

Production of recombinant human tissue transglutaminase using the baculovirus expression system, and its application for serological diagnosis of coeliac disease

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Background and objectives Tissue transglutaminase was identified as the main autoantigen in coeliac disease (CD) but enzyme immunoassays applying the commercially available antigen from guinea pig liver show insufficient specificity and sensitivity for diagnosis as compared with endomysium antibodies (EmA). The aim of this present study was to develop a new method for the cloning and expression of human tissue transglutaminase (hu-tTG) and to test hu-tTG in the serological diagnosis of CD.

Methods Hu-tTG was cloned and expressed using a baculovirus system and SF9 insect cells. The enzyme carried a C-terminal His-tag allowing efficient affinity purification from cell lysates. The recognition of hu-tTG by human sera was checked by using an enzyme linked immunosorbent assay (ELISA). For this, 35 patients with active CD were compared with 144 controls (18 patients with biologically excluded CD, 89 blood donors, 30 patients with inflammatory bowel disease, and seven patients with cystic fibrosis).

Results The ELISA using hu-tTG showed a sensitivity of 100% and a specificity of 98.6%. Titres of antibodies against hu-tTG (anti-hu-tTG) were positively correlated with EmA titres. All results negative for EmA were also negative for anti-hu-tTG. There were, however, EmA

positive results up to a titre of 1 : 80 below the cut-off for anti-hu-tTG. For comparison, antibodies against guinea pig tissue transglutaminase (anti-gp-tTG) were determined in parallel. All patients with anti-hu-tTG below the cut-off were also negative for anti-gp-tTG. However, there were eight patients positive for anti-hu-tTG but negative for anti-gp-tTG.

Conclusions The new test reaches and even exceeds diagnostic efficiency of EmA for coeliac diagnosis. *Eur J Gastroenterol Hepatol* 14:1217–1223 © 2002 Lippincott Williams & Wilkins

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Introduction

Coeliac disease (CD) is an immunologically mediated intolerance to wheat gliadins and related cereal proteins leading to damage of the small intestinal mucosa. Dermatitis herpetiformis (DH) is a skin disorder closely associated with CD. Both disorders are characterized by the appearance of autoantibodies conventionally determined as endomysial antibodies (EmA) by indirect immunofluorescence on monkey oesophagus tissue sections with high sensitivity and specificity. Tissue transglutaminase (tTG) has been identified as the predominant target antigen for the autoantibodies from coeliac patients [1]. tTG is a calcium activated enzyme which covalently cross-links the ϵ -amino group of a peptide bound lysine and the γ -carboxamide group of a

peptide bound glutamine, forming an ϵ -(γ -glutamyl) lysine isopeptide bond [2]. Commercially available tTG from guinea pig liver (gp-tTG) was used to establish enzyme immunoassays for autoantibodies [3–15]. Using a commercial test, in a recent, large, international, multicentre study the levels of antibodies against gp-tTG (anti-gp-tTG) were estimated in 391 coeliac patients with active disease and compared with 432 controls [16]. The assay of anti-gp-tTG has been shown to come close to the sensitivity and specificity described for EmA but, as described in many reports, does not reach it. A reason for this may be that crude preparations of tTG were used. Furthermore, the degree of homology between gp-tTG and human tTG (hu-tTG) is only about 80% [17,18]. Reports on the

application of hu-tTG for coeliac autoantibody determination are still rare. The human enzyme isolated from erythrocytes was applied [19], others used the tTG obtained from *in vitro* translation systems in radioligand assays [20–25]. The use of hu-tTG cloned and expressed in cell systems would be most desirable. Production of recombinant hu-tTG should be possible, and give highly reproducibly in high quantity. Until now, only two reports on the cloning and expression of hu-tTG and its use in coeliac serology have been available [26,27]. It was shown that hu-tTG was detected by coeliac autoantibodies with similar specificity and sensitivity as the EmA test or even exceeding it.

Our objective was to produce hu-tTG in the highest purity and high yield. Here, we describe a technique for expressing hu-tTG cloned from small intestinal fibroblasts by insect cells. The expressed protein carries a C-terminal His tag, thus simplifying its subsequent isolation and purification. This antigen was shown to react with high specificity towards CD and DH autoantibodies.

