Autoantibodies against the specific epitope of human tropomyosin(s) detected by a peptide based enzyme immunoassay in sera of patients with ulcerative colitis show antibody dependent cell mediated cytotoxicity against HLA-DPw9 transfected L cells

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Abstract

Background and aims—Recent studies suggest that tropomyosin (TM) may act as a putative autoantigen in ulcerative colitis (UC). Recently, we identified, by computer homology analysis, a specific peptide (HIAEDADRK) in human TM that can bind to HLA-DPw9. The aim of this study was to investigate the presence of autoantibodies against this peptide in UC.

Methods—Antibodies were measured by ELISA with a synthetic peptide in 20 healthy volunteers, 48 patients with UC, 26 with Crohn’s disease (CD), eight with primary sclerosing cholangitis (PSC), and six with primary biliary cirrhosis (PBC). The functional significance of antibodies was investigated by antibody dependent cell mediated cytotoxicity (ADCC) against DPw9 transfected L cells using a standard 51Cr release assay.

Results—Optical density values (mean (SD)) of sera from patients with UC (1.40 (0.52)) and PSC (1.65 (0.12)) were significantly higher than those from healthy volunteers (0.32 (0.28)) (p<0.05), CD (0.50 (0.34)) (p<0.05) and PBC (0.14 (0.09)) (p<0.05). Values in UC decreased with clinical improvement. The ADCC activity of UC sera correlated well with antibody titre against this synthetic peptide.

Conclusions—Anti-TM antibody was detected in UC sera by a specific peptide based ELISA with high reproducibility. This peptide may be an antigenic epitope of TM involved in the immunopathogenesis of UC and, perhaps, PSC.

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Keywords: tropomyosin; antibody dependent cell mediated cytotoxicity; HLA-DPw9; ulcerative colitis

Autoimmune phenomena have been implicated in the pathogenesis of ulcerative colitis (UC). Das et al have previously reported that IgG antibodies bound to the colon mucosa of patients with UC and showed antibody dependent cell mediated cytotoxicity (ADCC) activity. They subsequently identified the antigen recognised by these antibodies to be a protein with a molecular weight of 40 kD.

Recently, a 40 kD colonic protein was purified from the colon to homogeneity, partially sequenced after proteolytic digestions, and the amino acid sequences of the two peptides derived; the amino acid sequences showed 93–100% match with the cytoskeletal tropomyosins (TMs). TMs are microfilamentous proteins present in all eukaryotic cells with organ specific isoforms.

If these TMs play a role as autoantigens in patients with UC, specific antibodies against TMs might be expected to be present in these patients. In this context, Das et al detected the antibody reacting against porcine TM in UC sera, although the responsible epitope involved in immune recognition and the pathogenic implication of this antibody have not been clarified.

In our preliminary experiments using an assay system similar to that of Das et al, we confirmed that UC patients had the antibody against porcine TM. However, the incidence of positivity was lower than that of the previous report, and the reproducibility of the assay was rather poor, possibly because of the inconsistency of the porcine TM preparations.

In patients with UC in Japan, significant accumulation of HLA-DR2-DPw9 haplotype was noted by serological typing of HLA molecules. We have previously investigated the association of HLA class II molecules with TM peptides, and revealed that the tryptic peptide of TM bound to HLA-DPw9 expressing L cells but not to HLA-DR2 or DQ6 expressing L cells. On the basis of these findings we searched for a sequence which would bind to the HLA-DPw9 molecule in human skeletal muscle α-TM, that is, a sequence whose relative placement of hydrophobic amino acid anchors are in positions 2, 3 and 6; we identified the sequence HIAEDADRK.
Peptide based ELISA for antitropomyosin antibody

Table 1 Characteristics of the study groups

<table>
<thead>
<tr>
<th>Disease Category</th>
<th>No of patients (male/female)</th>
<th>Age (median (range)) (y)</th>
<th>Disease activity (active/inactive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ulcerative colitis</td>
<td>18/30</td>
<td>41 (18-70)</td>
<td>28/20</td>
</tr>
<tr>
<td>Crohn's disease</td>
<td>19/7</td>
<td>30 (16-51)</td>
<td>19/7</td>
</tr>
<tr>
<td>Primary biliary cirrhosis</td>
<td>6/9</td>
<td>22 (19-26)</td>
<td>2/4</td>
</tr>
<tr>
<td>Primary sclerosing cholangitis</td>
<td>3 (2/1)</td>
<td>34 (25-42)</td>
<td>3/0</td>
</tr>
<tr>
<td>Healthy volunteers</td>
<td>20 (10/10)</td>
<td>35 (25-42)</td>
<td>-</td>
</tr>
</tbody>
</table>

