

Cellular Immune Response against Tropomyosin Isoform 5 in Ulcerative Colitis

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We have reported an autoantibody response in ulcerative colitis (UC) against human tropomyosin isoform 5 (hTM5), the predominant colonic epithelial cell hTM isoform. In this report, we determined the number of IFN- γ -secreting cells (spot-forming cells, SFC) against hTM5 by an enzyme-linked immunospot (ELISPOT) assay. Another cytoskeletal protein, caldesmon, CaD40, was used as a control antigen. Peripheral blood mononuclear cells were separated by a Ficoll density gradient from 28 patients with UC, 13 patients with Crohn's disease (CD), and 9 healthy subjects (HS). The mean ($\pm$ SEM) SFC values against hTM5 in UC, CD, and HS were 48.8 ± 8.1 , 18.6 ± 4.6 , and 20.8 ± 8.6 , respectively. The value in UC was significantly higher than those in CD ($P < 0.005$) and HS ($P < 0.025$). SFC values in CD did not differ from those in HS. None of the 50 samples (except 1 UC) reacted to the CaD40 antigen. This study demonstrates, for the first time, a defined colon epithelial cell antigen, hTM5, that is capable of inducing a significant T cell response in UC but not in CD. © 2001 Elsevier Science

Key Words: ulcerative colitis; T cell response; tropomyosin isoform.

INTRODUCTION

Autoimmunity has been emphasized in the pathogenesis of ulcerative colitis (UC), although the autoantigen(s) involved in this process is unknown (1, 2). CD4⁺ T cells have been implicated in the initiation and perpetuation of organ-specific antigen-driven immune responses involved in several autoimmune diseases, such as multiple sclerosis and type I diabetes mellitus (3, 4). Using experimental models, the roles of CD4⁺ T cells were shown by adoptive transfer experiments or blocking experiments with several cytokines (5, 6). We earlier reported the presence of autoantibodies against tropomyosin (TM) in UC, but not in Crohn's disease (CD) (7, 8). Subsequently, using intestinal lymphocytes, we reported that the autoantibody response is mainly against human tropomyosin isoform 5 (hTM5), the predominant hTM isoform present in colon epithe-

lial cells (9). Using sera from patients with UC and their first-degree blood relatives, Biancone *et al.* demonstrated a significant ($P < 0.001$) autoantibody response against hTM5 in Italian patients with UC compared to those with CD and controls (10). Furthermore, first-degree healthy blood relatives of UC patients were more frequently seropositive against hTM than the relatives of CD patients and healthy controls ($P < 0.02$) (10). Autoantibodies against a specific TM peptide were also demonstrated in patients with UC as well as in patients with primary sclerosing cholangitis, but not in CD or in primary biliary cirrhosis (11). These investigators further reported an association of the TM peptide along with HLA Class II molecules and its expression on the cell surface (11).

TMs are microfilamental proteins present in all eukaryocytes with organ-specific isoforms (12). T cell receptor- α knock-out mice with spontaneous colitis developed a significant autoantibody response against TM (13). Furthermore, the severity of colitis in these mice was directly correlated with the titer of anti-TM autoantibodies produced by colonic mucosal B cells (13, 14). Thus, autoantibodies against TM have been demonstrated *in vitro* and *in vivo* in experimental colitis model as well as in patients with UC.

IFN- γ is a key cytokine produced by T-lymphocytes and NK cells that mediates major immune functions involved in the autoimmune effector mechanism (15). Among others, these include upregulation of major histocompatibility complex Class I and II antigens, Fc receptors, adhesion molecules (ICAM-1), complement components, and enhancement of several proinflammatory cytokines. Activation of T cells to secrete IFN- γ occurs following mitogenic and antigenic stimuli. While lamina propria mononuclear cells from UC tissue showed low levels of IFN- γ (16), a marked increase in IFN- γ as well as of IL-4 was reported in the colonic mucosa from T cell receptor- α mutant colitis mice (13). However, IFN- γ secretion by peripheral blood lymphocytes from UC has not been tested, particularly in response to a colon epithelial protein. In the present study, we report a hTM5-specific T cell response in UC.