Subjects and methods

Subjects

Serum samples were obtained from 35 patients with newly diagnosed, untreated CD, including two patients with CD and DH (group 1), 34 patients with CD and 12 patients with DH who were maintaining a gluten-free diet (GFD) including dietary transgressions (group 2), 18 patients with CD excluded bioptically (group 3), and from 89 blood donors (group 4). Group 5 comprised 30 patients with chronic inflammatory bowel disease and seven patients with cystic fibrosis. Diagnosis of coeliac disease was according to the ESPGAN criteria [28]. The age range of subjects was 2–81 years.

Culture of human intestinal fibroblasts

A small intestinal biopsy specimen was cut, extensively washed in Hank's Balanced Salt Solution (HBSS, Sigma Deisenhofen, Germany, Product Number H 9394) mixed with 5% fetal calf serum (FCS, Costar GmbH, Bodenheim, Germany, Cat. No. 01-01500) and digested in HBSS containing 0.1 mg/ml dispase type I (Roche, Mannheim, Germany) and 300 U/ml crude collagenase type XI (Sigma) for 30 min at room temperature. The material was disintegrated by pipetting and the tissue pieces allowed to sediment for 1 min. The resulting pellet was resuspended in complex medium M3:10 (In Cell Corp., San Antonio, TX, USA), transferred to a T25 culture flask (Greiner, Frickenhausen, Germany) and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. Outgrowing cells were trypsinized, washed in phosphate buffered saline (PBS, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.2, 136 mM NaCl, 2.7 mM KCl) and immediately shock frozen at –80°C.

cDNA cloning of hu-tTG

Total RNA was isolated from 1×10^6 cells with RNeasy kit (Qiagen, Hilden, Germany). After denaturation of RNA secondary structure at 70°C for 10 min, first strand cDNA synthesis was performed. For this, total RNA was incubated with Oligo d(T) primer and RT enzyme Superscript (Life Technologies, Karlsruhe, Germany) at 50°C for 50 min. Termination at 70°C was followed by RNase H incubation at 37°C for 20 min. The full length hu-tTG gene was amplified from cDNA using the Platinum Taq enzyme (Life Technologies) and primer designed from previously published hu-tTG sequence (gene bank accession number M55153). The forward primer, 5'-GGT GGT CCA TGG CCG AGG AGC TGG TCT TA-3', contains an NcoI site where the translation is initiated at the ATG codon. The reverse primer, 5'-TAC TAC CTC GAG GGC GGG GCC AAT GAT GAC ATT, contains an XhoI site. PCR products were gel purified and, after double digestion with NcoI and XhoI, directly ligated to a gel purified, NcoI-XhoI, double digested, pTriEx-1 Vector (Novagen, Schwalbach, Germany) yielding pTriEx-1-tTG. In this vector the tTG gene is fused at the 3' end with a sequence coding for a His tag. After transformation of competent *Escherichia coli* cells (strain Novablue, Novagen) with the ligation mixture, the recombinant pTriEx-tTG vector arose which was purified and the tTG gene was verified by sequencing.

Culture of insect cells, viral transfection, and expression of recombinant protein

Expression of hu-tTG in TriEx Baculovirus Expression System 1 (Novagen, Schwalbach, Germany) was performed according to the instructions of manufacturers. Briefly, co-transfection of a monolayer of SF9 insect cells was carried out using Triple cut BacVector™ 3000 and the transfer vector pTriEx-tTG. Three days after transfection, the supernatant was screened for recombinant baculovirus using a direct plaqueing protocol. Individual plaque were purified by replaqueing and amplified two to three times in order to reach a viral titre above 2×10^8 . Recombinant clones from individual plaque isolates were screened for expression of hu-tTG by infecting 2×10^6 SF9 cells in a 25 cm² T-flask (Greiner). Forty-eight hours post-infection the cell extracts were checked for fusion protein by western blotting. For large scale expression, SF9 cells were grown in a 75 cm² T-flask. Twenty million SF9 cells were infected at a multiplicity of infection between 5 and 7. The cells were kept in an incubator at 27°C and harvested 48 h post-infection after being washed with PBS.

