Autoantibodies of patients with coeliac disease are insufficient to block tissue transglutaminase activity

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Background and aims: Coeliac disease (CD) is characterised by the presence of autoantibodies against tissue transglutaminase (tTG), the endomysial autoantigen. This study was performed to determine the effect of purified autoantibodies on the enzymatic activity of tTG.

Methods: Total IgA and IgG class antibodies and purified anti-tTG autoantibodies were isolated from sera of untreated patients with CD and controls. The inhibitory capacity of the antibodies on tTG activity was checked by a fluorometric assay based on the incorporation of monodansyl cadaverine into casein by and/or tTG-catalysed cross linking of bioinylated cadaverine to gliadin.

Results: The enriched IgA and IgG fractions of five patients with CD and three controls resulted in no significantly different inhibition of enzymatic activity. In contrast, the use of affinity purified anti-tTG autoantibodies of 12 patients with CD led to a dose dependent reduction of tTG activity, compared to control immunoglobulins (n = 6). However, the remaining activity was sufficient for cross linking of cadaverine into gliadin, and enzymatic tTG activity was only blocked completely by high concentrations of a monoclonal antibody, which is directed to the active centre of tTG.

Conclusions: Despite a partial inhibitory effect of isolated anti-tTG autoantibodies from patients with CD, residual enzymatic activity remains sufficiently high to cast doubt on their in vivo relevance.

Isolation of human autoantibodies to tTG

The plasmid pJLP4 expressing human tTG (provided by JL Piper and Ch Khosla, Stanford, USA) was expressed in Escherichia coli Tuner DE3. The purification of recombinant human tTG was performed as described with minor variations. After dialysis against binding buffer (0.1 M sodium bicarbonate, 0.5 M NaCl, pH 8.3) the recombinant tTG was covalently coupled to CNBr-activated CL4B-Sepharose (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. For isolation of autoantibodies against tTG the sera of 12 different patients with active CD were diluted 1:5 in phosphate buffer, pH 7.4, and incubated with the tTG coupled Sepharose. Several washing steps were followed by elution of the anti-tTG autoantibodies with 0.1 M glycine pH 2.5, which were instantly neutralised with 1/5 volume of 1 M Tris-HCl, pH 8.0. The titre against tTG was checked by ELISA as described before.

All antibodies were intensively dialysed against 0.05 M Tris-HCl, pH 9.0. For isolation of IgG antibodies protein A Sepharose (Amersham Pharmacia Biotech, Freiburg, Germany) was swollen in 0.1 M Tris-HCl, pH 8.0, and incubated for 1 h at room temperature with sera from five untreated patients with CD and three control subjects adjusted to pH 8.0 by addition of 1/10 volume 1 M Tris-HCl, pH 8.0. After washing with 0.1 M Tris-HCl, pH 8.0, and 0.01 M Tris-HCl, pH 8.0, bound antibodies were eluted with 0.1 M glycine, pH 2.5, and immediately neutralised with 1/5 volume of 1 M Tris-HCl, pH 8.0.

Abbreviations: ab, antibody; CD, coeliac disease; ELISA, enzyme linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; tTG, tissue transglutaminase.
confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

For additional control experiments a polyclonal goat anti-guinea pig liver transglutaminase antibody (Biomol, Hamburg, Germany), and a monoclonal antibody against guinea pig transglutaminase (CUB 7402, Quartett, Berlin, Germany) were used.