MATERIALS AND METHODS

Patients and Controls

Blood samples were obtained from 28 out-patients with UC and 13 patients with CD at the Crohn's and Colitis Center of New Jersey. Ten of the 13 patients with CD had colonic involvement, with or without small intestinal inflammation. Nine healthy subjects (HS) were also included. The study was approved by the Institutional Review Committee. Patient demographics, disease characteristics, and current medications are summarized in Table 1. In all subjects, the diagnosis was made according to the clinical, endoscopic, radiological, and histological criteria. During collection of blood samples, disease activity was assessed in patients with UC by the criteria of Truelove and Witts (17) and in CD by the index of Harvey and Bradshaw (18). As shown in Table 1, 24 patients in the UC group and 11 patients in the CD group were symptomatic. Since all of these patients were included from the outpatient clinic, none had severe symptoms (17, 18). Symptomatic patients were mostly considered mild and hence further separation between mild and moderate was not done. Four patients with UC and 2 with CD were in remission. Treatment included sulfasalazine and 5-amino salicylic acid (5-ASA) (2–4.8 g/day) in most of the patients, steroids (10–20 mg/day), and purinethol (6-mercaptopurine, 6-MP), 25–75 mg/day, in some of the patients with UC or CD (Table 1).

Recombinant Human Tropomyosin Isoform 5 and Caldesmon

Recombinantly synthesized human tropomyosin isoform 5 and the N-terminal fragment (243 amino acid residues) from human fibroblast caldesmon, known as CaD40, were used for antigenic stimulation of peripheral blood mononuclear cells (PBMCs). The full-length complementary DNA encoding hTM5 and CaD40 was prepared from human fibroblasts, as reported previously (19). The cDNA clones were subcloned into prokaryotic expression vector pET8c. The resulting plasmids were transformed into the *Escherichia coli* BL21 (D3) LysS strain. Recombinant hTM5 and CaD40 were purified from bacterial lysates by ammonium sulfate fractionation, DE-52 ion-exchange chromatography, and hydroxyapatite column chromatography. The purity of hTM5 and CaD40 was examined by sodium dodecylsulfate–polyacrylamide gel electrophoresis, and the immunoreactivity was analyzed by enzyme-linked immunosorbent assay (ELISA) and transblot analysis using isoform-specific monoclonal antibodies.

Isolation of PBMCs and Antigen-Presenting Cells

Blood samples (10 ml) were obtained from healthy subjects and UC and CD patients and used within 6 h

TABLE 1

Demographic Data of Patients with Inflammatory Bowel Disease and Healthy Subjects

	UC (n = 28)	CD (n = 13)	HS (n = 9)
Sex (M/F)	19/9	7/6	5/4
Age, in years (mean $\pm$ SD)	44.6 $\pm$ 17	28.8 $\pm$ 14.2	40.3 $\pm$ 11
Location of disease	12—pancolitis 16—distal ^a or left-sided	6—ileocolitis 4—colitis 3—ileitis	NA
Disease state			
Symptomatic	24	11	NA
Remission	4	2	
Medications ^b			
SASP/5ASA	22	10	NA
Corticosteroid	4	4	
6-Mercaptopurine	10	3	

Note. UC, ulcerative colitis; CD, Crohn's disease; HS, healthy subjects; NA, not applicable.

^a Distal, proctosigmoiditis; left-sided, up to splenic flexure area.

^b No. of patients taking various medications; SASP, azulfidine (salicyl-azo-sulfapyridine); 5-ASA, 5-aminosalicylates, 2–4.8 g/day; corticosteroid, prednisone, 10–20 mg/day; 6-mercaptopurine or azathioprine, 25–75 mg/day.

of collection. PBMCs were purified by density centrifugation on Ficoll (Pharmacia, Uppsala, Sweden), isolated from the gradient interface, washed three times in phosphate-buffered saline (PBS, pH 7.4), and resuspended in culture medium (RPMI 1640) with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 20 mM Hepes buffer, pH 7.4, 100 U/ml penicillin, and 100 μ g/ml streptomycin sulfate. Sixty percent of the PBMCs were resuspended in cold medium (10% dimethyl sulfoxide, 90% fetal bovine serum), frozen in a liquid nitrogen freezer, and stored until used as antigen-presenting cells (APCs). Frozen PBMCs were treated with mitomycin C (Sigma Chemical Co., St. Louis, MO) for 30 min at 37°C and washed at least three times with culture medium before use.