Purification of recombinant hu-tTG

Harvested infected SF9 cells (10^9 cells) were resuspended in 40 ml of buffer A (20 mM Tris HCl, pH 8.5, 5 mM 2-mercaptoethanol, 100 mM KCl, 1 mM phenyl-

methylsulfonyl fluoride, 1% Nonidet P-40). The cell suspension was sonicated on ice for 30 s using Sonicator Sonopuls HD 70 (Bandelin electronic, Berlin, Germany) in pulsed mode at 50% duty cycle. The extract was centrifuged at $31\,000 \times g$ for 10 min. The supernatant was loaded on a Ni(II)-nitriloacetate agarose (NTA agarose) column equilibrated with buffer B (20 mM Tris HCl, pH 8.5, 500 mM KCl, 20 mM imidazole, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol) at 1 ml/min. The non-specifically bound proteins were washed away with four column volumes of buffer B. Elution of His tagged hu-tTG from the NTA-agarose was achieved using buffer C (20 mM Tris HCl, pH 8.5, 500 mM imidazole, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol). The eluate containing hu-tTG was dialysed for 2 days against 20 mM Tris HCl, pH 8.5 and was stored at -20°C .

DNA sequencing

The tTG gene in the pTriEx-tTG vector was sequenced using an automated sequencing apparatus *ABI PRISM*[®] 310 (PE Applied Biosystems, Weiterstadt, Germany).

Analytical electrophoresis and western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in discontinuous 10% gels [29]. Proteins were stained with Coomassie blue. For western blotting, after SDS-PAGE samples were electro-transferred semi-dry to nitrocellulose in a discontinuous system [30]. The membranes were saturated for 1 h with 2% Tween 20 in Tris buffered saline (TBST, 50 mM Tris HCl, pH 10.2, 150 mM NaCl, 0.05% Tween 20), then incubated for 2 h with rabbit polyclonal antibodies against guinea pig tTG (1:500, Covalab, Lyon, France, code 997-PtG). Bound antibodies were detected by incubation for 1 h with pig anti-rabbit antibodies conjugated with alkaline phosphatase (1:700, Dako Diagnostica; Hamburg, Germany, code D306), and colour was developed by using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium. Detection of His tagged tTG by western blotting was performed using the same technique as described above except that anti-His₆ mouse monoclonal antibody (1:100, Roche Diagnostics GmbH, Mannheim, Germany, code 1922416) was used as the first antibody and goat anti mouse immunoglobuline conjugated with alkaline phosphatase (Dako Diagnostica; Hamburg, Germany, code D486) as the second antibody.

Enzyme immunoassay of IgA class tTG antibodies

The detection of human antibodies using guinea pig tTG (Sigma, code T5398) (anti-gp-tTG) was performed as described previously [5]. The assay of human tTG antibodies using hu-tTG followed (anti-hu-tTG) the same procedure but with few modifications: dilution of

human sera 1:500 instead of 1:100, and concentration of hu-tTG for coating of microplates 5 mg/ml instead of 10 mg/ml. The results were compared to optical density of a reference serum which was set to 100 units. The optical density was linearly related to antibody titre up to 1.5. Sera producing higher optical densities were diluted more than 1:500. A positive control was included in every microplate. Its titre was about five times above the cut-off which was calculated in the course of the experiments.

Determination of anti-gliadin antibodies and of endomysium antibodies

IgA and IgG anti-gliadin antibodies (AGAs) were determined by enzyme immunoassay in microwells coated with gliadin as described previously [31]. A standard provided by Labmaster Diagnostics (Turku, Finland) was used. There are two cut-off values, indicating 'weak positive' and 'positive' results, which are different for patients above and below an age of 2 years. Human IgA class EmA were determined by indirect immunofluorescence [32] using sections of monkey oesophagus (Viro-Immun-Labor Diagnostica, Frankfurt, Germany). Sera with a titre of 1:10 were regarded as positive. One of the participating centres (M.S.) used a starting dilution of 1:5 for EmA but only minor discrepancies were observed [33].

