Missing endomysial and reticulin binding of coeliac antibodies in transglutaminase 2 knockout tissues

I R Kovács, S Fésüs, M Mäki

Background: Autoantibodies against transglutaminase 2 (TG2) are thought to be responsible for the endomysial (EMA), reticulin (ARA), and jejunal antibody (JEA) tissue binding of serum samples from coeliac patients but the exclusive role of TG2 in these staining patterns has not yet been established.

Aims: To evaluate whether antigens other than TG2 contribute to EMA/ARA/JEA reactions.

Methods: Serum samples from 61 patients with untreated coeliac disease, 40 dermatitis herpetiformis patients, and 34 negative non-coeliac controls were tested.

Results: None of the patient serum samples elicited EMA, ARA, or JEA binding in TG2 knockout (TG2−/−) mice, as these mice are viable and have no major organ abnormalities. Using such TG2 deficient tissue sections, all antibody binding other than that elicited by TG2 should remain visible.

Conclusions: EMA/ARA/JEA antibody binding patterns are exclusively TG2 dependent both in coeliac and dermatitis herpetiformis patients. Actin antibodies are responsible for some positive which are not part of the EMA/ARA/JEA reactions.

In a subgroup of individuals with HLA-DQ2 or DQ8 alleles, cereal gluten ingestion induces coeliac disease, which is accompanied by circulating autoantibodies against certain extracellular matrix structures. These autoantibodies are highly pathognomonic for untreated coeliac disease, are predominantly of the IgA class, and disappear on treatment with a gluten free diet. They also occur in cases of dermatitis herpetiformis where gluten sensitivity is manifested as both intestinal and skin disease. According to the tissue antigen used and the typical binding pattern observed, the coeliac autoantibodies have been named R, type reticulin (ARA), endomysial (EMA), and jejunal (JEA) antibodies.

Only since 1997 it was discovered that EMA react with the enzyme transglutaminase 2 (TG2, tissue transglutaminase), one of the eight currently known transglutaminase enzymes which form epsilon-gamma protein cross links between glutamine and lysine residues of proteins. Subsequently, TG2 based enzyme linked immunosorbent tests (ELISA) of high diagnostic value for coeliac disease were developed. Monoclonal antibodies against TG2 also bind to tissue sections in EMA, ARA, and JEA patterns under conditions similar to those in indirect immunofluorescent tests used in clinical diagnostics. Moreover, soluble TG2 from the guinea pig added to tissue fibronectin can replace the chemically removed endomysial target antigen which appears to be bound to fibronectin. However, a question remains as to whether other autoantigens contribute to the endomysial and related tissue binding patterns of coeliac serum samples. Autoantibodies against actin, calreticulin, desmin, and a 90 kDa dermal glycoprotein have also been described in coeliac patients.

In the present study we aimed to establish whether coeliac disease and dermatitis herpetiformis patient sera, giving the typical coeliac-type tissue staining pattern in normal primate and rodent tissues, contain autoantibodies to structures other than TG2. We took advantage of the recent development of TG2 knockout (TG2−/−) mice, as these mice are viable and have no major organ abnormalities. Using such TG2 deficient tissue sections, all antibody other than that elicited by TG2 should remain visible.

METHODS

Patient samples

IgA class EMA positive serum samples from 61 patients with untreated coeliac disease (aged 0.9–57.8 years; median 5.6) and from 40 patients with untreated dermatitis herpetiformis (aged 2.5–26.1 years; median 9.3), diagnosed according to current criteria by jejunal biopsy and skin immunofluorescent study, were investigated. Thirty four EMA negative serum samples from non-coeliac individuals with normal jejunal morphology (aged 1.2–60 years; median 6.8) were used as controls. Seven of these control serum samples were positive for IgA antibodies against gliadin. Serum samples were used with the permission of the ethics committee of the Heim Pál Children’s Hospital, Budapest.

Immunofluorescent studies

Oesophagus, jejunum, liver, and kidney tissues from TG2−/− C57BL/6 knockout mice and corresponding wild-type mice
TG2+/+) were snap frozen and kept at −70°C until testing. Unfixed frozen sections were used as substrates in indirect immunofluorescent studies with single and double labelling of patient IgA antibodies (green) and monoclonal mouse IgG1 class anti-TG2 antibodies CUB7402 and TG100 (red) (Neo-Markers, Fremont, California, USA), as previously described. Bound patient IgA antibodies were detected with fluorescein isothiocyanate conjugated secondary antibodies (Dako A/S, Glostrup, Denmark; diluted 1:120). Mouse tissue bound mouse anti-TG2 antibodies were detected with rabbit anti-mouse IgG1 (research antibody from Pharmacia Diagnostics AB, Uppsala, Sweden) and rhodamine conjugated swine antibodies against rabbit immunoglobulins (Dako), both diluted 1:100, to reduce background staining.

