Anti-transglutaminase antibodies and the serological diagnosis of coeliac disease

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ABSTRACT

Tissue transglutaminase (tTG) has recently been identified as the antigenic target recognised by anti-endomysial antibodies in patients with coeliac disease. In this study, an enzyme-linked immunosorbent assay (ELISA) is used to measure IgA, IgG and IgM antibodies to tTG in patients with coeliac disease and a variety of other inflammatory disorders; and is compared to the standard immunofluorescence test used to detect endomysial antibodies (EMA). In the samples tested, 3% control sera (n=146), 83% EMA-positive sera (n=29), 9% patients with Graves' disease (n=94), 12% antimitochondrial antibody-positive sera (n=53), 11% rheumatoid arthritis patients (n=53) and 22% systemic lupus erythematosus (SLE) patients (n=46) were positive for anti-tTG antibodies. In contrast, none of the controls, 1% of patients with Graves' disease, 2% antimitochondrial antibody-positive sera, 2% rheumatoid arthritis patients and none of the SLE patients were positive for EMA. Measurement of IgG or IgM antibodies to tTG was much less reliable than IgA anti-tTG antibody for the serological diagnosis of coeliac disease. The addition of calcium to the coating buffer improved the assay characteristics of the anti-tTG ELISA. However, the IgA anti-tTG ELISA, with and without calcium, performed less well than the standard EMA test used for the serological diagnosis of coeliac disease. In particular, the anti-tTG ELISA gave a higher rate of non-specific positive reactions.

KEY WORDS: Antibodies. Celiac disease. Endomysium. Transglutaminase.

Introduction

Coeliac disease is an inflammatory disease of the upper small intestine and is the result of gluten intolerance in genetically susceptible individuals.[1-3] Coeliac disease is thought to occur with an incidence of approximately one in 200 individuals. In its classic form, the histological lesion is characterised by raised numbers of T lymphocytes in the epithelium, crypt hyperplasia and villous atrophy. Treatment is a gluten-free diet for life.

The standard investigation for the diagnosis of coeliac disease is small intestinal biopsy; however, in recent years, much attention has focused on the value of serum antibody detection in the diagnosis and follow-up of the disorder. In particular, measurement of IgA anti-endomysial antibodies (EMA) is highly specific (≥ 99%) and sensitive (approximately 90%) in the diagnosis of coeliac disease.[4-6]

In 1997, Dieterich *et al*.[7] identified tissue transglutaminase (tTG) as the predominant, if not the sole, auto-antigen with which EMA react. This raised the possibility of detecting EMA in a specific tTG enzyme-linked immunosorbent assay (ELISA), instead of the standard method of EMA detection that employs indirect immunofluorescence using monkey oesophagus as substrate. Subsequent studies reported that the results of IgA anti-tTG ELISA correlated reasonably well with those obtained using the standard immunofluorescence technique.[8,9] In these two studies, a sensitivity of 95-98% and specificity of 94-95% for the diagnosis of coeliac disease was recorded.

Furthermore, it was noted that the addition of calcium ($CaCl_2$) to the coating buffer improved assay performance significantly. More recently, however, two separate studies gave conflicting results, with one finding very little difference in sensitivity and specificity between the assays[10] and the other indicating that the IgA anti-tTG is less specific than IgA EMA.[11]

Here, we investigate the performance of the IgA anti-tTG ELISA in a wide group of autoimmune diseases, examine the importance of incorporating calcium into the assay coating buffer and consider the role of IgG and IgM anti-tTG antibodies in the diagnosis of coeliac disease.

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Materials and methods

Study subjects

In IgA anti-tTG ELISA studies, serum samples from the following subjects were tested: 29 IgA EMA-positive patients, 94 patients with Graves' disease, 53 patients with rheumatoid arthritis, 46 patients with systemic lupus erythematosus (SLE), 53 patients with positive antimitochondrial antibody tests, and 30 healthy laboratory workers and 116 first-trimester healthy pregnant women as controls. In the IgG and IgM anti-tTG ELISA studies, sera from an additional 128 IgA EMA-positive patients were investigated. All sera were obtained from the Immunology

Table 1. IgA anti-endomysial antibody test results in patients and controls

Group	Number	EMA-positive	%
Controls	30	0	0
Pregnant women	116	0	0
IgA EMA-positive	29	29	100
Graves' disease	94	1	1
Mito-positive	53	1	2
Rheumatoid arthritis	53	1	2
SLE	46	0	0