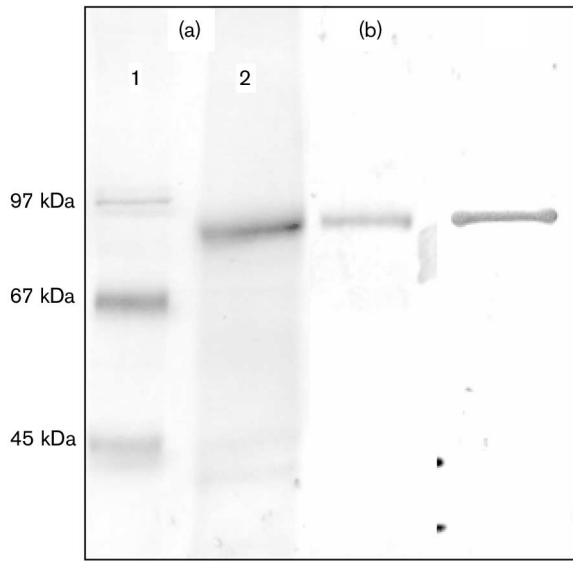
Results

This is the first report about cloning and expression of hu-tTG using baculovirus and insect cells. The hu-tTG sequence was identical to that previously reported [18] except for substitution of 13 base pairs. Seven base pair substitutions were without consequence for the amino acid sequence (at position 774 C instead of G, at position 806 T instead of G, at position 807 G instead of T, at positions 1008 and 1236 G instead of A, at position 1731 C instead of A, at position 2097 G instead of C). Six base pair substitutions caused amino acid substitutions (at positions 51 and 186 Glu instead of Gln, at position 509 Arg instead of Leu, at position 533 Asn instead of Thr, at position 629 Pro instead of Leu, and at position 655 Leu instead of Val).

Using the TriEx baculovirus expression system, a baculovirus was constructed which allows expression of hu-tTG with a poly-histidine tag at the C-terminus. Thus, the hu-tTG can efficiently be purified from SF9 insect cells by Ni affinity chromatography. Characterization of hu-tTG was performed using a commercially available antibody against guinea pig tTG (Fig. 1). A number of 10^9 infected SF9 cells yields 3.5 mg purified hu-tTG.

Recognition of the purified hu-tTG by human auto-antibodies was checked by enzyme immunoassay. The reactivity of sera with the antigen was very strong so

Fig. 1



SDS-PAGE and immunoblot of hu-tTG. (a) Coomassie stained gel. Lane 1 protein marker, lane 2 purified hu-tTG. (b) Immunoblot developed with anti-His₆ antibodies. (c) Immunoblot developed with anti-guinea pig tTG antibodies.

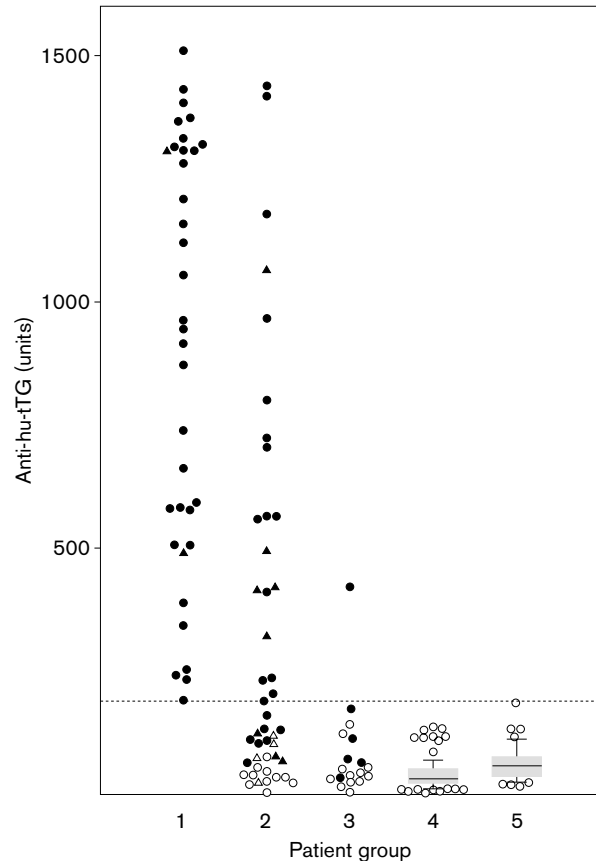
that a low amount of antigen per well (0.5 mg) and a high dilution of samples (1:500) could be used in the assay. All the newly diagnosed CD patients (group 1) were EmA positive. Their level of anti-hu-tTG was between 190 and 1510 units (Fig. 2). There was no overlap with sera from blood donors (group 4) and patients with chronic inflammatory bowel disease and cystic fibrosis (group 5). However, the minimum optical density of group 1 came very close to the maximum value (184 units) in group 5. All patients in groups 4 and 5 were EmA negative.