In vitro iTG activity test
Incorporation of biotinylated cadaverine into gliadin
Since gliadin is known as a good glutamine donor substrate for iTG, the enzymatic activity of iTG was checked by cross linking gliadin (glutamine donor) with biotinylated cadaverine (glutamine acceptor). Therefore, 1 μg of crude gliadin (Sigma, Taukirchen, Germany) was incubated with 200 ng biotinylated cadaverine (CovaLab, Lyon, France) and 0.5–1 μg of human recombinant iTG in 0.1 M Tris-HCl, 0.15 M NaCl, 5 mM CaCl₂, pH 7.5, in a total volume of 100 μl. Cross linking was allowed for 2 h at 37°C. For inhibition studies varying amounts of the corresponding antibodies were preincubated with iTG for 10 min at 37°C and the reaction was started by the addition of gliadin and cadaverine. The assay was stopped by addition of trichloroacetic acid at a final concentration of 10% at 4°C over night. Precipitated proteins were run under reducing conditions in SDS-PAGE and transferred to nitrocellulose. The blot was blocked with 3% bovine serum albumin in 0.1 M Tris-HCl, 0.15 M NaCl, pH 7.5, and further incubated with a covalently coupled streptavidine-alkaline phosphatase conjugate (Sigma). Colour was developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue-tetrazolium (SIGMA FAST tablets, Sigma).

Kinetic activity assay
The enzymatic activity of iTG in the presence of antibodies was quantified by measurement of the incorporation of monodansyl cadaverine (glutamine acceptor) into z-casein (glutamine donor). The incorporation of monodansyl cadaverine (N-(5-aminopentyl)-5-dimethylamino-1-naphthalinsulfonamide) (Sigma) into bovine z-casein results in an increased intensity of fluorescence of the dansyl group as described. 10 Although gliadin is also a suitable substrate for this assay, we used z-casein as substrate for iTG because of its better solubility in neutral buffers. z-Casein (17 μM) was incubated with 30 μM monodansyl cadaverine in a total volume of 100 μl 0.1 M Tris-HCl, 0.15 M NaCl, 5 mM CaCl₂, pH 7.5. For inhibition studies the antibodies were added at different concentrations ranging from 0.5–5.5 μg. The reaction was started by addition of 0.5 μg human recombinant iTG at 37°C. Excitation was set at 360 nm and the increase in fluorescence was measured at 550 nm with a fluorescence spectrophotometer (Photon Technology International, Canada). Since human iTG rapidly lost activity at 37°C the slope was determined for only 2 min after addition of iTG. iTG activity was calculated as the percentage of remaining activity in correlation to control experiments without addition of antibodies.

Statistics:
The Mann-Whitney U test was used to analyse the differences of inhibitory capacity of anti-iTG antibodies of patients with CD and immunoglobulins of controls.

RESULTS
Influence of serum on iTG activity
A concentration dependent inhibitory effect on iTG activity was noticed when the sera of patients with CD were added at concentrations of 1%, 5%, or 10% to the fluorometric activity assay. The rate of inhibition of iTG activity ranged from 25–58% with serum concentrations between 1% and 10% (data not shown). However, the same values were also noticed with the control sera, therefore indicating a non-specific inhibitory effect of serum on iTG activity in general.

Effect of isolated IgA and isolated IgG antibodies
Impurities of the isolated IgG with IgA and vice versa were negligible as checked in the IgG- and IgA-specific anti-iTG ELISA, respectively (data not shown).

There was a broad range of inhibition on iTG activity when using the purified IgA and IgG fractions of five patients with CD, reaching up to 60% at high concentrations (4.5–5.5 fold molar antibody excess relative to iTG) in some of the sera (mean 22% for IgA and 35% for IgG). However, there was no significant difference in comparison to total IgA or IgG fractions of three non-coeliac controls (data not shown). This rather suggests non-specific interactions than specific inhibition of iTG activity by total serum IgA or IgG.

Effect of purified anti-iTG autoantibodies
Whereas a commercially available polyclonal goat anti-guinea pig iTG antibody showed no relevant inhibition of iTG activity in the fluorometric assay, the application of the affinity purified anti-iTG autoantibodies of 12 untreated patients with CD (roughly equal quantities of which belong to the IgA and IgG class), caused a distinct and concentration dependent inhibition of iTG activity. Although the inhibition reached up to 80% at an antibody to enzyme ratio of 5.5:1(mol/mol), iTG activity could never be blocked completely and no positive correlation with anti-iTG titres was observed (table 1). In spite of a broad range of inhibitory capacity between different purified iTG autoantibodies from coeliac sera the inhibitory effect was significantly higher than that of immunoglobulin fractions of six non-coeliac controls. The remaining activity reached specific values with a mean of 66% when low (0.5–2 fold molar excess) and 46% when high antibody concentrations were used (4.5–5.5 fold molar excess of antibodies over iTG), thus demonstrating a distinct inhibitory effect of the anti-iTG autoantibodies on iTG activity (fig 1).

