Elevation of IgG antibodies against tissue transglutaminase as a diagnostic tool for coeliac disease in selective IgA deficiency

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Patients and Methods

Patients
To identify IgA deficient coeliac patients, the following policy was used at the Heim Pál Children’s Hospital, Budapest.

Abbreviations: AGA, antigliadin antibodies; ARA, R1-type reticulin antibodies; ELISA, enzyme linked immunosorbent assay; EMA, endomysial antibodies; ROC, receiver operating characteristics; TGF-β, transforming growth factor beta; tTG, tissue or type 2 transglutaminase
Serum samples submitted for IgA EMA analysis and found to be negative were analysed for IgG EMA in such cases where serum total IgA was low. Cut off values for IgG EMA testing were established from age related serum IgA ranges of normal Hungarian children<sup>23</sup>: <0.2 g/l in children aged 0–10 years and <0.4 g/l in older patients.

During the years 1989–2001, serum samples from 1807 new patients with low serum IgA levels were prospectively tested for IgG EMA, and a small intestinal biopsy was offered to patients with positive EMA results. IgA deficient patients with a clinical suspicion of coeliac disease underwent small intestinal biopsy also, independent of the EMA results. In total, 78 IgA deficient patients (median age 7.9 years (range 0.9–73), median serum IgA concentration 0.01 g/l (range 0.00–0.11)) were diagnosed with coeliac disease according to the revised ESPGHAN criteria,<sup>22</sup> and follow up samples were collected from 36 of these patients after a prolonged period on a gluten free diet (range 0.4–4.7 years).

Furthermore, serum samples from 73 IgA deficient non-coeliac patients (median age 2 years (range 1–40)) who were negative for both IgA and IgG EMA and had total serum IgA levels <0.2 g/l were included in the study. Forty six of these control patients (controls I) had a jejunal biopsy performed and all had normal villous architecture; the other 27 patients (controls II) had no small intestinal biopsy performed. Table 1 shows the clinical symptoms of the IgA deficient coeliac and non-coeliac patients in whom diarrhoea, weight loss, hypoproteinaemia, and vitamin K deficiency were equally distributed. IgA deficient coeliac patients had however a significantly higher frequency of iron deficiency, short stature, and hypocalcaemia (table 1).

IgG EMA and anti-tTG antibodies were also analysed in serum samples from 174 blood donors who had selective IgA deficiency with a total serum IgA level <0.2 g/l. These samples were collected during 1991–1992 at the Finnish Red Cross Blood Transfusion Service, Helsinki, Finland.<sup>24</sup>

### Measurement of serum autoantibodies

Anti-tTG antibodies were measured from number coded serum aliquots stored at −40°C. IgA anti-tTG specific antibodies were measured with the Celkey assay (Pharma Diagnostics Freiburg, Germany) according to the manufacturer’s instructions. IgG anti-tTG antibodies were measured with a research ELISA (Pharmacia Diagnostics Freiburg, Germany) reagents. In blood donors, serum IgA levels were measured by an enzyme immunoassay.<sup>25</sup>

### RESULTS

#### Serum antibodies on a normal diet

Serum levels of IgG anti-tTG antibodies of IgA deficient patients with coeliac disease, control patients, and blood donors are shown in fig 1A and 1B. The area under the ROC curve was 0.996, and with a cut off value of 10 U/ml, the sensitivity and specificity of the IgG anti-tTG assay in clinical patients were 98.7% and 98.6%, respectively. The frequency of values above 5 U/ml and 10 U/ml, respectively, were considered positive for IgA and IgG anti-tTG antibodies.

IgG EMA were assessed using an indirect immunofluorescent method on a composite block of monkey oesophagus, human jejunum, and appendix, or on human umbilical cord from premature newborns (blood donor samples), as described elsewhere,<sup>22</sup> Positive results (1:2 dilution) were quantified by further titration using appendix sections.

### Genetic typing

Whole blood was available from 19 IgA deficient coeliac patients and from 12 IgG EMA positive blood donors for determination of HLA-DQB1<sup>+</sup> alleles, which was performed with the Dynal AllSet SSP DQ low resolution (Dynal AS, Oslo, Norway) and Olerup SSP DQ low resolution (GenoVision, Saltsjöbaden, Sweden) kits.