Group 2 comprised CD and DH patients on differently strict GFDs. Of the 34 CD patients, 23 were EmA positive and 11 were EmA negative. Of the 12 DH patients, eight were EmA positive and four were EmA negative. All of the patients above the cut-off for anti-hu-tTG were EmA positive. Below this cut-off value, there were seven EmA positive CD patients and three EmA positive DH patients.

Group 3 comprised patients in which CD was excluded by biopsy. In this group, there was one EmA positive patient clearly above the cut-off for anti-hu-tTG. This patient had an EmA titre of 1:50. There were also EmA positives below the cut-off for anti-hu-tTG. Four of them were borderline positive (titre 1:5), one of them (a patient with inflammatory bowel disease) had an EmA titre of 1:40.

Titres of EmA and anti-hu-tTG were positively corre-

Fig. 2



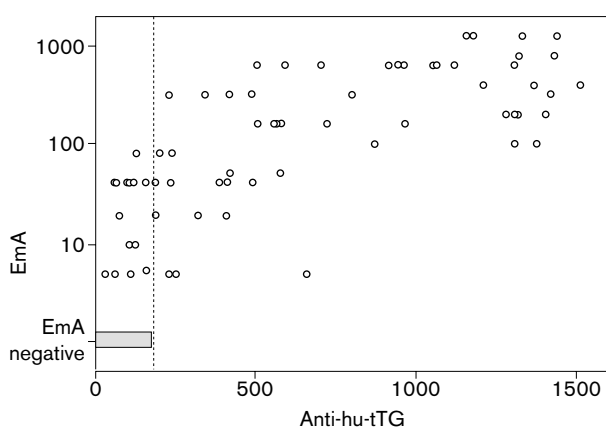
Assay of anti-hu-tTG in different groups of subjects. Black symbols indicate endomysial antibodies (EmA) positivity. Dermatitis herpetiformis (DH) patients are marked by triangles. DH patients in group 1 were newly diagnosed as coeliac disease (CD) patients. Due to the large number of subjects in groups 4 and 5, box plots are shown with the individual data points outside the confidence interval.

lated (Fig. 3). All EmA negatives were also negative for anti-hu-tTG. There were, however, EmA positives up to a titre of 1:80 below the cut-off for anti-hu-tTG. Most of them belong to patient group 2. Furthermore, among these patients there was one EmA positive but anti-hu-tTG negative subject with inflammatory bowel disease.

The titres of anti-hu-tTG were compared with titres of anti-gp-tTG (Fig. 4). All patients with anti-hu-tTG below the cut-off were also negative for anti-gp-tTG. However, there were eight patients with values for anti-hu-tTG up to 350 U which were negative for anti-gp-tTG. Five of these subjects were newly diagnosed EmA positive CD patients on a normal diet.