Serum samples from patients were initially tested in dilutions of 1:5 and 1:50 and, if necessary, titrated in serial twofold dilutions until the end point was reached. In double immunostainings, five coeliac, three dermatitis herpetiformis, and five control samples were used together with the commercial monoclonal anti-TG2 antibodies.

The EMA binding pattern was evaluated on the muscular layers of the oesophagus and jejunum, and on blood vessels, the ARA binding pattern on liver and kidney, and the JEA binding pattern on jejunal villi, as described elsewhere. In control double staining experiments, polyclonal rabbit antibodies against fibronectin (Dako; diluted 1:120), calreticulin (Alexis Biochemicals, San Diego, California, USA; diluted 1:200), and monoclonal mouse IgG2a antibodies against smooth muscle actin (MS-113; NeoMarkers; diluted 1:400, plus rabbit antibodies against mouse IgG2a; Zymed, San Francisco, California, USA) recognised with rhodamine conjugated antirabbit antibodies comprised the red component and human IgA binding (recognised as before) the green. In other control experiments, TG2−/− tissues were incubated prior to the indirect immunofluorescent study with soluble human recombinant TG2 (Pharmacia), 2 µg/ml in 0.05 M Tris, 0.15 M NaCl, pH 7.4 (Tris buffered saline (TBS)) with 0.005 M CaCl2 for 30 minutes.

Serum antibodies were also investigated on primate tissues, monkey oesophagus, normal human jejunum, and human appendix, respectively, as previously described. ELISA tests IgA serum antibodies against gliadin and natural human TG2 antigen purified from normal red blood cells were measured by ELISA, as described elsewhere. The cut off level for TG2 antibody positivity was previously established at 10% of the absorbance of an international reference sample. IgA

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Coeliac-type endomysial (EMA), reticulin (ARA), and jejunal antibody (JEA) binding of serum samples from gluten sensitive and control patients to transglutaminase 2 knockout (TG2−/−) and corresponding wild-type (TG2+/+) mouse tissues</th>
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<tbody>
<tr>
<td></td>
<td>Coeliac disease (n=61)</td>
</tr>
<tr>
<td>EMA/ARA/JEA+ on TG2+/+ mouse tissues</td>
<td>57*</td>
</tr>
<tr>
<td>EMA/ARA/JEA+ on TG2−/− mouse tissues</td>
<td>0</td>
</tr>
<tr>
<td>EMA/ARA/JEA+ on TG2−/− mouse tissues</td>
<td>61</td>
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<tr>
<td>Antihuman TG2 antibodies (ELISA)</td>
<td>61</td>
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*Two additional patients showed EMA in the oesophagus, but not JEA or ARA in other tissues.
antibodies against calreticulin were investigated as follows: 0.2 µg/well purified calreticulin (Sigma Chemicals, St Louis, Missouri, USA) were coated overnight at 4°C on ELISA plates (Nunc-Immuno Maxisorp, Nunc, Roskilde, Denmark) in 0.03 M NaHCO₃ buffer, pH 9.6. After three washes with TBS containing 0.1% Tween 20, patient samples diluted 1:100 in TBS were incubated for one hour. After extensive washing, bound IgA antibodies were measured with peroxidase conjugated rabbit antibodies against human IgA (Dako), similar to TG2 antibody detection. Commercial antibodies against calreticulin (Alexis) were used as positive controls.

Statistical differences between study groups were estimated using the Student’s t test and χ² test, with two tailed probabilities. The median was used to characterise discontinuous antibody titres in immunofluorescence.

RESULTS
Coeliac-type positivity
On wild-type mouse tissues, serum samples of both coeliac and dermatitis herpetiformis patients but not controls showed EMA-, ARA-, and JEA-type IgA binding (table 1). The positivity clearly colocalised with the tissue distribution of TG2 shown by monoclonal TG2 specific antibodies (fig 1A, 2A). In contrast, in TG2 knockout mouse tissues, no EMA-, ARA-, or JEA-type binding was seen in any of the coeliac, dermatitis herpetiformis, or controls samples, even at low serum dilutions of 1:5. Similar results were obtained with IgG antibodies from IgA deficient coeliac patients (data not shown). No tissue positivity was seen with the CUB7402 (fig 1B, 2B) or TG100 monoclonal TG2 specific antibodies in TG2−/− knockout mice, thus confirming that they lack TG2. Tissues of TG2−/− mice were of normal morphology, and also the small intestinal mucosa, showing a villus height-crypt depth ratio of more than 5.