Human muscle tropomyosin
Peptide No 17 LKEAKHIAEDADRKYEEVARVLTIIESDLSRA
O volvulus tropomyosin (IgG binding epitope) GKEAQALLAEADDRKYDEVARKLAMVEADLE
Shrimp tropomyosin (IgE binding epitope) FLAEEADRKYDEVAR
Shuffled synthetic peptide ADEIRKHD

Figure 1 Comparison of the amino acid sequence of synthetic peptide with the equivalent region of other tropomyosins (TM). Aligned are representative sequences from the human skeletal muscle α-tropomyosin, IgE binding epitope of shrimp TM, peptide No 17 from p40 which Das et al reported, and the antigenic epitope of O volvulus TM. The peptide with the sequence in the shaded area is the synthesised peptide. There is 66.7% match in nine amino acids between the synthetic peptide (peptide No 17 from p40), the IgE binding epitope of shrimp TM, and the antigenic epitope of O volvulus TM. Shuffled synthetic peptide was composed of the same nine amino acids as the synthetic peptide in a different order.

In this investigation, we established an ELISA with a high reproducibility for the antibodies against TM using a synthesised peptide with a specific sequence of TM (HIAE- DADRK) as the antigen. Elevation of antibody levels demonstrated by this assay was specific for active UC and primary sclerosing cholangitis (PSC) but not for Crohn’s disease (CD) or primary biliary cirrhosis (PBC). In addition, we demonstrated that ADCC activity in UC sera, when plotted against levels of antibody against the TM peptide, showed a positive correlation.

Materials and methods

PATIENTS

Serum samples were obtained from the subjects listed in table 1, and informed consent was obtained from each patient. Diagnoses of UC, CD, PBC, and PSC were made according to established criteria, including clinical symptoms, radiological results, blood examination, endoscopy, and pathological demonstration of either inflamed intestinal mucosa for UC and CD or liver tissue for PBC and PSC. Disease activity was assessed in patients with UC by clinical history and endoscopy findings and in patients with CD by Bristol’s simple index. Mucosal inflammation in patients with UC was assessed during colonoscopy according to the criteria of Matts.

Twenty eight UC patients with active disease were treated with steroids and mesalaminas (Hoechst Marion Roussel, Inc., Kansas City, Missouri, USA) while 14 inactive UC patients were given mesalamines alone. All patients with CD had small intestinal involvement. Nineteen of the patients with active CD received steroids and/or mesalaminas. Seven CD patients with inactive disease were treated with mesalaminas alone. Two of six PBC patients showed PBC symptoms, and the other four were asymptomatic; all were taking ursodeoxycholic acid (Axcan Pharmaceutical US Inc. Minneapolis, Minnesota, USA). All eight PSC patients were symptomatic, having intermittent jaundice and pruritus, but they had no colonic symptoms or histological evidence of UC (table 1).

TM PEPTIDE

In accordance with the report analysing the ligand of the HLA-DP molecule, the sequence in which hydrophobic anchoring amino acids (AA) are located in positions 2, 3 and 6, was searched for in human skeletal muscle α-TM using the ProFound Amino Acid database searching program. The peptide spanning the sequence of human TM (153–161AA), which has 66.7% homology with the IgE binding epitope of Shrimp TM (fig 1),13 was identified and synthesised in a peptide syntheser (Excels; Millipore Corp., Milford, Massachusetts, USA). A shuffled peptide composed of the same nine peptides but in a different order was also synthesised.

