 s and placed in dry ice/ethanol bath for 5 min. The tube was spun in a microcentrifuge for 2 min at 4°C. The supernatant was discarded, the pellet containing the eluted protein was redried and dissolved in PBS, 7.4. The protein concentration was measured using the method described by Bradford (10). The homogeneity of the eluted proteins was checked in SDS-PAGE followed by silver nitrate staining, and immunoreactivity was examined using antitropomyosin antibodies by an ELISA and by transblot analysis. The reactivity with

Table I
Immunoreactivity of fractions of purified colon extracts against antibodies and patients' sera

	Colon Extracts	
	0.35 M NaCl eluate from FPLC Mono Q column	20 mM phosphate eluate from the HIC column
Antilaminin	0.124	0.00
Antiactin	0.043	0.00
Antitropomyosin	1.068	1.74
UC sera ($n = 9$ (1:200))	^a 0.175 $\pm$ 0.043	^b 0.262 $\pm$ 0.057
NL sera ($n = 9$ (1:200))	0.005 $\pm$ 0.002	0.012 $\pm$ 0.003
CD sera ($n = 9$ (1:200))	0.051 $\pm$ 0.011	0.059 $\pm$ 0.012

UC, Ulcerative colitis; CD, Crohn's disease; eight of these nine patients had colonic involvement; NL, normal subjects.

^a vs NL $p < 0.001$, ^a vs CD $p < 0.05$.

^b vs NL $p < 0.001$, ^b vs CD $p < 0.01$.

Table II
Amino acid composition of M, 40K protein

	Mole%
Aspartic acid	8.0
Glutamine or glutamic acid	23.9
Serine	3.7
Glycine	3.5
Histidine	0.9
Arginine	5.1
Threonine	1.7
Alanine	9.5
Proline	0.4
Tyrosine	0.9
Valine	5.2
Methionine	1.1
Isoleucine	2.7
Leucine	8.3
Phenylalanine	17.1
Lysine	8.0

patients' sera was examined in an ELISA, as described above, and by immunoprecipitation experiments (11) using purified IgG from serum of patients with UC and control IgG.

Results

The proteins purified from colon extracts at different fractions from FPLC and HIC columns are shown in Figure 1. The 0.35 M NaCl eluate from the FPLC-Mono Q column enriched for P40 (Fig. 1B, lane 3) included a laminin fragment and actin in addition to the P40, which, as shown by immunoblot and ELISA analysis was reactive to UC serum (Table I). After phenyl Sepharose hydrophobic interaction chromatography, P40 was purified as a doublet (Fig. 1B, lane 5). This eluate reacted with UC serum and antitropomyosin antibodies, but not with antiactin and antilaminin antibodies (Table I).

The amino acid composition of the P40 is shown in Table II. Figure 2 presents the chromatograph obtained after tryptic digestion of the P40 (purified P40 doublet). Sequencing

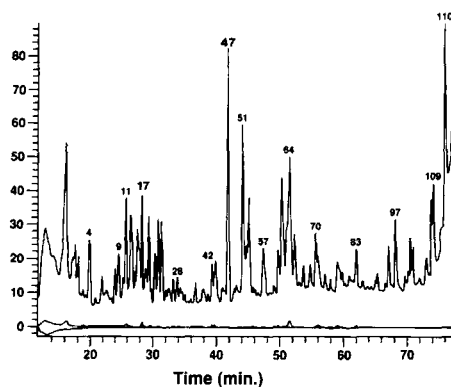


FIGURE 2. Chromatogram of the trypsin digested (in situ) P40 (both bands) and separated by narrow-bore reverse-phase HPLC. Peaks 17 and 47 are sequenced as shown in Figure 3.

of two of the tryptic fragments (*peaks 17 and 47*) demonstrated that the peptides in both peaks had 93 to 100% identity in 14 through 16 amino acids with α -tropomyosins from different sources (Fig. 3). Furthermore, HIC- purified P40 (both bands) reacted with antitropomyosin antibody when examined by immunotransblot analysis (Fig. 1C).

Figure 4 shows the ELISA data with all of 130 subjects. The highest background value of this ELISA with a non-UC patient was an OD of 0.125 yielded by serum from a patient with SLE. Thirty-five serum samples from 41 (85%) UC patients produced an OD higher (>0.125) than that of all other sera, clearly differentiating these patients from all other groups. The mean value for the UC group was 0.300 ± 0.040 . The difference between this value and the mean value for each non-UC group is highly significant ($p < 0.0001$). No sera from patients with intestinal disorders other than UC or from normal control subjects had values above 0.110. If the 0.110 OD value is taken as the cut-off line, 39 of 41 (95%) UC patients had antitropomyosin antibody activity above 0.110.