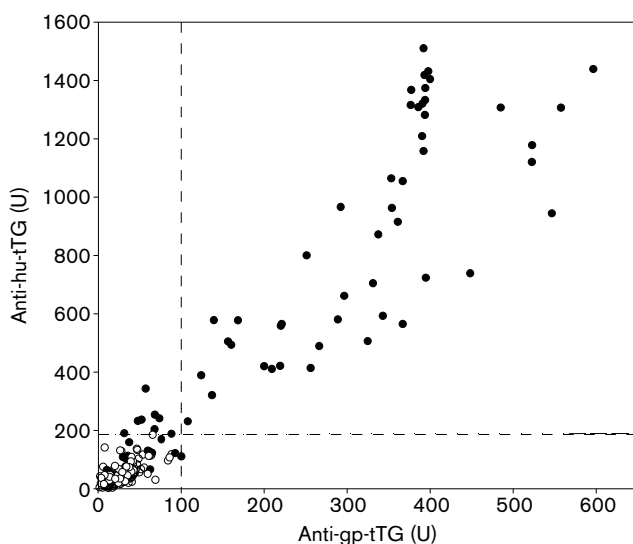
Taking the data of patients of groups 1, 3, 4 and 5 together, diagnostic efficiency of anti-hu-tTG was higher than that of EmA and anti-gp-tTG (Table 1). The

Fig. 3



Relation between anti-hu-tTG and endomysial antibodies (EmA). The grey box indicates the range of anti-hu-tTG of EmA negatives. The dotted line marks the cut-off value for anti-hu-tTG.

Fig. 4



Relation between anti-hu-tTG and anti-gp-tTG. Broken lines indicate cut-off values for both assays. Black symbols for endomysial antibody (EmA) positive and white symbols for EmA negative subjects.

receiver operating characteristic (ROC) analysis is shown in Fig. 5.

The intra-assay variation of determination of anti-hu-tTG was calculated as the variation of triplicate measurements made with the same microplate on the same day. The mean intra-assay variation of seven triplicate measurements performed on consecutive days was 2.3%. The inter-assay variation was calculated as the variation of seven measurements performed on different days. The mean inter-assay variation of three

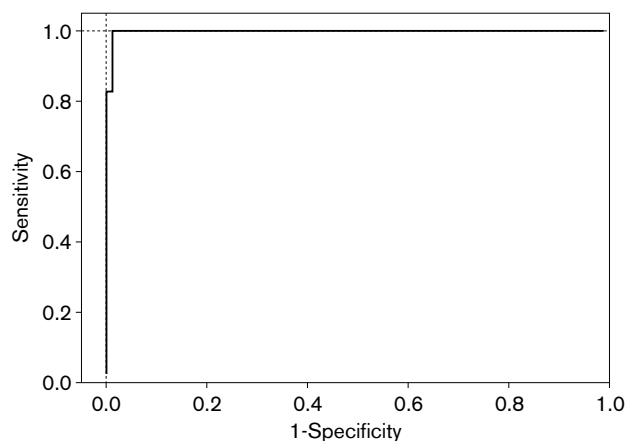
Table 1 Diagnostic sensitivity, specificity, and efficiency of different assays for coeliac autoantibodies

Autoantibody	Diagnostic sensitivity (%)	Diagnostic specificity (%)	Diagnostic efficiency (%)
EmA	100.0	96.5	97.2
Anti-gp-tTG	85.7	99.3	96.6
Anti-hu-tTG	100.0	98.6	99.3
IgA-AGA (upper cut-off)	71.4	88.9	85.5
IgA-AGA (lower cutoff)	85.7	67.4	70.9
IgG-AGA (upper cut-off)	65.7	89.7	86.0
IgG-AGA (lower cut-off)	77.1	83.3	82.1

For calculation of diagnostic sensitivity only newly diagnosed coeliac disease patients (group 1) were used. Diagnostic specificity was estimated from patient groups 3, 4 and 5.

EmA, endomysium antibodies; gp-tTG, antibodies against guinea pig transglutaminase; hu-tTG, human tissue transglutaminase; IgG, immunoglobulin G; AGA, anti-gliadin antibodies.

Fig. 5



Receiver operating characteristic (ROC) analysis using the anti hu-tTG data from group 1 (sensitivity) and groups 3, 4 and 5 (specificity).

experiments on consecutive days each with seven measurements was 16.1%.