ELISA FOR DETECTION OF ANTI-TM PEPTIDE ANTIBODY

Each of the 96 wells of microtitre plates (high binding capacity, flat bottomed microplate; Costar, Cambridge, Massachusetts, USA) was coated at 4°C overnight with 100 µl of the synthetic peptide at a concentration of 3 µg/ml in 50 mM phosphate buffered saline (PBS) (pH 7.4). The wells were incubated initially with 200 µl of a blocking reagent (Super block in TBS, Pierce, Rockford, Illinois, USA) for 30 minutes at room temperature and subsequently with 100 µl of patients’ or normal subjects’ sera (x100 diluted with PBS) in triplicate for two hours at 37°C. As a positive control, serum from a rabbit immunised with the synthetic peptide was added in separate wells in the same plate at dilutions x400 to x6400. After washing with 20 mM Tris HCl containing 0.5 M NaCl, 0.05% Tween 20, and 0.1% NaN3, pH 7.5 (T-TBS), the wells were treated with 100 µl of alkaline phosphatase (ALP) conjugated goat antimouse IgG (Dako, Carpinteria, California, USA) or goat antirabbit IgG (Dako) in ELISA buffer (0.05% Tween 20 in PBS, pH 7.4), respectively, and incubated for one hour at room temperature. After washing with ELISA buffer, the wells were further incubated for two hours at 37°C in 50 µl of substrate solution (1 mg/ml of P-nitrophenyl phosphate in PBS). The reaction was stopped by adding 50 µl of 0.3 M H2SO4. The colour intensity was measured by an ELISA plate reader (ImmunoMini NJ-2300; Intermed, Japan) with a measuring filter of 405 nm.

The specific immunoreactivity of the immune rabbit serum and UC sera was verified by examining their reactivity against the synthetic peptide before and after preincubation with the synthetic peptide or unrelated shuffled synthetic peptide.14

ADCC ASSAY

ADCC of UC sera (TM antibody positive sera from five UC patients, TM antibody negative sera from four UC patients) and normal sera

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(sera from three healthy volunteers) were assessed using the standard four hour \(^{51}Cr\) release assay following the method described previously.\(^{17}\) HLA-DPw\(^{9}\) gene transfected L cells\(^{18}\) which were shown to express TM antigens on their surface\(^{6}\) were used as targets. Briefly, target cells (1×10\(^6\)/ml) were incubated with synthetic peptide (3 \(\mu\)g/ml) for three hours. After washing with PBS, cells were labelled for two hours at 37°C with 100 \(\mu\)Ci of \(Na_2^{51}CrO_4\) (Daichi Radioisotope Laboratory, Tokyo, Japan) and washed five times with PBS. Radiolabelled target cells (5×10\(^4\)) were incubated with 50 \(\mu\)l of UC or healthy volunteer sera at 37°C in humidified 5% CO\(_2\) for 60 minutes. The cells were washed three times and resuspended in RPMI 1640 medium. Peripheral blood mononuclear cells (PBMNC) were obtained from healthy volunteers using the standard Ficoll-Isopaque density gradient separation method. Each of the above mentioned target cells was incubated with PBMNC at a final volume of 0.2 ml in each 96 well round bottomed culture plate (Corning 25850, Corning, New York, USA). Thereafter, the plates were centrifuged at 200 \(g\) for five minutes. After four hours of incubation at 37°C, 5% CO\(_2\), the supernatants were collected and radioactivity was measured using a gamma ray counter. Percentage cytotoxicity was calculated as follows: % cytotoxicity= ((experimental release−spontaneous release)/(maximum release−spontaneous release))×100. Spontaneous release was determined by incubating target cells alone in the medium. Maximum release was obtained by lysing target cells with 1% Nonidet P-40 (Nakarai Chemical Co., Kyoto, Japan). Values above the mean +2 SD of healthy volunteers were defined as positive.

**HLA-DPB1 TYPING**

Genotyping of all DPB1 alleles was performed using a polymerase chain reaction-sequence specific oligonucleotide probe (PCR-SSOP) approach according to the protocols described in the 11th International Workshop.\(^{19}\) Generic primers were used for DPB1 typing; 25 SSOs were used to type 36 DPB1 alleles. Details of the hybridisation conditions have been published previously.\(^{20}\)

**STATISTICAL ANALYSIS**

The Mann-Whitney U test was used to assess the standard error of the difference between two means; values were significant if \(p<0.05\). Analysis of variance (ANOVA) parallel line assay was used to determine statistical
Table 2  Levels of IgG antibodies against synthetic tropomyosin peptide in sera from patients with ulcerative colitis, Crohn’s disease, primary biliary cirrhosis, primary sclerosing cholangitis, and healthy volunteers