Enzyme-Linked Immunospot Assay

The enzyme-linked immunospot (ELISPOT) assay was performed as described before, (13, 20, 21) with some modifications (Fig. 1). PBMCs from each subject (1×10^5 cells/well) were cultured in six replicate wells in the presence of recombinant hTM5 or medium alone. CaD40, an actin-binding protein purified in a manner similar to that of hTM5, served as a control antigen. On day 5, rIL-2 (10 U/ml) was added, and on day 7, the cells were cocultured with APCs and antigen, either hTM5 or CaD40 (second boost). A 96-well nitrocellulose plate (Millipore Co., Bedford, MA) was coated overnight with murine anti-human IFN- γ antibody (10 μ g/

- Day 0** Ficoll whole blood → Isolate PBMCs
Plate 1×10^5 PBMCs / well; add Ag (hTM5
or CaD40) to 6 replicate wells
- Day 5** Add 1 U of rIL-2 / well
- Day 7** Add 1×10^5 mitomycin treated PBMCs /
well $\pm$ Ag
- Day 9** Pool replicate → Re-aliquot
PBMCs to anti-IFN- γ
capture plate
- Day 12** Add detecting → Add BCIP / NBT
anti-IFN- γ Ab substrate
- Day 14** Examine plate by dissecting microscope

FIG. 1. Steps of the ELISPOT assay.

ml) in 0.1 M carbonate buffer (pH 8.2) at 4°C. After the wash, each well was blocked with 1% bovine serum albumen/PBS at 37°C for at least 1 h. On day 9, re-stimulated PBMCs (after the second boost) were added to each well in 200 μ l of culture medium and incubated at 37°C for 72 h. Subsequently, the wells were washed with 0.05% Tween-PBS at least five times, and then biotinylated mouse anti-human IFN- γ monoclonal antibody was added to each well and incubated overnight at 4°C. The next day, alkaline phosphatase-conjugated streptavidin was added to each well and incubated at room temperature for 2 h, washed, and then developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. After a wash with tap water, all of the spots (spot-forming cells, or SFC, producing IFN- γ) were counted under the zoom stereomicroscope (Olympus, New Hyde Park, NY). The mean value of SFC from 6 wells containing medium only was subtracted from the mean SFC values obtained either with hTM5 or CaD40 for each patient.

Enzyme-Linked Immunosorbent Assay

PBMCs (2×10^5 cells/well) were incubated with either of the antigens or with medium alone, similar to the ELISPOT assay. rIL-2 (10 U/ml) was added on day 5 and mitomycin C-treated PBMCs and antigens (0.1 μ g/ μ l) on day 7. After culture for 5 additional days, the supernatant was collected and tested for IFN- γ using the OptEIA test (Pharmingen, San Diego, CA), a solid-phase sandwich ELISA. The detection limit of this assay was 4 pg/ml.

Statistical Analysis

All data are presented as means $\pm$ SEM as well as SD. All values are compared among UC and CD individuals and healthy subjects by using the Fisher exact test. Responses against hTM5 and the control antigen CaD40 are also analyzed. Mann-Whitney statistical analysis is used to calculate the significance of spot numbers observed in antigen-stimulated samples versus medium control (no antigen). Relationships between ELISPOT data and ELISA data are analyzed with the Spearman correlation coefficient.

RESULTS

Enzyme-Linked Immunospot Assay

T cells producing IFN- γ in response to the antigens (hTM5 and CaD40) were estimated by counting the spot-forming cells in an ELISPOT assay and also by quantitating IFN- γ secreted by T cells in an ELISA (Opt EIA).