#### Immunoglobulin levels

Total serum IgA was determined in clinical patients using the Boehringer Mannheim/Hitachi 912 Analyzer (Boehringer Mannheim Corporation, Indianapolis, Indiana, USA) with Tina-quant IgA (Roche Diagnostics GmbH, Mannheim, Germany) reagents. In blood donors, serum IgA levels were measured by an enzyme immunoassay.<sup>25</sup>

### Statistics

Antibody levels are expressed as median (5th and 95th centiles) values. The Mann-Whitney U test (two tailed) was used to estimate differences in antibody levels between groups, the Wilcoxon signed rank test to compare antibody levels before and after the diet, and the Spearman rank method (two tailed) to calculate the correlation between IgG EMA and anti-tTG antibody levels. The cut off level of the anti-tTG assay was calculated from receiver operating characteristics (ROC) analysis<sup>21</sup> carried out with coeliac disease patients and all of the control subjects (controls I+controls II).

| Table 1 Presence of clinical symptoms (signs) and associated diseases in IgA deficient coeliac patients and non-coeliac control patients (%) |
|---|---|---|---|---|---|---|
| **Clinical symptoms and signs** | Coeliac patients (n = 78) | Controls I (n = 46) | Controls II (n = 27) | All controls* (n = 73) | p Values† |
| Chronic or recurrent diarrhoea | 53 (67.9%) | 32 (69.6%) | 11 (40.7%) | 43 (58.9%) | 0.248 |
| Distended abdomen, bloating | 34 (43.6%) | 14 (30.4%) | 6 (22.2%) | 20 (27.4%) | 0.038 |
| Slow weight gain or weight loss | 57 (72.1%) | 32 (69.6%) | 11 (40.7%) | 43 (58.9%) | 0.057 |
| Short stature | 34 (43.6%) | 11 (23.9%) | 3 (11.1%) | 14 (19.2%) | 0.001 |
| Iron deficiency | 65 (83.3%) | 21 (45.7%) | 9 (33.3%) | 30 (41.1%) | <0.0001 |
| Hypocalcaemia, tetany | 10 (12.8%) | 1 (2.2%) | 0 | 1 (1.4%) | 0.007 |
| Hypoproteinaemia | 9 (11.5%) | 5 (13.0%) | 2 (7.4%) | 8 (11.0%) | 0.910 |
| Vitamin K deficiency | 5 (6.4%) | 1 (2.2%) | 1 (3.7%) | 2 (2.7%) | 0.284 |
| **Associated diseases** | | | | | (0.18) |
| Insulin dependent diabetes mellitus | 4 (5.1%) | 1 (2.2%) | 0 | 1 (1.4%) | |
| Other autoimmune diseases | 2 (2.6%) | 1 (2.2%) | 0 | 1 (1.4%) | |
| Gastro-oesophageal reflux disease (GORD) | 0 | 4 (8.7%) | 0 | 4 (5.5%) | |
| Recurrent abdominal pain | 2 (2.6%) | 6 (13.0%) | 1 (3.7%) | 7 (9.6%) | |

*Final diagnoses: cow’s milk and/or soy protein enteropathy (n = 24), jejunal bacterial overgrowth (n = 6), Crohn’s disease (n = 5), toddler’s diarrhoea (n = 5), Giardia lamblia infection (n = 4), Giardia lamblia infection (n = 2), cystic fibrosis (n = 2), lymphphacteosis, Helicobacter pylori infection, cytomegalovirus infection (n = 1 each), no gastrointestinal disease (n = 14), asymptomatic IgA deficient family members of known coeliac patients (n = 8).

†Controls I had a jejunal biopsy performed and all had normal villous architecture; controls II had no small intestinal biopsy performed.

‡The p values refer to the difference between coeliac disease patients and all control patients using the χ² test.
IgG anti-tTG autoantibody positivity was 9.8% among IgA deficient blood donors. There was a positive correlation ($r_s = 0.91, p < 0.0001$) between IgG anti-tTG antibody and IgG EMA levels (fig 2).

The youngest coeliac disease patient (11 months), who initially was negative for all autoantibodies, displayed elevated levels of IgG EMA and IgG anti-tTG antibodies during later gluten exposure. Two of the control patients had IgG anti-tTG antibody levels close to the cut off level and one of the coeliac disease patients (aged three years) had positive IgA anti-tTG antibody levels (12 U/ml) despite low total IgA levels (0.04 g/l). This patient and three additional coeliac patients negative for IgA anti-tTG antibodies and total serum IgA <0.05 g/l resumed normal serum IgA levels on a gluten free diet. None of the other subjects had elevated levels of IgA anti-tTG antibodies.

