



Investigating the potential impact of post translational modification of auto-antigens by tissue transglutaminase on humoral islet autoimmunity in type 1 diabetes



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ABSTRACT

Background: Post-translational modification (PTM) of antigens plays a role in the pathogenesis of many autoimmune disorders. In coeliac disease (CD), tissue transglutaminase (tTG) deamidates gliadin peptides to activate the immune response against the gut endomysium. CD is six times more prevalent in type 1 diabetes (T1D) patients than in the general population.

Hypothesis: tTG also modifies auto-antigens implicated in the pathogenesis of T1D, leading to an autoimmune response to pancreatic β -cells.

Methods: tTG PTM was investigated in the following auto-antigens, which had been previously shown to have high importance in the development of T1D: glutamic acid decarboxylase isoform 65 (GAD65), full length islet antigen (IA-2), intracellular portion of IA-2 (IA-2ic), and both isoforms of zinc transporter 8 (ZnT8W and ZnT8R), on antibody binding. Radiolabelled antigen was incubated with tTG for 20 h at 37 °C in 100 mM Denver buffer, 3.33 nM CaCl₂, at pH 7.3. Antibody binding in 20 mixed samples from the Bart's-Oxford (BOX) cohort was measured by radiobinding assay.

Results: Results varied between serum samples. Generally, tTG treatment of ZnT8W, ZnT8R and IA-2ic showed no significant change in antigen: autoantibody binding, while increases in binding were observed with tTG-treated GAD65 and full length IA-2.

Conclusion: In the case of GAD65, full length IA-2, the strength of antibody: antigen binding increased after incubation with tTG. However, the exact tTG-modification events that occurred requires further elucidation.

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1. Introduction

1.1. Insulin-dependent diabetes mellitus (IDDM)

Diabetes mellitus describes a spectrum of disease, with a wide variety of etiologies, but each resulting in gradually diminishing pancreatic β -cell mass and function [10]. Insulin-dependent diabetes mellitus, otherwise known as type 1 diabetes (T1D) arises when the extent of destruction of pancreatic β -cells leads to insulin deficiency, resulting in the deficient storage of fats and carbohydrates throughout the body.

1.2. Autoantibodies in type 1 diabetes

Prior to the onset of clinical symptoms of disease, antigens (Ag) in the pancreatic β -cells are targeted by antibodies (Abs) produced by B cells [9]. The exact role of autoantibodies in the pathogenesis of T1D, however, remains elusive [17].

Over 35 years ago, the first Abs associated with T1D were discovered, and were called islet cell antibodies (ICAs) [4]. Decades of research have since identified many other T1D associated autoantibodies, including insulinoma-associated protein 2 (IA-2) antibodies (IA-2A) and glutamic acid decarboxylase 65 (GAD65) antibodies (GADA), which were later shown to contribute to results in initial ICA tests [3]. Other pancreatic β -cell targeted Ags include zinc transporter protein 8 (ZnT8). There are two isoforms of ZnT8 against which T1D autoantibodies can be generated: ZnT8R and ZnT8W, which have polymorphisms at amino acid (aa) position 325

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of arginine (R) and tryptophan (W) respectively.

The presence of one or more types of autoantibody can precede the full development of T1D by a few months to decades; one patient in the Bart's-Oxford (BOX) study developed T1D 25 years after the first appearance of T1D autoantibodies. Nevertheless, this prolonged length of time between autoantibody and clinical establishment of disease is uncommon [2]. Not everyone with autoantibodies in their blood serum will develop T1D - and so this prodrome is not a definitive indicator that T1D will develop. Even so, the risk of developing the autoimmune disorder increases with increasing numbers of autoantibodies [5].

1.3. Autoantibody assays

The radiobinding assay, also known as a radio-immunoprecipitation assay or radioimmunoassay, is an established and sensitive serological technique used to detect autoantibody levels in the patients blood [3]. Consequently, this technique can be used to investigate the patient's humoral response to pancreatic β -cell antigens, and enabling estimates of the patients' risk for developing T1D.

A radiobinding assay involves creating an *in vitro* transcribed and translated radiolabelled antigen associated with T1D, adding the antibody, washing away the excess antibody, and detecting the levels of radioactivity. To give the most accurate prediction of risk, radioimmunoassays are often used to test for three or four specific antibodies in patient serum. GADA, intracellular portion of IA-2A and Abs directed against insulin (IAA) and zinc transporter 8 (ZnT8A) are considered characteristic of T1D, and can be accurately detected using refined immunoassay techniques. Subsequently, these antibodies are often tested for, and used as T1D risk markers.

1.4. Post-translational modification of auto-antigens in type 1 diabetes

Post-translational modification (PTM) of proteins, through enzymatic or spontaneous PTM, is commonplace within the cell. It is estimated that 50–90% of proteins produced by human tissues are post-translationally modified [7].

