Anti-transglutaminase immunoreactivity and histological lesions of the duodenum in coeliac patients

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Abstract

Coeliac disease (CD) is characterized by several markers, including anti-transglutaminase auto-antibodies (tTGAb) directed against multiple epitopes of the gliadin protein. We aimed to investigate the correlation among CD duodenal lesions, tTGAb titres and the immunoreactivity against tTG constructs. A total of 345 CD patients (209 females, 136 males, overall median age: 7.3 years) were tested for full-length (fl) tTGAb with a fluid-phase radioimmunoassay. Out of the total, 231 patients were also tested for immunoreactivity against tTG fragments (F1: a.a. 227–687 and F2: a.a. 473–687). Patients were classified according to diffuse (D), patchy (P) or bulb (B) histological lesions. All sera were found fltTGAb positive. Patients with D, P and B lesions had a mean Ab index of 0.84 ± 0.39, 0.57 ± 0.39 and 0.45 ± 0.24, respectively. Mean tTGAb titre varied between D and localized (P+B) patients (0.84 ± 0.39 versus 0.52 ± 0.34, P < 0.0001). Overall, 86.1% of patients were F1 auto-antibody (F1Ab) positive (D: 89%, P: 75%, B: 40%; D versus P+B: P = 0.004) and 49% of patients were F2 auto-antibody (F2Ab) positive (D: 53%, P: 19%, B: 10%; D versus P+B: P = 0.0006). Of the D patients 50.7% showed combined F1Ab-F2Ab (D versus P+B: P = 0.001), whereas 60% of B patients were negative for both F1Ab and F2Ab (B versus D: P < 0.0001). Coeliac-specific tTGAb immunoreactivity correlates with the grading and extension of histological duodenal lesions in CD patients at diagnosis. The immunoreactivity against single and combined tTG fragments is significantly higher in patients with D lesions. This is the first evidence of a distinct coeliac-specific immunoreactivity in patients with different duodenal involvement.

Keywords: anti-transglutaminase antibodies, coeliac disease, histological lesions, tTG target domains

Introduction

Coeliac disease (CD) is a chronic, autoimmune, gluten-dependent enteropathy that occurs in genetically predisposed subjects. CD can be revealed in a typical form (with gastrointestinal symptoms), or in an atypical form (in patients with iron-deficiency anaemia, headache, and recurrent aphthous stomatitis), or it can be asymptomatic (silent form).

Among the coeliac-specific serum auto-antibodies (Ab), which can be used as a screening method, tissue transglutaminase (tTG) represents the dominant auto-antigen (1). tTG proved to play a crucial role in CD pathogenesis by modifying gliadin peptides that become strongly bound to HLA-DQ2/DQ8 and are recognized by gut-derived T cells (2). Several studies demonstrated that anti-tTG auto-antibodies (tTGAb) are directed against multiple epitopes of the protein (3–7).

The gold standard for CD diagnosis is the demonstration of typical changes of the small bowel mucosa, classified according to Marsh and modified by Oberhuber et al. (8). In all CD children, the duodenal bulb is involved (9), but a patchy appearance of the intestinal mucosa may be present.
Moreover, villous atrophy limited to the bulb has been described in 2.4% of CD children (10). Similar findings have been reported in adult CD patients (11).

To date, no information is available concerning immunoreactivity against the whole tTG or its specific domains according to the different histological CD patterns. For this purpose, the aim of this study was to evaluate the possible correlation between the immunoreactivity and three human recombinant constructs of the tTG protein (fl: full-length a.a. 1–687, F1: a.a. 227–687 and F2: a.a. 473–687; Fig. 1) with the grading and extension of CD duodenal lesions.

Methods

Subjects

The study was designed as a retrospective chart review of 345 unrelated Caucasian coeliac patients, diagnosed over the period 2001–11 at an Italian centre. The database was anonymized to protect patient confidentiality. Sera from each biopsy-proved coeliac patient (136 males, age range 0.7–45 years; median age 7.33 years) at diagnosis, on a gluten-containing diet, were analyzed for fl tTGAb with a fluid-phase radioimmunoassay (RIA). No patients with IgA deficiency participated in the study.