Mito-positive: antimitochondrial antibody-positive patients

SLE: patients with systemic lupus erythematosus

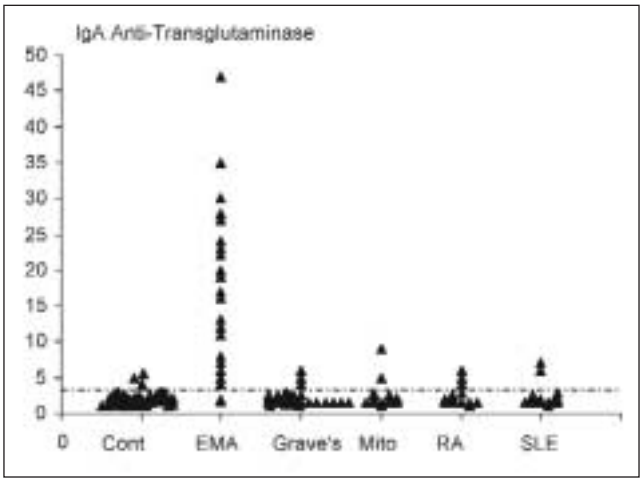

Fig. 1. Levels of IgA anti-transglutaminase antibodies, after the addition of CaCl2 to the coating buffer, in the normal population and disease study groups. The dotted line indicates the positive cut- off value (3.89 arbitrary units) for this assay.

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Endomysial antibody test

Serum IgA EMA were measured by an indirect immunofluorescence technique as previously described,[12] using human umbilical cord as substrate. In brief, 5 μm cryostat sections were attached to glass microscope slides coated with poly-L-lysine (Sigma). Serum diluted 1 in 10 in 0.5 mol/L phosphate-buffered saline (PBS; pH 7.2) containing 0.2% bovine serum albumin (BSA) was applied to the sections and incubated for 20 min at room temperature (RT). Fluorescein isothiocynate (FITC)-conjugated rabbit anti-human IgA (Dako, Denmark), diluted 1 in 50 with PBS, was then applied to each section and specific staining was examined under a fluorescence microscope.

All sera considered positive with the umbilical cord substrate were confirmed using commercial monkey oesophagus tissue sections (Medica, California). Sera and the FITC conjugate were diluted in PBS/BSA at dilutions of 1 in 5 and 1 in 20, respectively. An IgA EMA-positive serum sample was tested with each batch to confirm reproducibility of the techniques.

Tissue transglutaminase with calcium

Guinea pig tissue tTG (Sigma, USA; 5 µg/well) in bicarbonate buffer containing 5 mmol/L $CaCl_2$ (pH 9.6) was coated on to Nunc maxisorp microtitre plates.[6] After each subsequent step, the plate was washed (x4) with PBS/0.1% Tween (pH 7.3). Following overnight incubation at 4°C, the plate was blocked with PBS/1% HSA for 2 h at RT. Serum (100 µL) diluted 1 in 20 in PBS/Tween/HSA was added in duplicate and incubated for 2 h at RT. Bound IgA, IgG or IgM was detected by the addition of 100 mL peroxidase-conjugated rabbit anti-human IgA, IgG or IgM at a dilution of 1 in 1000 in PBS/Tween/HSA for 2 h at RT.

Colour was developed by adding 100 µL o-phenylenediamine in distilled water containing 0.012% hydrogen peroxide for 15 min at RT. The reaction was terminated by adding 100 µL 0.5 mol/L sulphuric acid. Absorbance was read at 492 nm on a Titertek Multiskan Plus MKII Type 313. Using the healthy control subjects, a reference range was established from the mean concentration in arbitrary units (AU), plus or minus two standard deviations (+2SD), and a positive cut-off value of 2.89 AU was determined. All values below this were considered negative for IgA anti-tTG antibodies.

Tissue transglutaminase without calcium

Similar experiments were performed using a coating buffer that did not contain calcium. As previously described, both reference range and positive cut-off value (3.02 AU) were calculated from the control population (mean + 2SD).

Statistical evaluation

Fisher's exact test was used to determine the significance of difference between various control and patient groups.

Results

IgA endomysial antibody test

Results of the EMA tests are shown in Table 1. All samples obtained from the healthy control population, including the pregnant women, were EMA-negative. In contrast, 2% of patients with rheumatoid arthritis, 2% of antimitochondrial antibody-positive patients and 1% of patients with Graves' disease were EMA-positive. All individuals with SLE were EMA-negative.

Comparison of IgA anti-tTG positivity before and after adding calcium to the coating buffer

Figures 1 illustrates the level, in arbitrary units (AU), of IgA anti-tTG antibodies in all groups in the IgA anti-tTG ELISA, with calcium added to the coating buffer. Table 2 shows the percentage of IgA anti-tTG antibody positivity before and after adding calcium to the coating buffer, and compares it with IgA EMA positivity. Based on the normal, healthy control population, a positive cut off of 3.02 AU (mean + 2SD) was assigned to the assay without calcium, and 2.89 AU (mean + 2SD) assigned to the assay with calcium.