The 17 IgA deficient blood donors positive for IgG EMA had lower anti-tTG antibody levels (median 162 (40–990) U/ml) than the 78 coeliac disease patients with clinically manifest disease (median 602 (58–4330) U/ml). Also, IgG EMA titres of the blood donors (median 1:160 (1:20–1:2560)) were lower than for coeliac disease patients (median 1:640 (1:<2.5–1:10 240)).

**Figure 1** Serum levels of IgG antibodies against recombinant human tissue transglutaminase (tTG) in (A) coeliac disease patients, control subjects who had a small intestinal biopsy performed (controls I), and non-biopsied controls (controls II) and in (B) 174 IgA deficient blood donors. Positivity (titre 1:≥2.5) and negativity (titre 1:<2.5) for serum IgG antibodies against endomysium (EMA) are represented. Vertical and horizontal lines represent cut off levels. Cut off = 10 U/ml.

**Figure 2** Serum levels of IgG antibodies against recombinant human tissue transglutaminase (tTG) plotted against the reciprocal serum titre of IgG antibodies against endomysium (EMA). Spearman correlation coefficient was 0.91 (95% confidence interval 0.877–0.935; p < 0.0001). Vertical and horizontal lines represent cut off levels.

**Figure 3** Serum levels of IgG antibodies against tissue transglutaminase (tTG) in 36 coeliac disease patients before and during a gluten free diet. IgG antientdomysium (EMA) titres 1:≥2.5 (positive) and IgG anti-endomysium titres 1:<2.5 (negative) are represented. Cut off = 10 U/ml.

**Serum antibodies during a gluten free diet**

Initial IgG anti-tTG antibody levels of 36 coeliac patients and levels after various times on a gluten free diet are shown in fig 3. Median level of IgG EMA antibodies decreased from 1:640 (range 1:80–1:10 240) to 1:10 (range 1:<2.5–1:640) in the follow up sample (p<0.001). Initial IgG anti-tTG antibody values (median 928 (92–1346) U/ml) also showed a significant decrease in the follow up sample (median 40 (3–384) U/ml; p<0.0001).

Only 10 of 36 patients regained normal IgG EMA titres and eight of 36 normal IgG anti-tTG antibody levels after a long term gluten free diet. However, control jejunal biopsies performed after a gluten free diet in 17 coeliac patients with negative (n = 9) or low (1:640 (range 1:80–1:10 240) to 1:10 (range 1:<2.5–1:640) in the follow up sample (p<0.001). Initial IgG anti-tTG antibody values (median 928 (92–1346) U/ml) also showed a significant decrease in the follow up sample (median 40 (3–384) U/ml; p<0.0001).

The 17 coeliac patients with unchanged IgG EMA titres displayed a
fiveness in positive and negative results between IgG EMA and anti-tTG antibodies was observed for 32 of 36 patients after a gluten free diet.

Genetic typing

Eighteen of 19 coeliac patients and 10 of 12 IgG anti-tTG positive blood donors were positive for HLA-DQ2, and one in each group was positive for HLA-DQ8. The single DQ2 and DQ8 negative antibody positive blood donor had only the DQB1*02 allele (that is, only the DQB1 part, but not the DQA1 part of the DQ2 heterodimer).

Discussion

Circulating IgA class anti-tTG autoantibodies and EMA are highly prevalent and characteristic in active coeliac disease.15 20 Our results show that in as many as 98.7% of IgA deficient coeliac patients, disease specific IgG anti-tTG antibodies and EMA can be detected. Therefore, the humoral response targeting tTG as an autoantigen should be considered as a regular and diagnostically useful feature in all coeliac patients, both IgA competent and IgA deficient.

The human recombinant tTG based ELISA used in this study clearly out performs the guinea pig substrate based IgG class anti-tTG antibody test, earlier shown to be highly unspecific.13 Immunofluorescent investigation of serum IgG EMA has severe technical drawbacks because secondary antibodies against human IgG often bind to the tissue connective fibres in a similar non-specific manner.24 While the positive EMA reaction is easy to recognise, up to 50% of negative samples may show endomysial staining when monkey oesophagus substrate is used.26 For more specific results, human IgG1 specific or monkey immunoglobulin preadsorbed secondary antibodies,10 20 21 or substrates with low background (human fetal tissues, jejunum, or appendix) are needed.14 15 Given these technical requirements, only few laboratories offer IgG class coeliac antibody testing on a routine basis. In contrast, although the performance of IgG AGA tests is not optimal, they are widely accessible because they can be performed with simple ELISA methods. Introduction of an efficient ELISA for IgG anti-tTG antibody testing could clearly enhance the feasibility of detection of IgG coeliac autoantibodies for laboratories that have not mastered the IgG EMA method.