The CD diagnosis was made according to the North American Society for Paediatric Gastroenterology, Hepatology and Nutrition criteria (12) and to the guidelines of the American Gastroenterological Association (13). Patients with Marsh 1–2 histological lesions were diagnosed as having CD in the presence of high tTGAb levels, responsive to gluten-free diet.

All patients, fasting overnight after narcosis, had undergone upper endoscopy, using GIF-E for children over six, XP-10 or GIF-P140 gastrosopes for younger children and GIF-H180 or GIF-Q140 gastrosopes (Olympus Italia S.r.l. Segrate, Milan, Italy) for adult patients. During each endoscopy, four biopsies from the distal duodenum and two biopsies from the duodenal bulb were taken, oriented on filter paper, fixed in 10% foral bulb were taken, oriented on filter paper, fixed in 10% foral bulb were taken, oriented on filter paper, fixed in 10% formalin and separately embedded in paraffin blocks. Sections were serially cut, stained with haematoxylin and eosin, and assessed under a light microscope. The histological lesions of the intestinal mucosa were evaluated according to the Marsh classification modified by Oberhuber et al. (8): type 0 = normal mucosa; type 1 = infiltrative (with >40 intra-epithelial lymphocytes/100 epithelial cells); type 2 = crypt hyperplasia; type 3a = mild villous atrophy; type 3b = marked villous flattening; and type 3c = total villous atrophy.

According to the extension of the intestinal lesions, coeliac patients were divided into three groups:

• Group 1: 299 patients with diffuse duodenal lesions (D, when histological lesions were found in all biopsies).
• Group 2: 25 patients with patchy lesions (P, when one or more specimens were found to be normal).
• Group 3: 21 patients with only the bulb involved (B, when biopsies from the distal duodenum were normal).

Results were also reported comparing patients with diffuse lesions (D) with patients having localized lesions (P+B).

A total of 231 sera of these patients (D, 205; P, 16 and B, 10), which had been stored, were also tested for F1 (a.a. 227–687) and F2 (a.a. 473–687) immunoreactivities.

The study was performed according to the Declaration of Helsinki.

Auto-antibody radioimmunoassay

The fl, F1 and F2 tTG cDNAs (kindly provided by Prof George Eisenbarth, Barbara Davis Center for Childhood Diabetes, University of Colorado Denver, Aurora, CO, USA) were transcribed and translated in vitro in the presence of [35S]-methionine (NEN™ Life Science Products Inc.) using the TNT-coupled rabbit reticulocyte system (Promega). The presence of serum IgA tTGAb against each of the three tTG constructs was detected by previously published RIA methods (6, 14) and antibodies bound with labelled products were separated from free ones utilizing goat anti-human IgA-agarose (Sigma). Results were expressed as an autoantibody index calculated, for each single tTG fragment, as follows: autoantibody index = (sample cpm – negative standard control cpm)/(positive standard control cpm – negative standard control cpm). Serum samples were considered IgA-fl, F1 or F2 autoantibody positive when the autoantibody index values were above 0.050, 0.166 and 0.126, respectively, as calculated in our previous study (6). We took part in the first International Transglutaminase Auto-antibody Workshop for Coeliac Disease (15), using the RIA method, which resulted in the most sensitive (93%) and specific (100%) assay among the 20 laboratories participating in the Workshop.

Statistical analysis

A standard statistical package was used for comparisons between groups (SPSS version 16.0, Chicago, IL, USA). The χ² test was used to compare frequency distributions obtained from the two groups. Spearman’s correlation was used to compare continuous variables. Type 1, 2, 3a, 3b, 3c histological lesions, according to Marsh modified classification (8), and B (bulb only), P (patchy) and D (diffuse) were considered as continuous variables.

Quantitative variables, means, medians and standard deviations were computed and analysis of variance was used.
to compare continuous data across independent ordinal variables.

Linear regression analysis and multivariate analysis were used to evaluate the relationship between variables. A two-tailed $P < 0.05$ was considered significant.