Figure 2 demonstrates positive spot-forming cells that secreted IFN- γ . The background SFC values (mean $\pm$ SEM) in all of the three groups for patients with UC and CD and for HS with medium alone (without any antigen) were 15.4 ± 2.2 , 13.1 ± 2.9 , and 21.4 ± 7 , respectively, and these values were not significantly different among the three groups. The mean $\pm$ SEM (as well as SD) SFC values in the presence of the antigens, namely hTM5 or CaD40, are shown in Table 2 and Fig. 3. The mean $\pm$ SEM values of IFN- γ -producing SFC against hTM5 in UC, CD, and HS were 48.8 ± 8.1 , 18.6 ± 4.6 , and 20.8 ± 8.6 , respectively. The value in UC was significantly higher than CD ($P < 0.005$) and HS ($P < 0.025$). However, there was no difference between CD and HS. The SFC values against the CaD40 antigen among the three groups were 11.6 ± 4.6 , 4.3 ± 1.5 , and 3.3 ± 2.2 , respectively. There was no statistical difference in the SFC values

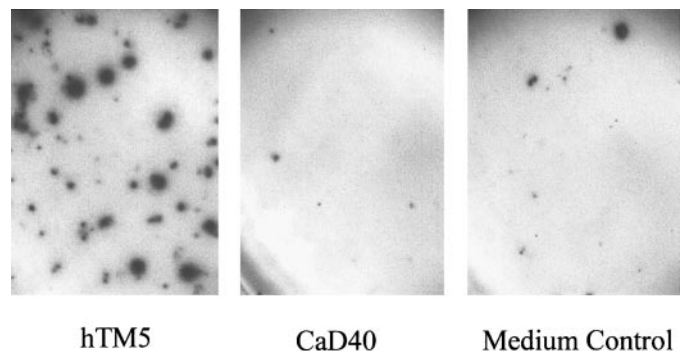


FIG. 2. Typical spots of T cells producing IFN- γ seen under the Zoom stereomicroscope with hTM5, CaD40, and medium alone (magnification, 40 $\times$).

TABLE 2

IFN- γ -Producing T Cell Response against hTM5 and CaD40 in Patients with UC and CD and in HS

	No. of subjects	SFC against hTM5 (mean $\pm$ SEM)	SFC against CaD40 (mean $\pm$ SEM)
UC	28	48.8 $\pm$ 8.1 ^a	11.6 $\pm$ 4.6 ^b
CD	13	18.6 $\pm$ 4.6 ^c	4.3 $\pm$ 1.5 ^d
HS	9	20.8 $\pm$ 8.6 ^e	3.3 $\pm$ 2.2 ^f

Note. Fisher exact test. *a* vs *c*, *P* < 0.005; *a* vs *e*, *P* < 0.025; *c* vs *e*, not significant; *b* vs *d* or *f*, not significant; *d* vs *f*, not significant. UC, ulcerative colitis; CD, Crohn's disease; HS, healthy subjects.

against the CaD40 antigen between UC and CD or UC and HS. The SFC values in UC were significantly (*P* < 0.05) higher with hTM5 than with the CaD40 antigen.

Among the CD patients, the highest SFC value against hTM5 in one of the 13 patients was 47 (Fig. 3). If this value is taken as a cutoff line, 10 of 28 (36%) patients with UC had SFC values higher than 47 (Fig. 3 and Table 3). Eight of these 10 patients had pancolitis. This difference in the extent of the disease with distal (rectosigmoid colon) and left-sided colitis was statistically highly significant (*P* < 0.01). Eight of the 10 UC patients were symptomatic and none was treated with corticosteroids during the collection of blood samples. Five of the 10 patients were, however, treated with 6-MP (25–75 mg/day) (Table 3). A total of 4 UC patients and 4 CD patients were treated with corticosteroid. Most patients were also treated with 5-ASA compounds. Only 1 of the 9 healthy subjects had a positive response to hTM5, with an SFC value of 76 (Fig. 3). None of the 50 subjects (except 1 UC) showed any positive response against CaD40 antigen (Fig. 3).