However, PTM can become deregulated and aberrantly over-activated, leading to autoimmune disease [1]. Under cellular stress, as a result of infection or trauma, the cellular environment may change; for example, Ca^{2+} levels increase. This can lead to the aberrant activation of enzymes, modifying amino acid residues on target proteins, thus creating neo-epitopes that are not recognized by immune cells.

Could PTM of self-antigens play a role in the destruction of pancreatic β -cells? PTM of auto-antigens has been implicated in the pathogenesis of many autoimmune diseases, including coeliac disease and T1D [6].

1.5. Coeliac disease

Coeliac disease (CD), also known as gluten intolerance, is another form of chronic autoimmune disease mediated by tissue transglutaminase. CD shares HLA susceptibility genes with T1D – HLA-DR3 DQ2.5 and DR4 DQ8 - as well as many of the same environmental initiating factors [5]. Approximately 95% of celiac patients carry the HLA DQ2.5 haplotype, while the majority of the remaining 5% carry the HLA DQ8 loci. Coeliac disease is relatively common in T1D patients, with an average incidence of 7% in those with T1D; compared to the general public, who have an average incidence of 1% [4].

1.6. Transglutaminase in type 1 diabetes?

Studies investigating transglutaminase indicate that cell stress, viral infection or immunization may provide the correct environment for tTG activation. Therefore, it is possible that initiating factors may activate PTM of auto-antigens by tTG in inflamed tissues, creating new epitopes that are not tolerated, and activating the immune response in the correct HLA context. Could this occur in the initiating stages of T1D?

1.7. Objectives and aims

A full understanding of type 1 diabetes pathogenesis will only come when we understand how and why T1D antigens are targeted in the β -cells of the pancreas. This study aims to investigate the potential impact of transglutaminase PTM on auto-antibody binding, and therefore investigate whether the tissue transglutaminase affects the humeral response in T1DM.

2. Methods and materials

2.1. Subjects

The effects of tTG PTM antigen: autoantibody binding were studied in 20 samples taken from the BOX study cohort. The BOX study was carried out in Oxfordshire, Buckinghamshire and Berkshire, and is one of the longest running and most successful type 1 diabetes studies.

Our laboratory had access to 868 samples from this cohort. Samples were positively selected if serum was obtained from a diagnosed T1D patient within 3 months of their initial diagnosis, and if sufficient serum was available for our investigations. Of the samples that did fit our selection criteria, 20 samples were randomly selected to give a mixed population of samples.

The samples included 11 males and 9 females, with a median age of 9.5 years (range 1–19 years). Samples were all from white Caucasians. HLA haplotypes of patients were not known.

Sera from one healthy volunteer and three positive sera with high, medium and borderline levels of antibody were used as quality controls for each assay. The radiobinding values for these controls were pre-determined.

2.2. Methods

GAD65, full length IA-2, IA-2ic, ZnT8W and ZnT8R binding with antibody were measured in all patients, based on their theoretical ability to be altered by tTG.

2.2.1. IA-2A, GADA and ZNT8A radio-binding assay

The effects of tTG incubated antigen on autoantibody binding were studied, and compared with wild-type (WT) antigen using radio-binding assay, as previously described [12].

2.2.2. Transglutaminase incubation

Commercially available guinea pig liver tTG shows high sequence homology (>80%) with human tTG; thus, this enzyme was used for tTG incubation of serum samples [15].

For tTG-treated samples, *in vitro* translated [^{35}S]-methionine labeled antigen was incubated with 1 μM guinea pig TG2 enzyme (Sigma Aldrich Company Ltd., Gillingham, Dorset, U.K.), for 18 min at 37 °C in 100 mM Denver buffer, 3.33 nM CaCl_2 , and pH 7.3 according to a protocol previously described [11]. tTG-treated samples were desalted to remove calcium so that antigen: autoantibody binding was not affected by excess salt. Illustra NAP-5 Columns (GE Healthcare, Little Chalfont, Bucks, U.K.) were used to remove

calcium, and levels of auto-antigen: antibody binding measured using the radio-binding assay [12]. Radiobinding assays for WT and tTG treated antigen were carried out alongside one another to minimize sub-conscious differences in assay protocol.

2.2.3. Predicted tTG deamidation sites within T1D auto-antigens

tTG deamidates proteins at Q-X-P sites, where X is any amino acid. IA-2 contains 5 Q-X-P sites, IA2-ic contains 2 Q-X-P sites, ZnT8R contains 1 Q-X-P site, ZnT8W contains 1 Q-X-P site and GAD-65 contains 2 Q-X-P sites according to GenBank [14]. As pre(pro) insulin contains no Q-X-P sites, we did not include this antigen in our investigations [8].

2.3. Analysis

The thresholds for positivity of ZnT8RA, ZnT8WA, IA-2A, GADA, and competitive islet antigen antibody assay (cIAA), are 1.8, 1.8, 0.94, 1.6, and 0.2 arbitrary units respectively. Only samples with positive sera for each auto-antigen were included in the analysis.