Figure 5A shows that the immunoreactivity of four UC

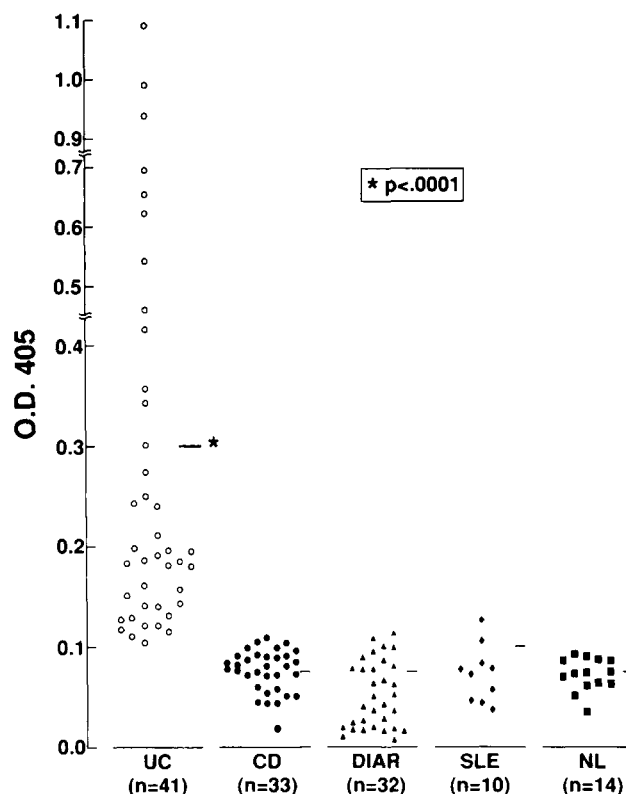
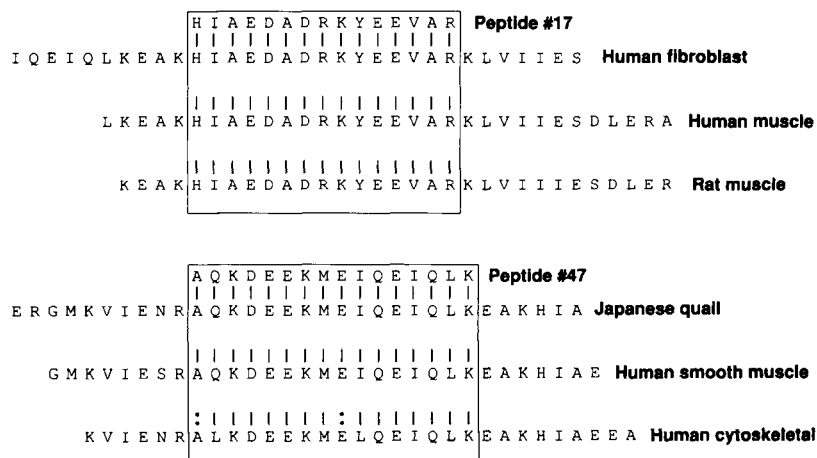


FIGURE 4. Scattergram showing the OD of each of 130 subjects' serum (1/200) reacted against tropomyosin by ELISA. The immunoreactivity of UC sera was significantly ($p < 0.0001$) higher than each of the non-UC group.

sera (nos. 1–4) against P40 could be serially diluted. Sera from two patients with CD, two with bacterial diarrhea, and two normal subjects (nos. 5–10) showed only background reactivity (OD >0.100) at dilutions from 1/200 to 1/3200. Each of the four UC serum was absorbed with porcine tropomyosin covalently coupled with Tresyl-activated agarose (Schleicher & Schuell, Inc., Keene, NH). The immunoreactivity of the postabsorbed sera was substantially reduced

FIGURE 3. Amino acid sequence of the peptides eluted in *peaks 17 and 47* compared with tropomyosin isoforms from various sources: peptide 17 from P40 showing 15 amino acid sequence, human fibroblast-derived tropomyosin (HuT-14), human skeletal muscle α -tropomyosin (3' end), rat-striated muscle α -tropomyosin mRNA (3' end); peptide 47 from P40 showing 16 amino acid sequence, Japanese quail α -tropomyosin (clone cC401), human smooth muscle tropomyosin mRNA (exon 6–9 part), and human 2.5-kb mRNA for cytoskeletal tropomyosin TM30 (nm). A 93 to 100% identity in 14 to 16 amino acids overlap is seen.