The results demonstrate that hTM5 induced a cellular immune response, with increased IFN- γ production, in UC, but not in CD and healthy subjects. This hTM5-specific response in UC was not seen with cytoskeletal protein CaD40. All of the patients were outpatients with relatively mild symptoms of disease or in remission, and, hence, they were classified as symptomatic or in remission, thus further gradation of the disease state was not done. Among the 4 UC patients in remission, 2 were in the hTM5-positive group and 2 were in the hTM5-negative group. Age of the patients, sex, and duration did not influence the hTM5 response (Table 3). With a limited number of patients treated with corticosteroid, it appears that although statistically not significant, prednisone may have had a suppressive effect on the hTM5 response, since none of the 10 hTM5-positive UC patients was taking prednisone. However, 6-MP treatment in the dosage (25–50 mg/day) used did not influence the SFC values (Table 3).

Enzyme-Linked Immunosorbent Assay

To confirm reproducibility and accuracy of the SFC, we also measured IFN- γ levels in culture supernatant by ELISA. SFC numbers in ELISPOT assay were compared to IFN- γ levels of culture supernatant from 12 of these patients, as shown in Fig. 4. There was a significant (*P* < 0.0001) correlation between the two methods.

DISCUSSION

This study shows that there is a cellular immune response against hTM5 by PBMCs in patients with UC. The specific antigen-induced IFN- γ production was measured by two sensitive assays, ELISPOT and Opt EIA. This T cell response, as evidenced by IFN- γ production, is more pronounced in patients with pancolitis. Indeed, as shown in Fig. 3, 10 of 28 (36%) patients with UC had a clear heightened response to hTM5 compared to that of each of 13 CD patients. Such a response was, however, not seen with another cytoskeletal protein, CaD40, with similar size (243 amino acid residues) as hTM5 (248 amino acids) and prepared following the same method (19). TM-related autoanti-

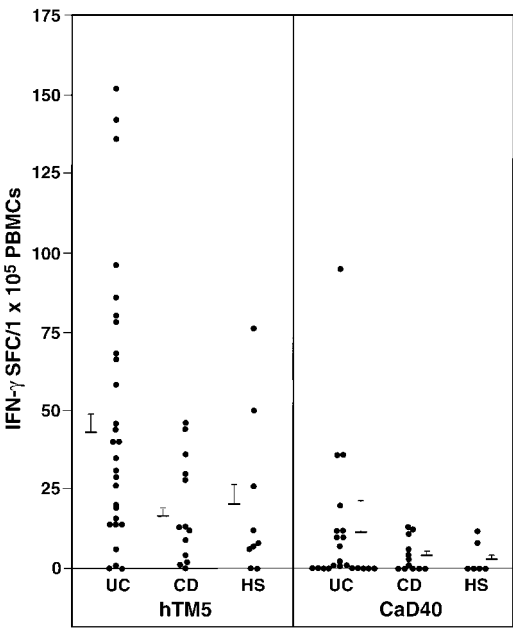


FIG. 3. Scatterogram showing the individual values (mean of six replicates) of spot-forming cells (SFC) producing IFN- γ with PBMCs from patients with ulcerative colitis (UC) or Crohn's disease (CD) and healthy subjects (HS) in response to human tropomyosin isoform 5 (hTM5) and the control antigen, myosin-binding site of another actin-binding protein, caldesmon (CaD40). The mean value is identified by the horizontal bar and the standard deviation by the vertical bar for each group against both antigens. The detailed statistical analysis is shown in Table 2.

TABLE 3

hTM5 Response and Clinical Parameters of Patients with Ulcerative Colitis

	hTM5 response		P value
	Positive ^a (n = 10)	Negative (n = 18)	
Age in years (mean $\pm$ SD)	42.4 $\pm$ 16.5	46.5 $\pm$ 16.9	NS ^b
Sex (M/F)	9/1	10/8	NS ^c
Extent (pan/distal or left-sided)	8/2	4/14	$P < 0.01^c$
Duration of disease in years (mean $\pm$ SD)	9.5 $\pm$ 9.8	7.5 $\pm$ 5.4	NS ^b
Disease state (symptomatic/remission)	8/2	16/2	NS ^c
Medications			
SASP/5-ASA (+/-)	8/2	14/4	NS ^c
Corticosteroid (+/-)	0/10	4/14	NS ^c
6-Mercaptopurine (+/-)	5/5	5/13	NS ^c

Note. NS, not significant.