Discussion

Very soon after the discovery of tTG as the main autoantigen in CD, enzyme immunoassays were established applying guinea pig antigen. However, in many reports a lower sensitivity and specificity of the test was described, when the rodent enzyme was used as antigen, compared with EmA immunofluorescence techniques on monkey oesophagus sections. This was ascribed to three possible reasons: (1) poor purity of the guinea pig antigen; (2) species differences in amino acid sequences between guinea pig and human tTG; and (3) the existence of further, non-tTG autoantigens which are missing in the antigen preparation used for immunoassay. The purity of the human antigen used in our assay was over 95% as judged from SDS-PAGE. The amino acid sequence calculated from the DNA sequence agrees well with the published sequence of

hu-tTG [18]. We found differences in base sequence which would result in seven amino acid exchanges. Five of the disagreeing amino acid positions were also described earlier [21,34]. The remaining differences might represent polymerase chain reaction mutations.

Both improved purity and the human origin of the antigen may be the reason for the enhanced diagnostic efficiency of anti-hu tTG compared with anti-gp-tTG. A comparison was reported [35] between an in-house ELISA using a commercially available crude gp-tTG, a test kit containing a purified gp-tTG, and another test kit containing hu-tTG. The authors found a higher sensitivity and specificity of the purified gp-tTG ELISA when compared with an assay using the crude antigen. Hu-tTG produced slightly better results than the purified guinea pig enzyme. However, due to the different origin of the test kits (home made versus two commercial kits of different manufacturers) these data cannot be compared directly. In another study, gp-tTG and hu-tTG were compared for recognition by human sera of different patient groups including liver disease [36]. A high rate of false positives was seen with gp-tTG for autoimmune liver disease due to the presence of impurities in the antigen preparation. The high specificity of anti-gp-tTG (99.3%) in our investigation may partly be due to the lack of such patients in our control groups.

The high diagnostic efficiency of the assay using hu-tTG as the antigen is not in contradiction with the existence of minor non-tTG autoantigens [37]. These autoantigens have been demonstrated to co-exist with tTG in the endomysial tissue. All newly diagnosed CD patients of group 1 were positive for both EmA and anti-hu-tTG. Thus, there was no case with exclusively coeliac autoantibodies against non-tTG epitopes.

Contrary to the complete agreement between anti-hu-tTG and EmA in the group of newly diagnosed CD patients (group 1) there was a large overlap between EmA positives and anti-hu-tTG negatives in the group of patients with different strict gluten-free diets (group 2). The anti-hu-tTG negatives in this group had EmA titres up to 1:320. A reason for the different behaviour of antibodies in group 1 and 2 may be that, under conditions of a less strict diet and of dietary transgressions, the amount of gluten taken up was lower than under the conditions of the normal diet in group 1. This, however, may indicate that the EmA assay is more sensitive than the anti-hu-tTG test. However, it should be kept in mind that there are no data on the state of intestinal mucosa in this group and, therefore, data cannot be correlated with histology.

Surprisingly, there was a fraction of EmA positives in the group of individuals with bioptically excluded CD.

Only one of these patients was also positive for anti-hu-tTG. This patient, with an EmA titre of 1:50, had slightly shortened villi and slightly enhanced numbers of intraepithelial lymphocytes (IEL) and might represent a case of latent CD [38,39]. Four of the five EmA positive patients below the cut-off for anti-hu-tTG were only borderline positive for EmA (1:5). One of them had a slightly and partially villous atrophy but normal IEL counts and another had insulin dependent diabetes mellitus but did not show any mucosal alterations. Several of these patients might represent very early (latent) stages of CD, in which immunofluorescence detection of autoantibodies may precede mucosal changes [40]. However, further investigations are necessary to compare sensitivity of immunofluorescence and ELISA technique in subtle cases of CD.

In conclusion, it could be shown that the use of hu-tTG as the antigen in ELISA for the detection of autoantibodies reaches diagnostic efficiency of EmA for diagnosis of CD. The new assay is easier to perform and might be a substitute for the conventional and time consuming immunofluorescence based technique of determining CD autoantibodies.

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