As expected, a highly significant increase in the incidence of IgA anti-tTG antibodies was seen in the EMA-positive patients ($P \leq 0.001$) As can be seen in Table 2, there is a highly

significant increased incidence of IgA anti-tTG antibodies in the inflammatory groups as a whole (34/200), in comparison with EMA positivity (5/200; $P \leq 0.001$). This finding suggests that the anti-tTG assay is less specific than the EMA assay. In overall terms, the addition of calcium to the coating buffer significantly improved the sensitivity of the anti-tTG assay by increasing the number of EMA-positive individuals who were also positive for anti-tTG (93% versus 83%; $P=0.04$). Furthermore, the addition of calcium reduced the number of individuals positive for anti-tTG in the inflammatory disease groups, but this did not reach statistical significance ($P=0.17$).

Comparison of IgA anti-tTG and IgG and IgM anti-tTG antibody positivity in the different groups

Figures 2 and 3 show the AU levels of IgG and IgM anti-tTG antibodies, respectively, in the normal population and disease study groups. A positive cut off of 4.9 AU was assigned to the IgG anti-tTG assay and 12.5 AU to the IgM assay. Table 3 shows the number and percentage positivity of IgG and IgM anti-tTG antibodies compared with IgA tTG antibodies for the different patient groups studied. In the Graves' disease group, 13% and 34%, respectively, had raised IgG and IgM anti-tTG antibody levels ($P=0.03$ and $P<0.001$, respectively, compared with controls) but only 9% were IgA anti-tTG antibody-positive. In the rheumatoid arthritis group, 9% and 25%, respectively, had increased IgG and IgM anti-tTG antibodies ($P=0.1$ and $P<0.001$, respectively, compared with controls), while 11% were IgA anti-tTG antibody-positive. Interestingly, none of the three IgA EMA-positive patients in the inflammatory disease groups examined had raised IgG or IgM anti-tTG antibody levels.

Discussion

Serological tests have played a central role in screening for coeliac disease over the past decade.[4-6] Initial tests relied on the measurement of IgG and IgA anti-gliadin antibodies, but it was gradually appreciated that these assays had unacceptably low sensitivity and specificity profiles.[6,13] The advent of the EMA test improved our ability to screen for coeliac disease and, through the use of this assay, a high prevalence rate has been discovered in many European populations and in a Saharan ethnic group.[14-16] Moreover, EMA testing has demonstrated a high prevalence rate for coeliac disease in patients with other autoimmune disorders, most notably in insulin-dependent diabetes mellitus.[17-21]

Although the EMA test is highly specific (≥ 99%) and sensitive (approximately 90%) for the diagnosis of coeliac disease,[4-6] it has the disadvantages common to any immunofluorescence assay. In contrast, an ELISA-based assay system can be automated and subjective interpretation of results is replaced by quantitation. Following the discovery of tTG as the principal autoantigen in the EMA test, several groups have reported high specificity and sensitivity rates for an IgA anti-tTG ELISA system,[8,9,22-27] with reported sensitivity of 85-100% – values similar to the immunofluorescence EMA test. However, the ELISA assay

Table 2. The percentage of IgA anti-tTG antibody positivity before and after the addition of $CaCl_2$ to the coating buffer, compared with IgA EMA positivity

Group	Number	IgA tTG pos (%) without calcium	IgA tTG pos (%) with calcium	IgA EMA pos (%)
Pregnant women	116	3	2	0
IgA EMA-positive	29	83	93	100
Graves' disease	94	9	6	1
Mito-positive	53	12	4	2
RA	53	11	13	2
SLE	46	22	11	0

Mito-positive: antimitochondrial antibody-positive patients

RA: rheumatoid arthritis patients

SLE: patients with systemic lupus erythematosus.

Table 3. The percentage of patients with IgG and IgM antibodies to transglutaminase compared to IgA antibodies to transglutaminase

Group	IgG tTG pos (%)	IgM tTG pos (%)	IgA tTG pos (%)	%IgA EMA pos (%)
Controls	4	5	3	0
IgA EMA-positive	13	39	83	100
Graves' disease	13	34	9	1
RA	9	25	11	2

RA: rheumatoid arthritis patients

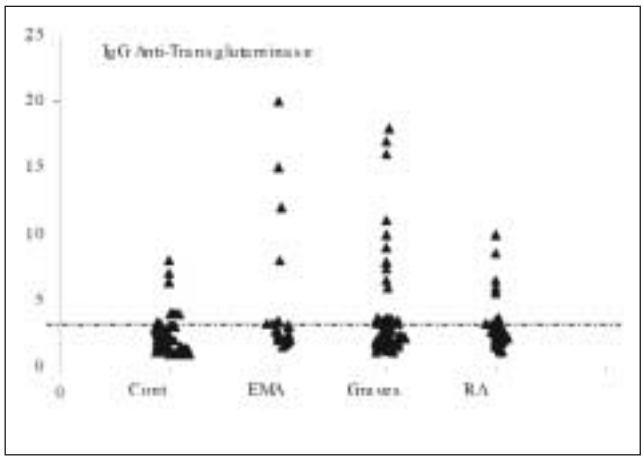

Fig. 2. Levels of IgG anti-transglutaminase antibodies in the normal population and disease study groups. The dotted line indicates the positive cut-off value (4.9 arbitrary units) for this assay.