<table>
<thead>
<tr>
<th>Disease</th>
<th>Antibodies against synthetic peptide (active, inactive disease)</th>
<th>Positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ulcerative colitis</td>
<td>1.40 (0.52)** (1.63 (0.52), 1.00 (0.44))**</td>
<td>68.8%</td>
</tr>
<tr>
<td>Crohn’s disease</td>
<td>0.50 (0.34) (0.52 (0.34), 0.46 (0.37))</td>
<td>15.4%</td>
</tr>
<tr>
<td>Primary biliary cirrhosis</td>
<td>0.14 (0.09)</td>
<td>0.0%</td>
</tr>
<tr>
<td>Primary sclerosing cholangitis</td>
<td>1.61 (0.22)</td>
<td>100.0%</td>
</tr>
<tr>
<td>Healthy volunteers</td>
<td>0.32 (0.28)</td>
<td>5.0%</td>
</tr>
</tbody>
</table>

Values represent optical density (OD) values at 405 nm.

*Positivity: (number of patients whose OD value is higher than mean + 2 SD of healthy volunteers/number of patients examined)×100.

*P<0.05 compared with Crohn’s disease; †P<0.05 compared with active disease; ‡P<0.05 compared with healthy volunteers.
When serially examined, in 11 of 12 patients with active UC, the OD value decreased when they were in remission (p<0.05).

![figure depicting changes in optical density (OD) values of sera from patients with ulcerative colitis (UC) before and after remission.](image)

When UC was active, OD values were lower than in remission. Conversely, when UC became inactive, OD values were higher than in active disease.

**Figure 6 Changes in optical density (OD) values of sera from patients with UC before and after remission.**

When serially examined, in 11 of 12 patients with active UC, the OD value decreased when they were in remission (p<0.05).

![figure depicting changes in antibody dependent cell mediated cytotoxicity (ADCC) activity against synthetic peptide of sera from patients with UC.](image)

A similar but slightly different modality was presented by Senyk and colleagues who showed that glucagon contains both the antigenic determinant for serum antibody and the epitope peptide for T cell receptor side by side, and that the B cell epitope near the T cell epitope can be recognised by an antibody. This is an alternative possibility which we find less likely than the former because ADCC was observed when DPw9 transfected L cells treated with the synthetic peptide were used as target cells.

The specificity of the ELISA for the peptide was proved in two ways. Firstly, the ELISA specifically detected antibodies in immune rabbit serum but not in preimmune rabbit serum. Secondly, the antibody titre in positive sera decreased after preincubation with the synthetic peptide but not with the unrelated shuffled synthetic peptide. The clinical validity of this ELISA for the diagnosis of UC was apparent as high levels of the antipeptide antibody were detected only in UC and PSC but not in normal subjects or in patients with CD or PBC. Furthermore, in UC, levels decreased as the disease state became inactive. It may be
particularly noteworthy that the levels of antibodies in PSC were as high as those in active UC because both diseases are often associated with each other, and HLA class II expression is commonly found in colon and bile duct epithelium in these diseases. Some OD values in healthy volunteers may represent the presence of low titre antibody in normal individuals as reported in other autoimmune diseases. Despite the usefulness of ELISA in differential diagnosis and monitoring, some limitations in its sensitivity have been observed; almost 30% of active UC patients showed normal levels of the antibody. This indicates that the epitope recognised by this antibody may not be the only antigen involved in immune recognition in UC. Further elucidation of antigens other than this peptide and its application for combined assay with the present ELISA may be needed to increase the sensitivity.

Another point of special interest is the positive correlation between the anti-TM peptide antibody titre and ADCC activity, which suggests that the antibody we detected in this assay has a pathogenic role in UC.

Recently, Jenkins et al reported that TM antibodies play an important role in resistance against microfilariae in patients with onchocerciasis through an ADCC mechanism. They identified the B cell epitope of O volvulus TM which shows 66.7% homology with the synthetic TM peptide we used for the ELISA. They also revealed that the dominant isotype of the antibody induced by vaccination was IgG1. These data support the idea that the antibody against the TM peptide detected in UC sera may cause tissue destruction through an ADCC mechanism. However, this does not necessarily rule out the possibility that other immunological responses, including T cell mediated effects related to the TM epitope, may also be involved in the pathogenesis of UC.

In this study, we revealed that a specific epitope of TM was capable of a humoral immune response in patients with UC and PSC. This may provide important biochemical information to explain the autoimmune mechanism in UC and PSC. From the experiments described here, this peptide based ELISA could provide a valuable tool for detecting TM antibody in sera because of its reproducibility and specificity. Measurements of the levels of TM antibody may help to understand disease activity and also to identify the pathognomonic character of UC.

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