iTG undergoes conformational changes in the presence of calcium ions. 15 To determine to what extent the inhibitory effect of anti-iTG autoantibodies depends on the structural changes we preincubated iTG with 5 mM calcium chloride for 5 min on ice. However this procedure had no influence on antibody inhibition (data not shown).

| Table 1 Effect of varying concentrations of affinity purified autoantibodies from six different patients with CD to iTG. |
|-----------------|---|---|---|---|
| Patients with CD | 0.5 | 1 | 2 | 5.5 |
| CD 1 (163)* | 59 | 32 | 25 |
| CD 2 (70) | 30 | 45 | 22 |
| CD 3 (717) | 83 | 50 | 33 |
| CD 4 (100) | 70 | 35 | 44 |
| CD 5 (91) | 123 | 51 | 42 |
| CD 6 (91) | 123 | 65 | 63 | – |

*In IgA anti-iTG titres as determined by EUSA are shown in parenthesis. The values indicate the percentage of remaining iTG activity compared to basal iTG in the absence of inhibitory antibodies.

CD, coeliac disease; iTG, tissue transglutaminase.
Inhibitory effect of mouse monoclonal anti-tTG antibody

The CUB 7402 antibody is described to map amino acids (aa) 447–478 of tTG and inhibit its activity. Accordingly, the antibody clearly inhibited tTG activity resulting in a mean activity of 33% at high antibody concentrations (4.5–5.5 fold molar excess over tTG) compared to purified immunoglobulins of non-coeliac controls (n = 6). The data were calculated as percentage of remaining activity in relation to basal activity. The p values for significant differences of mean values are indicated.

In vitro assay to determine the effect of residual tTG activity

The fluorometric assay was most helpful to quantify the extent of the autoantibody inhibition. We observed a spontaneous decline in tTG activity up to 50% after preincubation of the enzyme without antibodies at 37°C for a short period of 5–15 min. This effect was more pronounced when the preincubation was done in a calcium free buffer (data not shown). Consequently, we performed the test without preincubation of tTG with the antibodies and the enzymatic activity was determined for only 2 min after addition of tTG, since the relation of fluorescence intensity to time was linear in this time frame. However, this assay yields no information about the remaining enzymatic activity.

Therefore, the tTG catalysed incorporation of biotin-labelled cadaverine into gliadin in the presence of the autoantibodies was determined. After preincubation of tTG with the antibodies for 10 min at 37°C, the assay was performed for 2 h at 37°C. Here, cross linking of cadaverine with gliadin results in protein complexes with molecular weights covering the whole range above 30 kDa in SDS-PAGE. The patterns of cross linked gliadins did not differ if tTG was preincubated in the test buffer for 10 min at 37°C or not, irrespective of the presence of increasing amounts of inhibitory antibodies (fig. 2). This indicates that when the cross linking reaction is allowed for a sufficient time the remaining overall enzymatic tTG activity is indistinguishable between uninhibited and partially inhibited enzyme.

In contrast, the monoclonal anti-tTG antibody CUB 7402 markedly reduced the catalytic activity of tTG and almost completely inactivated the enzyme at 5.5 fold molar excess over tTG (fig. 2). This can be explained by the preincubation of tTG with CUB 7402 in this assay for 10 min which appears to be sufficient for the antibody-epitope recognition, while the fluorometric assay which shows 33% residual activity reflects the beginning situation without preincubation.