**Results**

**Full-length anti-tTG immunoreactivity**

All the 345 CD patients were found to be full-length tTGAb (flAb) positive (mean ± SD, 0.82 ± 0.42 autoantibody titre). A positive correlation was observed between flAb titer and both Marsh modified histological grading of duodenal lesions ($r$: 0.292, $P < 0.0001$) and duodenal lesions’ extension ($r$: 0.284, $P < 0.0001$). Mean ± SD flAb titres were 0.30 ± 0.32, 0.15 ± 0.26 and 0.03 ± 0.06 in patients with D, P and B lesions, respectively. A significantly higher mean of flAb titres was found in D compared with localized (P+B) patients ($P = 0.002$) and in D versus B patients ($P = 0.009$; Fig. 4A).

Autoantibodies recognising F2 (F2Ab) were found in 113 out of 231 CD patients (48.9%) and in 53.2, 18.8 and 10% of D, P and B patients, respectively (Fig. 3). A significantly higher F2Ab frequency was found in localized (P+B) compared with D sera (53.2 versus 15.4%, $P = 0.0006$; Fig. 3). A positive correlation was observed between F2Ab titres and duodenal lesions’ diffusion ($r = 0.211$, $P < 0.001$).

Mean ± SD F2Ab titres were 0.04 ± 0.17, 0.005 ± 0.13 and −0.09 ± 0.15 in patients with D, P and B lesions, respectively (Fig. 4B).

A positive correlation was observed between flAb titres and both F1Ab ($r$: 0.541, $P < 0.0001$) and F2Ab ($r$: 0.421, $P < 0.0001$) titres. A positive correlation was observed between F1Ab and F2Ab titres ($r$: 0.624, $P < 0.0001$).

**Combined F1 and F2 immunoreactivities**

Out of 231 patients, 108 sera (46.8%) were positive for both F1Ab and F2Ab, 90 sera (38.9%) were positive for F1Ab but negative for F2Ab, 27 (11.7%) were negative for both fragments and only six (2.6%) were positive for F2Ab but negative for F1Ab (Fig. 5A).

The percentage of positive sera for both F1Ab and F2Ab in D, P or B patients was 50.7, 18.8 and 10%, respectively. A significant difference was found between localized (P+B) and D lesions (15.4 versus 50.7%, $P = 0.0014$). Among patients with D, P and B lesions, 38, 56.2 and 30% of sera were positive for F1Ab but negative for F2Ab, respectively. The percentage of D, P and B patients negative for both F1Ab and F2Ab was

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**Fig. 2.** fl mean auto-antibody titres in coeliac patients divided according to the diffusion of the histological lesions in the duodenum. D: histological lesions in all biopsies; P: one or more normal duodenal specimens; B: only the duodenal bulb involved.
8.3, 25 and 60%, respectively. A significant difference was found between localized (P+B) and D patients (38.5 versus 8.3%, \(P = 0.0017\)). Only 6 out of 205 patients (3%), all with diffuse lesions, were negative for F1Ab and positive for F2Ab (Fig. 5B).

Discussion

CD is a multi-factorial disease with a wide clinical spectrum. It is characterized by a multiformal combination of immunological, genetic, clinical and histological pictures that interact in a unique and sometimes surprising way.

Although a correlation between RIA tTGAb titres and HLA typing (16) in coeliac patients has been demonstrated, as well as a high probability of duodenum damage in patients with high ELISA tTGAb (17–19), as far as we know CD histopathology has never been linked with the clinical or serological data. In this study, we demonstrated, in a large cohort of CD patients, a correlation between the humoral coeliac-specific immune response and histological duodenal lesions. In particular, higher tTGAb titres were found in CD patients with the worst grade of histological lesions and in patients with a diffuse duodenal involvement.