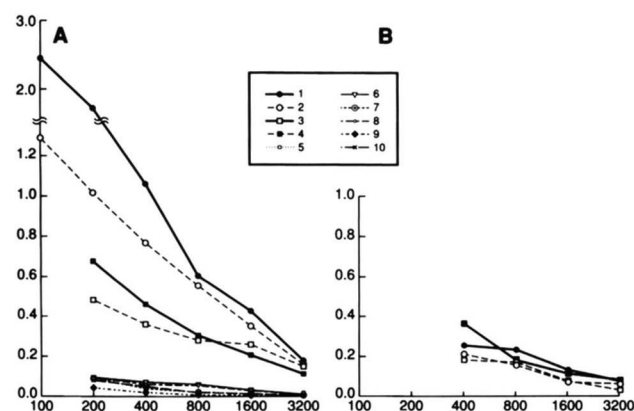


FIGURE 5. A, Immunoreactivity of 4 UC sera (curves 1–4) at serial dilutions from 1/100 through 1/3200 against tropomyosin tested by the ELISA. Six additional sera from two patients with Crohn's disease (nos. 5 and 6), two bacterial diarrhea (nos. 7 and 8), and two normal subjects (nos. 9 and 10) were also included in these experiments. A significantly higher immunoreactivity is evident with each of the four UC serum that could be serially diluted. The background reactivity of all six non-UC sera was 1 OD less than 0.100. B, After absorption of each of the four UC serum with tropomyosin, the reactivity was substantially reduced.

as shown at different dilutions (Fig. 5B).

To further determine if the two bands in P40-doublet are both tropomyosin and if UC serum reacts with either or both bands, we purified the two proteins individually by electroelution after SDS-PAGE. Figure 6A shows the homogeneity of the two electroeluted proteins when examined by silver stain. Both proteins also reacted with antitropomyosin antibody in the ELISA (Table III) and in immunoblot analysis (Fig. 1C). IgG purified from UC serum reacted with both bands (Fig. 6B) as shown by immunoprecipitation analysis. Electroeluted protein from each band was further examined for immunoreactivity against 48 of 130 sera by the ELISA and results are shown in Table III. These sera included 22 patients with UC, 11 patients with Crohn's disease, 8 patients with bacterial diarrhea, 3 patients with SLE, and 4 normal subjects. The immunoreactivity of the UC sera was higher against the eluted protein from the upper band when compared to the protein extracted from the lower band. The mean OD by UC sera for the upper band protein was 0.626 ± 0.093 and for the lower band protein it was 0.343 ± 0.040 . However, the difference between either of this value and the mean value for each non-UC group is highly significant ($p = <0.0001$) (Table III).

Discussion

The tropomyosins are a large group of closely related actin-binding proteins that can be found in virtually all cell types. These proteins are critical in the regulation of cytoskeletal structure and various functions involving cellular contrac-

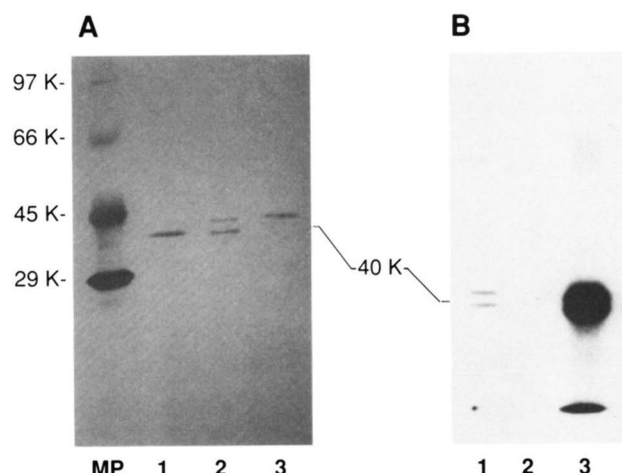


FIGURE 6. Purification of tropomyosin doublet by SDS-PAGE followed by electroelution. A shows purified proteins stained with silver nitrate. B is an autoradiogram that contained iodinated (^{125}I -Na) tropomyosin doublet that was immunoprecipitated with UC serum-IgG. A, MP, marker proteins. Lane 1 Lower band protein; lane 2, tropomyosin doublet; lane 3, upper band protein. B, lane 1, iodinated tropomyosin doublet immunoprecipitated with UC serum-IgG, the reactivity with both proteins is evident; lane 2, tropomyosin doublet immunoprecipitated with control serum-IgG, no reactivity is seen; lane 3, strong immunoreactivity with antitropomyosin mAb.

tility. Although there are structural overlaps among tropomyosin isoforms, organ-specific isoforms of tropomyosins have been clearly identified (12–15). The mechanism of this specificity has been attributed to alternative RNA splicing and polyadenylation (14, 16). Molecular masses of 30 to 45 kDa have been reported for various isoforms of tropomyosin in different cells (13). Although the function of tropomyosin in muscle has been well characterized, its function in nonmuscle cells, particularly different epithelial cells, is unclear.