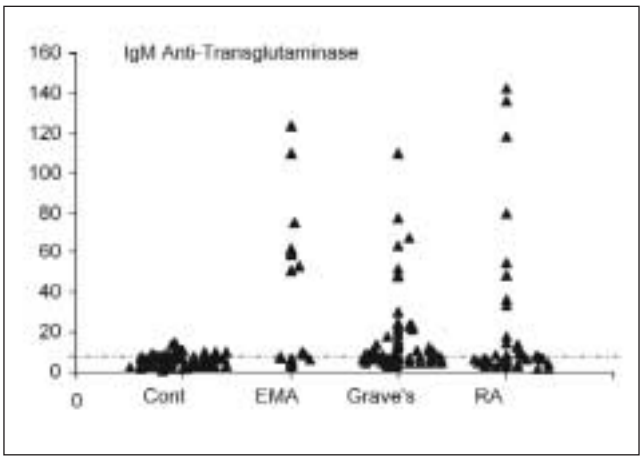

Fig. 3. Levels of IgM anti-transglutaminase antibodies in the normal population and disease study groups. The dotted line indicates the positive cut-off value (12.5 arbitrary units) for this assay.

was less specific than the EMA test, with values of approximately 95% reported.

The purpose of the present study was to assess further the value of measuring IgA anti-tTG antibodies in an ELISA system in patients with various autoimmune diseases. An initial finding was that assay sensitivity and specificity was improved by the addition of calcium to the coating buffer (Table 2), which supports the results of earlier studies.[8,9] In spite of this, however, high rates of anti-tTG positivity were found in all the autoimmune groups studied.

Although small intestinal biopsy findings were not available on most of these patients, the high overall positive anti-tTG antibody incidence (8%) and low EMA positivity rate (1%) makes it probable that the majority of these were false-positive results. This suggests that the anti-tTG ELISA assay did not display the same high specificity as the EMA test, at least in these patient populations. To some extent, experience with the anti-tTG ELISA mirrors ELISA detection of autoantibodies in other disorders. In many instances, these assays are highly sensitive but a significant false-positive rate is observed.[28,29]

The sensitivity of the IgA anti-tTG test also was evaluated. Of 29 EMA-positive individuals, 93% had raised anti-tTG antibodies. Thus, 7% of these patients were anti-tTG antibody-negative, and other groups have described similar results.[7,23,25-27] In addition, it is known that up to 10% of patients with coeliac disease may not be identified by the EMA test. These findings indicate that both the anti-tTG ELISA assay and the EMA test may significantly underestimate the presence of coeliac disease in population screening studies, which raises the vexed question of how to detect such individuals and whether or not the addition of IgG and IgA anti-gliadin assays have a role to play.[4,6,13-15]

The present study also established that IgG and IgM anti-tTG assays are of very little value in the diagnosis of coeliac disease, as they give a large number of false-positive results (Figures 2 and 3). In addition, a significant number of the EMA-positive patients were IgG and/or IgM anti-tTG antibody-negative (Table 3). These findings contradict some recent studies which used a radioimmunoassay to measure IgG and IgA anti-tTG antibodies and claimed high sensitivity[23,26] and specificity[23] for both antibody isotypes.

Guinea pig transglutaminase was used as the antigen in the anti-tTG assays in the present study, but several smaller studies have used recombinant tTG and some improvement in assay sensitivity and specificity claimed.[22,23] However, one recent study reported similar findings to our own and showed that the IgA anti-tTG ELISA is less specific for coeliac disease than the IgA EMA.[11] It seems unlikely that the ELISA assay will be able to match the almost 100% specificity reported for the IgA EMA. [8-9,23-27]

An interesting finding of the present study was the relatively high incidence (1%) of EMA- positive subjects in the inflammatory disease groups examined (Table 2). All three of these patients had raised anti-tTG antibodies. Two of the patients (one with Graves' disease and another with primary biliary cirrhosis) had been biopsied and both showed histological evidence of coeliac disease. Thus, it is very likely that the third patient (with rheumatoid arthritis) also had coeliac disease. These findings are in concordance with those of other studies that showed an increased prevalence of coeliac disease in specific autoimmune disorders.[18-21]

In conclusion, the present study demonstrated that the IgA anti-tTG ELISA compares less favourably with the EMA test as a serological aid to the diagnosis of coeliac disease. In addition, IgG or IgM anti-tTG assays appear to have a limited role in the serological diagnosis of coeliac disease. □

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