^a A positive response is defined by an SFC value higher than 47, which was the highest value observed in Crohn's disease.

^b Mann-Whitney U test.

^c Fisher Exact Test.

bodies have been reported to be present in UC patients, but not in CD, by us in American patients (9) and by two investigators involved with the initial work who performed independent studies with Italian and Japanese patients with UC and CD (10, 11). Recently, we also reported that hTM5, which is the predominant hTM isoform in colon epithelial cells (9), is externalized specifically in colonic epithelial cells and not in small intestinal enterocytes (22). Using the isoform-specific monoclonal antibody, it was intriguing to demonstrate that hTM5 could be detected on the surface of colon epithelial cells by FACS analysis and in the culture supernatant as determined by ELISA and immunoblot analysis (22). Thus, hTM5, although an intracellular microfilament protein, is capable of inducing effector immune responses because of its expression on the colon epithelial cell surface and possible release from the colonocytes (22). In a separate study, utilizing colonoscopic biopsy specimens, we further reported that in UC, but not in CD or in controls, a large number of lamina propria B cells produced IgG against hTM5 (median values: UC, 42%; CD, 2.5%; other controls, 0%). This difference, between UC and CD and other controls, was highly significant ($P < 0.0001$) (23). On the basis of these observations, we studied whether hTM5 was able to induce an antigen-specific T cell response.

Previous reports showed that lamina propria T cells in healthy subjects and in inflammatory bowel diseases show diminished induction of T cell activation through the TCR/CD3 pathway, whereas the CD28/CD2 co-stimulation pathway was preserved (24–26). There-

fore, we focused on peripheral blood mononuclear cells for detecting an antigen-specific T cell response against the colon epithelial protein hTM5. For the measurement of T cell activation *in vitro*, investigators utilized several methods, including cytokine release, surface markers, and thymidine incorporation (27–29). The ELISPOT assay is very sensitive and measures the actual number of cells secreting the specific cytokine. To increase the sensitivity and specificity, we used six replicate wells. We have also used Opt EIA to confirm the data from an ELISPOT assay. There was a significant ($P < 0.0001$) correlation between the two methods.

In the preliminary experiments while establishing the ELISPOT assay, the second boost (APC + antigen) on day 7 was not included. In these experiments, SFC values, although relatively higher in UC than in CD and HS, were low (less than 20 SFC). Therefore, we performed restimulation using mitomycin-treated autologous PBMCs as APCs and the antigen (hTM5 or CaD40) to determine antigen-specific cytokine production. After restimulation, we detected significantly increased IFN- γ secretion, with hTM5 increasing the sensitivity of the assay. This restimulation on day 7 and culture for another 2 days might have increased the possibility of heightened expansion of the limited progenitor cells producing IFN- γ in response to hTM5. However, the results clearly demonstrate that hTM5 is capable of inducing a significant cellular immune response in 36% of the UC patients compared with patients with CD, suggesting that this is not a secondary phenomenon. Indeed, 10 of the 13 CD patients had symptomatic Crohn's colitis. Taken together, with significant humoral immune responses against hTM, as shown by using both sera and mucosal B cells, in UC but not in CD (9–11, 23), the current studies focusing