Fibronectin, the main protein anchoring TG2 to the extracellular matrix, was found in both TG2+/+ and TG2−/− tissues (fig 2D, E). However, in TG2 deficient mice it did not bind IgA from sera of coeliac or dermatitis herpetiformis patients (fig 2E).

To further confirm that EMA positivity elicited by serum samples from coeliac disease and dermatitis herpetiformis patients is TG2 dependent, we incubated TG2−/− tissues with recombinant human TG2 to insert TG2 into empty fibronectin binding sites. When these TG2 coated sections were used as

Figure 2  Wild-type (A, D) and transglutaminase 2 knockout mouse (B, C, E, F) jejunum double stained with coeliac patient IgA (green) and monoclonal transglutaminase 2 specific (TG2) antibodies (red) (A, B, C), and with coeliac patient IgA (green) and antibodies to fibronectin (D, E, F). Coeliac IgA binding completely colocalised with tissue TG2 (A) and partially colocalised with fibronectin (D) in wild-type jejunum and in TG2 knockout mouse tissues incubated with human recombinant TG2 (C, F). Coeliac IgA and TG2 monoclonal antibodies did not bind to TG2 knockout jejunum (B) where fibronectin was normally present (E). (Bar=30 µm.)
substrates, both coeliac and dermatitis herpetiformis patient samples, as well as monoclonal TG2 specific antibodies, showed typical EMA-, ARA-, and JEA-type binding (fig 1C, fig 2C, F).

A total of 57 (93.4%) of 61 coeliac samples and 39 (97.5%) of 40 dermatitis herpetiformis samples showed clear coeliac-type positivity with all four rodent TG2+/+ organs. Two additional coeliac serum samples were positive only for the oesophagus. However, all 101 samples reacted in the EMA, ARA, and JEA pattern with TG2−/− mouse tissues coated with human TG2 and also reacted with natural TG2 from human red blood cells in ELISA (table 1). These findings demonstrate that some coeliac patients have only human TG2 specific antibodies.

**DISCUSSION**

Coeliac disease is an autoimmune disorder characterised by production of a large array of antibodies against tissues structures, matrix, and cellular antigens.1 3 8–11 19 According to the present findings, TG2 specific autoantibodies would appear to be of primary importance. Our results with TG2 knockout tissues for the first time directly prove that the diagnostically relevant EMA, ARA, and JEA binding patterns in serum samples from both coeliac and dermatitis herpetiformis patients are clearly and exclusively TG2 autoantibody dependent and target the fibronectin bound extracellular TG2 in normal human and rodent tissues. In the large series of serum samples we tested on TG2 knockout mouse tissues, TG2 unrelated non-specific EMA positivities were not observed. Complementing these results, we also experienced the same with EMA positive subjects in whom coeliac disease remained unconfirmed (data not shown). Thus the typical coeliac-type immunofluorescent positivity has an outstanding specificity for TG2 antibodies. Non-TG2 antibodies that produce EMA binding, albeit theoretically possible, seem to be very rare.

Serum samples from some seriously ill coeliac patients often show complex antibody binding. Due to high background binding to epithelial or smooth muscle cells, such “difficult” samples may appear negative for EMA in low serum dilutions20 but react with TG2 in ELISA. Smooth muscle cells contain high amounts of TG2. However, our results do not support the notion that reactivity with smooth muscle cells represents antibodies to intracellular TG2, as it was similarly observed in TG2 deficient tissues. We identified a subtype of smooth muscle antibodies as antibodies to actin. When present together with EMA, actin antibodies bound to the contractile bundles of the villi may modify the appearance of the coeliac-type positivity on the jejunum and may be responsible for the additional non-TG2 related components observed by other investigators.12 However, actin antibodies alone were not able to elicit EMA-type positivity in the absence of TG2.

We frequently found non-TG2 related antibodies in serum samples from coeliac and dermatitis herpetiformis patients but in low titres compared with endomysial autoantibodies. Apart from antibodies against actin, their binding lacked any specific morphological appearance and they occurred in less than half of all patients. Gliadin antibodies were associated both in coeliaes and controls with a higher rate of antibody binding to jejunal epithelial cells. These features support the

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**Table 2 Non-endomysial-type autoantibody binding patterns observed in transglutaminase 2 knockout mouse tissues with serum samples from gluten sensitive and control patients**