An advantage of the measurement of IgG anti-tTG antibodies with ELISA is the more precise quantification of serum antibody concentrations which can make monitoring a gluten free diet effective and more convenient. In the present study, a clear decrease was detected, even in patients where IgG-EMA was still considerably positive.

Due to impaired responses to mucosal immunostimuli, IgA deficient subjects frequently present with enteral complaints but coeliac disease combined with IgA deficiency accounts in general for only a few of cases in each gastroenterology centre.7 The prospective use of IgG EMA enabled us to identify a high number of IgA deficient coeliac patients, and also those with moderate and mild symptoms, and therefore we included all IgA deficient patients evaluated by biopsy during the same period to avoid significant bias. The ELISA used here identified coeliac patients effectively whereas none of the non-coeliac subjects had IgG anti-tTG antibody levels in the range of untreated coeliac patients (fig 1).

Furthermore, the decrease in IgG autoantibodies seems to be very slow in IgA deficient coeliac patients and most of our patients were still positive after more than two or three years on a gluten free diet. In contrast, IgA competent coeliac patients became negative for both IgA and IgG anti-tTG antibodies by one year on a gluten free diet.31 Dietary interviews in our centre did not reveal more lapses in coelicals with IgA deficiency than in those without (data not shown), and mucosal healing was observed at low levels of IgG autoantibody positivity. In fact, all of these patients had several fold reductions in IgG anti-tTG antibody concentrations on a gluten free diet, clearly showing that IgG coeliac autoantibodies are indeed gluten dependent. Specific antibody production is dependent on T helper lymphocyte function, and T cell priming, cytokine profiles, and B cell responsiveness show alterations in IgA deficiency.23 24 Therefore, the slow disappearance kinetics of IgG coeliac antibodies may be part of the immunoregulatory defect seen in IgA deficiency. However, there are a number of reports showing that individuals with HLA B8 DR3 haplotypes have alterations in their immune response, regardless of their IgA status, and hence it is not clear whether these features are directly due to IgA deficiency as such or associated HLA genes.25

We also found that 9.8% of apparently healthy IgA deficient adult blood donors were positive for both IgG EMA and anti-tTG antibodies and may have undetected coeliac disease. Biopsies were not performed in these patients but they were genetically similar to the clinically diagnosed IgA deficient coeliac patients. Given the high specificity of the EMA and anti-tTG antibody tests in our clinical cases, the results further support the fact that known IgA deficient subjects have at least a 10-fold increased risk of coeliac disease and should be an important target group for case finding.

However, IgA deficiency is often not known at the time of coeliac antibody testing and the firstline use of IgG EMA instead of serum IgA measurements was successful in population screening.14 The IgG anti-tTG antibody ELISA seems to be an easy tool to evaluate samples with negative IgA EMA or anti-tTG antibodies and unknown serum IgA levels. However, the cost effectiveness of this approach remains to be established.

Humoral IgA deficiency is usually defined as a total serum IgA of <0.05 g/l but levels may fluctuate as a response to mucosal antigens and cytokines.19 Four of our IgA deficient patients resumed normal serum IgA levels on a gluten free diet although IgA in IgA competent coeliac patients usually decreases on a gluten free diet.26 One prominent regulator of the IgA class switch is transforming growth factor b (TGFb), and mice lacking the TGFb receptor type II develop serum IgA deficiency.21 Although TGFb1 is increased in the coeliac mucosa,28 its action to promote epithelial cell differentiation on the crypt-villus axis seems to be impaired.29 The action of TGFb1 on the IgA switch also may be altered in genetically susceptible individuals who may also benefit from early recognition and treatment of coeliac disease in terms of IgA production and related pathology.

In conclusion, IgG anti-tTG antibodies measured with human tTG are highly reliable serum markers of coeliac disease in IgA deficient subjects. To ensure the detection of these patients, IgG anti-tTG measurements should be integrated into diagnostic and screening strategies and coeliac disease should be considered in all IgA deficient individuals.

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