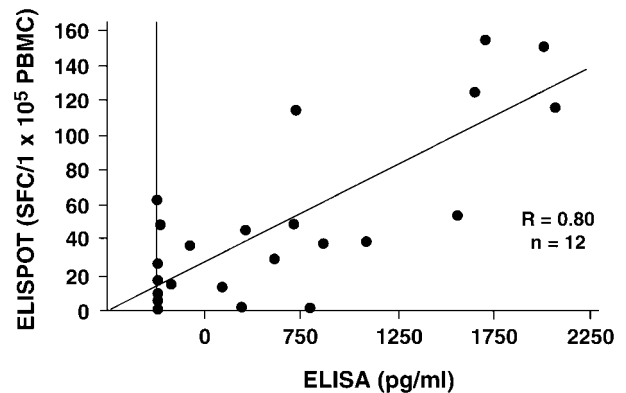


FIG. 4. Correlation between the enzyme-linked immunospot assay (ELISPOT) to measure the spot-forming cells producing IFN- γ (SFC per 1×10^5 PBMCs) and the enzyme-linked immunosorbent assay (ELISA) to quantitate IFN- γ (pg/ml) in patients with UC ($n = 10$) and CD ($n = 2$). The line represents the best-fit correlation between ELISPOT data and ELISA data ($P < 0.0001$).

on the antigen-specific cellular immune response further add to an important autoantigenic role of hTM5 or related peptide(s) in UC. In a computer-based physicochemical analysis of the structure of 109 human autoantigens, it was observed that sequences longer than 27 residues with coiled-coil α -helices were the forerunner autoantigens for various autoimmune diseases. Among them, several sequence segments of TM were found to be the most probable autoantigen(s) (30). It is unknown whether the response seen with the hTM5 may have molecular mimicry with bacterial peptide(s). This is particularly relevant, since TM has a motif that is identical to an α -helical domain of streptococcal M protein (31). Clonal T cell specificity and cross-reactivity toward enteric microflora (32) and cross-reactivity between the P-ANCA antigen and colonic bacterial proteins in UC have also been recently reported (33).

IFN- γ has been a critical cytokine in several autoimmune experimental models, such as the experimental allergic encephalomyelitis model (34). In psoriatic patients, using PBMCs, a significant Th-1 response producing IFN- γ was found and considered to be pathogenetically important (35). In the current study, using PBMCs from patients with UC, we also demonstrate a Th-1 type response. IL-4 production was investigated and was similar to that in CD and HS (data not shown). It is unknown, however, whether IL-4 production by PBMCs does not change in UC, or if this is due to the fact that exogenous IL-2 used in the ELISPOT assay inhibited IL-4 production, or antigen dose, or whether the length of response for the Th-2 profile may not have been adequate.

There are several reasons to explain why only 36% of UC patients showed a significant positive cellular immune response against hTM5. One factor, which was clearly shown to be important, is the extent of the disease. Eight of 12 pancolitis patients had a significantly ($P < 0.01$) higher hTM5 response than with distal and/or left-sided colitis ($n = 16$). The sensitivity of the assay to detect the antigen-specific T cells from PBMCs may be limited, the precise quantity of the antigen needed may be a factor, and there may be other autoantigenic proteins involved in UC. The positive response may also depend on the HLA haplotype of the patients, which was not examined in this study. As mentioned above, these were all outpatients, most of whom had relatively mild disease, and, hence, the frequency of hTM5-specific T cells in the peripheral blood of these patients compared to that of colonic mucosal T cells may be low. Interestingly, 2 of the patients with UC also had associated primary sclerosing cholangitis. Both of them were positive against hTM5. Indeed, 8 of 8 patients with primary sclerosing cholangitis with UC and none of the 6 primary biliary cirrhosis patients showed a significant ($P < 0.05$) high humoral immune response against a specific tropomyosin peptide (11). In

a recent study, the restricted V_H gene usage in anti-colon autoantibodies producing B cell clones suggests that a particular colon epithelial antigenic stimulus contributes to the pathogenesis of UC (36). However, the specific epithelial antigen involved has not been studied.

In summary, we, for the first time, demonstrate that a defined colon epithelial antigen, hTM5, is capable of inducing cellular immune response in many patients with UC. Further characterization of hTM5-specific epitope(s) involved in the T cell response, using both PBMCs and mucosal T lymphocytes from UC, may provide an important clue in the understanding of the pathophysiologic process in UC. Identification of the immunologically active hTM peptide(s) may also have therapeutic implications.

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