The extraction of disease-specific colonic mucosal-bound IgG antibodies from patients with UC (CCA-IgG) led us to identify the P40 in both normal and diseased colon (5). CCA-IgG extracted from autologous UC colon also reacted with P40 from the same colon, further supporting the autoantigenicity of P40. In this study, more than 90% of sera from patients with UC reacted with purified tropomyosin, whereas sera from patients with acute colitis caused by various pathogens and chronic CD, as well as SLE, another autoimmune disease, did not. Indeed, the difference between the reactivity of the UC group and that of each non-UC group was highly significant ($p < 0.0001$).

In intestinal epithelial cells, tropomyosin has been localized in the apical cytoplasm at the rootlet and in the brush border (17, 18). It is interesting that deposition of specific IgG1 antibody with activated complement products along the apical part of colonic epithelial cells has recently been reported in patients with UC (4), but not in those with CD

Table III

Immunoreactivity of sera from patients and normal subjects against each of two bands of tropomyosin (M, 40K), purified by SDS-PAGE followed by electroelution

Subjects	OD at nM 405		Statistical Analysis
	Upper band protein	Lower band protein	
a) Ulcerative colitis (n = 22)	0.626 ± 0.093	0.343 ± 0.040	a vs b: $p < 0.0001$ against either protein a vs c: $p < 0.0001$ against either protein a vs d: $p < 0.0001$ against either protein a vs e: $p < 0.0001$ against either protein
b) Crohn's disease (n = 11)	0.094 ± 0.011	0.089 ± 0.008	
c) Specific diarrheas (n = 8)	0.091 ± 0.010	0.047 ± 0.008	
d) Lupus erythematosus (n = 3)	0.110 ± 0.007	0.073 ± 0.004	
e) Healthy subjects (n = 4)	0.040 ± 0.008	0.021 ± 0.006	
Antitropomyosin		1.921 1.445	

or in normal controls (17). Among our UC patients, there was an approximately 10-fold variation in the antitropomyosin antibody titer. This may reflect heterogeneity among patients with UC; alternatively, the titer may be influenced by the severity and extent of the disease. Although, as determined clinically and sigmoidoscopically, all of our UC patients had active disease, the precise extent of colonic involvement and the severity of the disease were not determined during the collection of serum samples, which was usually done at an outpatient clinic. Further studies correlating antibody titers with the anatomical extent of the disease and its clinical severity might provide clinically important information. There may also be diversity of antibodies against different isoforms of tropomyosins (12–15). Although there are structural overlaps among them, distinct peptides exist in each isoform (14) related to different cell types and some antibodies may react largely to the unique peptide(s). After absorption of UC serum with muscle tropomyosin, there was substantial reduction of OD (Fig. 5B). However, complete absorption did not occur, which may be due to isoform differences of tropomyosins between muscle and epithelial cells.

It is currently unknown why tropomyosin or tropomyosin-related peptide(s), acts as an autoantigen in UC. Although we have identified two peptides in P40 having 93 to 100% identity with α -tropomyosins, the complete structure of colonic epithelial cell tropomyosin is unknown. The amino acid composition of the P40, particularly the presence of proline and relatively large amount of glycine and phenylalanine are different compared to the known tropomyosin isoforms. Because of the nonreactivity of the tropomyosin with sera from either acute diarrheal syndromes due to specific pathogens or with sera from patients with CD, it is unlikely that its antigenicity is a secondary effect of inflammation. However, its antigenicity may be related to differences between the structure of colonic epithelial tropomyosin or related peptide(s) in patients with UC and in normal individuals.

Tropomyosin-related antigenic epitopes have been described in other chronic diseases, including such autoimmune diseases as the neurofibrillary pathology of Alzheimer's disease (20) and autoimmune hepatitis (21). It has

been shown that tropomyosins extracted from human cardiac muscle have immunologic epitopes that cross-react with group A streptococcal M proteins (22), which cause autoimmune myocarditis. The antigenicity of colonic epithelial tropomyosin may be related to molecular mimicry (23) of viral or bacterial proteins such as *Escherichia coli* or other colonic microbial agents; if so, this may explain the chronicity of UC.

Our data provide the first evidence that a cytoskeletal microfilament protein belonging to tropomyosin family acts as an autoantigen in patients with UC. Indeed, more than 90% of patients with UC had circulating antibodies against this protein. Future studies focusing on the structural and molecular aspects of tropomyosin isoforms in the colonic epithelium may lead to further understanding of autoimmunity in UC. We plan to undertake such studies, focusing on the polymorphism of these isoforms in normals and patients with UC, identification of their antigenic epitopes and cellular immunologic responses, and their cross-reactivity to other organs such as skin and biliary epithelium, which are known to be involved in UC (24).

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