In order to gain new insights on these findings, sera from a subgroup of the coeliac patients were also tested for the immunoreactivity against two tTG fragments that account for protein domains shown to be targets of tTGAb in CD (3–7). Human tTG plays a key role in CD pathogenesis (2, 20) and represents the main target of CD specific auto-antibodies (1). Evidence from literature suggests that human tTG has a bi-functional role. In its inactive state, tTG can function in G-protein inside signal-transduction pathways, reflecting the involvement in programmed cell death (21), whereas its activation by calcium ions leads to catalyzation of \(\gamma\)-glutamyl-lysine bonds (22). A three-dimensional model of tTG revealed that it consists of four domains: the N-terminal domain with a \(\beta\)-sandwich structure; the enzyme core domain, containing a

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**Fig. 3.** F1 (a.a. 227–687) and F2 (a.a. 473–687) auto-antibody frequencies in coeliac patients divided according to the diffusion of histological lesions in the duodenum. D: histological lesions in all biopsies; P: one or more normal duodenal specimens; B: only the duodenal bulb involved.

**Fig. 4.** F1 (a.a. 227–687) (a) and F2 (a.a. 473–687) (b) mean Ab titres in coeliac patients divided according to the diffusion of the histological lesions in the duodenum. D: histological lesions in all biopsies; P: one or more normal duodenal specimens; B: only the duodenal bulb involved.
series of α-helices; and two C-terminal domains, consisting of two β structures arranged in barrel-like conformations. The transglutaminase activity is localized in the N-terminal domain, the enzyme core domain contains the GTP/ATP hydrolysis activity. tTG also contains a calcium-binding region, consisting of a short amino acid sequence (a.a. 446–453), and an active site located at Cys 277 of the protein. At least four critical amino acid residues are involved in the preservation of dominant tTG epitopes (a.a. 1–13, a.a. 228–347, a.a. 473–496, a.a. 649–687) (3). Nakachi et al. (5) identified the N-terminal and the core of the protein as the major immunodominant domains of the protein. A recent paper showed the key role, as a coeliac epitope, of Glu153 and 154 on the first α-helix of the core domain and of the Arg19 on the first α-helix of the N-terminal domain, both in closed and in open conformations of tTG. As an alternative to Arg19, Glu153 also may cooperate with Met659 on the C-terminal domain (7).

In our study, patients with diffuse histological lesions showed, at the time of diagnosis, a stronger immunoreactivity in terms of both autoantibody titres and frequency compared with patients with localized duodenal involvement. The single and the combined immunoreactivities of tTG (227–687) and tTG (473–687) constructs were significantly higher in patients with diffuse lesions, whereas the absence of both immunoreactivities was significantly more frequent in coeliac patients with lesions confined in the bulb. A difference in extension of histological lesions has been extensively reported in both children (10) and adult CD patients (11, 23).

In our study, autoantibody titres showed a trend downward in line with the extent of duodenal involvement. These results could be explained based on the assumption that the bulb is the first duodenal tract to be reached by gluten and possibly the first tract to initiate the intestinal damage in coeliac subjects. If this was true, patients with a shorter gluten exposure would be those with only the bulb involved. This assumption accords with our results, where patients with only the bulb involved showed a lower immunoreactivity at diagnosis. Moreover, this group of patients react against the N-terminal tract of the protein, which is one of the immunodominant domains of the molecule (5, 7). On the other hand, coeliac patients with D lesions that were supposed to have a longer gluten exposure

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**Fig. 5.** Serum auto-antibody frequency of combined F1 (a.a. 227–687) and F2 (a.a. 473–687) in all the coeliac patients investigated (a) and in coeliacs divided according to the diffusion of the histological lesions in the duodenum (b). D: histological lesions in all biopsies; P: one or more normal duodenal specimens; B: only the duodenal bulb involved.
showed a broad immune response and reacted against all the tTG epitopes.

However, our study has some limits. First, the immunoreactivity against F1 and F2 was possible only in a subgroup of patients, due to limited availability of patients’ sera. Second, it would be interesting to perform immunofluorescence on biopsy specimen since we did only a serological evaluation. Finally the folding of F1 and F2 could expose epitopes quite different from fltTG.

This is the first study, to our knowledge, demonstrating that tTGAb immunoreactivity correlates with CD histological grading and diffusion of duodenal lesions. Our study represents a first attempt to understand what might influence the natural history of the disease. However, further studies on the coeliac-specific immunoreactivity are necessary in order to clarify CD pathogenesis.

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