DISCUSSION

Since tTG was identified as a CD autoantigen several studies stressed the importance of this enzyme in the pathogenesis of the disorder. Thus tTG expression is upregulated in the subepithelial lamina propria, where tTG-induced deamidation of certain gliadin peptides can enhance their T cell stimulatory capacity via presentation by HLA-DQ2 or -DQ8.

In this context the potential inhibitory capacity of the autoantibodies against tTG is of major interest. A former study suggested that total serum IgA of coeliac patients prevented tTG-induced activation of transforming growth factor β, resulting in a disturbed epithelial cell differentiation which is implied in the pathological damage of CD.
Recently, using a crude lysate of IgT-transfected Madin-Darby canine kidney cells as source of IgT, the enzyme inhibitory capacity of total serum IgA and IgG and of recombinant monoclonal anti-IgT antibodies was underlined.\(^\text{23}\) In contrast, another study failed to demonstrate any inhibitory effect of total serum IgA and IgG of patients with CD when tested with purified human IgT.\(^\text{24}\) These results with total IgA and IgG class antibodies were confirmed by our data. Therefore, the question of whether CD autoantibodies to IgT inhibit the enzymatic activity and thus have major physiological significance remains unresolved. Firstly, different IgT sources were used. Secondly, when measuring IgT activity, buffer reagents like glycine or acetic acid which are normally used to isolate the antibodies, have to be completely removed since even small amounts of these reagents strongly inhibit IgT activity. Thirdly, bioactive IgT is a very unstable protein. Thus incubation at 37°C leads to a marked decrease in enzymatic activity particularly in calcium free buffers (data not shown). In this context it must be emphasised that both the enriched IgA and IgG fractions as well as total serum of patients with CD and of non-coeliac controls block IgT activity (up to 60% in particular cases, possibly due to substrate competition). We could exclude human serum albumin as inhibitor but could not identify the (unspecific) inhibitory serum component(s).

However, when using the sensitive fluorometric assay and affinity purified anti-IgT autoantibodies, which belong to the IgA and IgG class in a nearly equal proportion, we could show a distinct and specific inhibition of IgT activity. This inhibition was dose-dependent and led to a mean residual activity of 46% when used at the highest autoantibody concentrations (4.5–5.5 fold molar excess over IgT). We obtained similar results when IgT was preincubated in calcium containing buffer on ice, but complete inhibition could never be achieved with the purified anti-IgT autoantibodies. This is in agreement with a report which demonstrated that the catalytic region of IgT is only of minor antigenicity and that most of the autoantibodies are directed against the non-catalytic amino and carboxyterminal region.\(^\text{12}\) One reason for this could be that in vivo the enzyme is usually substrate-bound and thus the catalytic centre is concealed from the immune system. Thus, only preincubation of IgT with the monoclonal antibody CUB 7402, which is directed to the catalytic centre of IgT, almost completely blocked IgT activity.

The results with purified autoantibodies seem relevant for the situation in vivo, since no significant high molecular serum components are expected in the lamina propria. Therefore, we suggest that the inhibitory effect of anti-IgT autoantibodies is of minor biological importance in vivo. This conclusion is underlined by the fact that incomplete inhibition of IgT activity still allows highly efficient incorporation of cadaverine into gliadin when the reaction was performed for a longer time period. The increased mucosal IgT activity seems to play a central role in the pathogenesis of CD. Since this enhanced enzymatic activity in CD can not be completely blocked by patients’ anti-IgT autoantibodies, the search and use of efficient inhibitors to reduce the pathologically increased IgT activity may be a viable novel treatment approach for CD.\(^\text{11}\)

ACKNOWLEDGEMENTS

We thank Dr Chaitan Khosla for the kind gift of the recombinant IgT plasmid and Dr Norbert Blank, Medical Department III, FAP Erlangen-Nuernberg for the introduction in measurement with the fluorometric PTI instrument.

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This study was supported by grants Schu 666/11-2 from the Deutsche Forschungsgemeinschaft and by grants of the European Communities, Celiac EU-cluster program “Quality of Life and Management of Living Resources, QLRT-1999-00037”, as well as NIH grant DK063158.