<table>
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<tr>
<th>Serum dilution</th>
<th>Reactivation with smooth muscle cells</th>
<th>Reactivation with epithelial cells</th>
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<tbody>
<tr>
<td>Coeliac disease (n=61)</td>
<td>1.5 [31.1%]</td>
<td>16 [26.3%]*</td>
</tr>
<tr>
<td>Dermatitis herpetiformis (n=40)</td>
<td>1.5 [14.8%]</td>
<td>2 [5.0%]</td>
</tr>
<tr>
<td>Controls (n=34)</td>
<td>1.5 [1.6%]</td>
<td>3 [8.8%]</td>
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*P=0.002 versus dermatitis herpetiformis patients; p=0.02 versus controls.
view that these antibodies may be secondary and variably induced by tissue damage facilitating increased presentation of different self antigens to the immune system. As we now observed, coeliac disease specific autoantibodies react primarily with human TG2. Cross reaction with mouse TG2 was prevalent (93.4%) but not universal in our coeliac patients. The slightly different rate of coeliac autoantibody positivities in primate and rodent tissues was for a long time a subject of diagnostic debate and led to the misconception that ARA (rodent specific) and EMA (primate specific) tissue autoantibodies represent different antibody populations. However, with demonstration that a recombinant human TG2 protein inserted into mouse tissues comprises as good a coeliac autoantigen as natural primate tissues, we provide additional evidence that the “reticulin” and “endomysial” binding patterns are tissue-type and not species dependent detection methods of the same TG2 autoantibodies.

Our current understanding of the differences in clinical outcome between classical coeliac disease and dermatitis herpetiformis, where apart from gluten enteropathy dermal IgA deposits also occur, remains incomplete. It has been hypothesised that dermal IgA may represent immune complexes or additional antibodies against dermal proteins. Recent interest has focused on potential cross reaction with other structurally related transglutaminases, of which several are expressed in the epidermis (TG1, TG3, TG5, TG7). While serum antibodies from coeliac and dermatitis herpetiformis patients do not recognise TG1, it has been shown recently that a subset in both diseases cross reacts with TG3 (epidermal transglutaminase). In dermatitis herpetiformis, patients also develop high avidity antibodies specific for TG3, and TG3 is a component of dermal deposits and seems to be the major autoantigen involved in skin manifestations. However, dermatitis herpetiformis patients also produce autoantibodies that primarily react with TG2 and relate to the enteropathy. As we have shown here, TG3 specific antibodies do not contribute to the EMA positivity of these sera which requires targeting of TG2.

In similar ways, other particular presentation types of coeliac disease may potentially be associated with targeting of both TG2 and other transglutaminases. Studies with mRNA suggest that TG5,7 as well as TG3 are widely distributed in various tissues but the protein amounts and their histochemical localisation in non-dermal tissues are at present still unknown. Tissues of TG2 knockout mice are particularly suitable in the search for such autoantibody targets because the apparently normal condition of these mice is most probably maintained by other upregulated transglutaminases. As we also observed, these mice do not develop jejunal villous atrophy even in the absence of TG2 function. However, with the conventional immunofluorescent technique, we did not find any staining differences between samples from coeliac and dermatitis herpetiformis patients. This is not in contrast with previous results because the autoantibodies in both conditions can cross react with TG3 in ELISA and they differ only in their relative avidity and

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**Figure 3** (A, B) Serum sample from a coeliac patient containing IgA antibodies (green) which bound to transglutaminase 2 knockout (TG2−/−) mouse jejunum along the axis of the villi (arrows) and colocalised to yellow with smooth muscle actin stained in red (C). Note the absence of endomysial (EMA) or jejunal (JEA) antibody staining. In wildtype mouse jejunum (D), the axial positivity merged with the IgA coeliac-type JEA of the same serum sample (arrow), colocalised with actin stained in red (E) but its cross sections remained discordantly green (arrows) after double staining with TG2 in red (F). (G) Actin bundles (red) were similarly arranged in the human jejunum but did not merge with the typical coeliac-type jejunal positivity (green) elicited by a coeliac patient serum sample containing only EMA. (Bar=30 µm.)
specificity which, however, may not lead to visible differences in immunofluorescence. We were also unable to demonstrate staining differences between patients and controls in TG2 deficient tissues. Several explanations are possible, such as the small fraction of TG3 reactive autoantibodies or the low amounts of TG3 protein. In fact, TG3 specific rabbit antibodies, working in western blot or on skin sections, also failed to show jejunal immunofluorescent positivity in other studies. Therefore, further search for additional transglutaminase targets will necessitate more sensitive detection tools or unmasking of antigens.

In conclusion, our results confirm the identity of EMA, ARA, and JEA with TG2 specific autoantibodies and directly prove the exclusive role of TG2 in the specific and diagnostic coeliac-type immunofluorescent reactions.

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