Values from duplicate samples were compared to give the mean, and to establish the percentage difference in cpm values. Individual samples were scrutinized for duplicate cpm differences. Percentage differences greater than 30% were also discounted from the results.

The percentage reduction in binding in accepted samples was calculated as: $100 \times (\text{domain cpm of WT} - \text{cpm of mutant}) / \text{cpm of WT}$. A negative percentage reduction signifies increased binding in mutant, in the case of mutated Q357E ZnT8, or with tTG-treated Ag, compared to tTG-untreated WT Ag [13]. Percentage reductions greater than 20%, positive or negative, were considered significant, as well as samples with a cpm reading difference greater than 1000.

Intra-assay coefficient of variation (c.v.), (IAc.v.), values were calculated to indicate the reproducibility of the assay. Assays with IAc.v.'s greater than 10% were discounted. The IAc.v. was calculated

as follows: $(\text{sum of duplicate c. v. \% for each sample}) / (\text{no. of samples})$.

For the radiobinding methods we adopted, the historical c. v. values for moderate/high and low positive quality control (QC) samples, respectively, were 20 and 16% for ZnT8RA, 23 and 27% for ZnT8WA, 16 and 17% for GADA, and 14 and 21% for IA-2A [16].

The non-parametric test, the Wilcoxon signed-rank test two-tailed hypothesis, was used to test for significant differences between tTG treated and non-treated sample cpm values for each assay. P values less than 0.05 were considered significant, while assays with a p value (P) greater than or equal to 0.05 were considered insignificant.

3. Results

3.1. The effect of transglutaminase incubation on binding of autoantibodies to treated antigen

Treatment of antigens with tTG altered autoantibody binding differently in all patient serum samples. Collective findings from these assays have been presented as a box and whisker plot (Fig. 6).

3.1.1. ZnT8W and ZnT8R

11 patient sera were positive for ZnT8W auto-antibodies. Treatment of ZnT8W with tTG, compared with untreated Ag, overall caused a significant median percentage reduction (Med.PR) in binding of 2% (range -29 to 37%; $p < 0.05$) in the positive sample population (see Fig. 1) (see Fig. 2).

Similarly, ZnT8R was positive in 11 patients. Patients positive for ZnT8R antibody showed a Med. PR of 20% (range -15 to 35%; $P < 0.05$) in tTG-treated versus wild type antigen, indicating a decrease in binding of ZnT8R antibody to tTG-incubated ZnT8R.

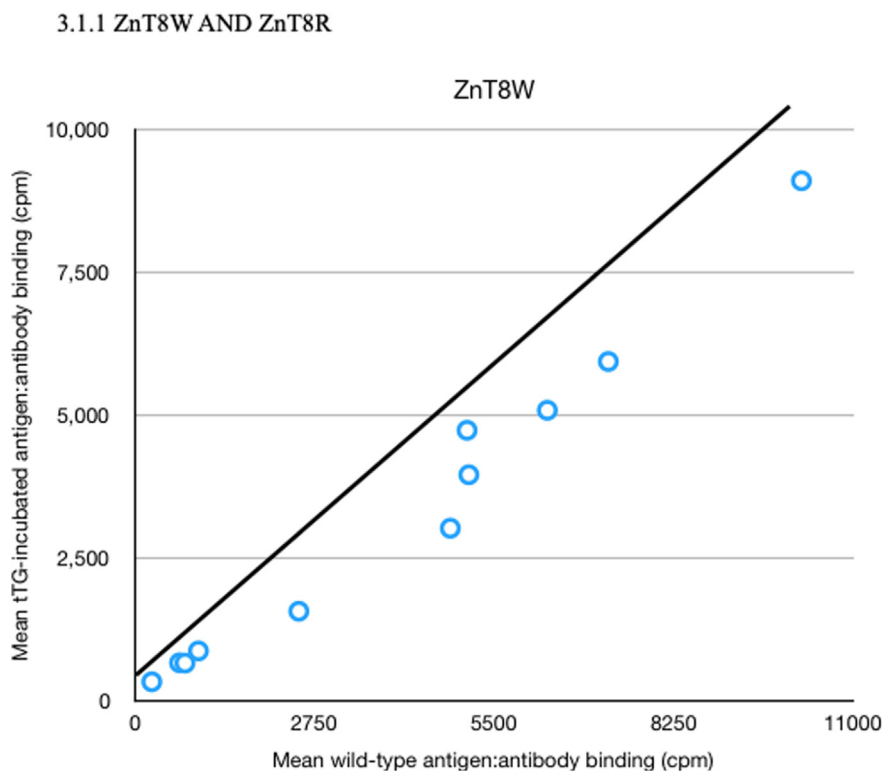


Fig. 1. Binding (cpm) of BOX sera to ZnT8W treated with tTG versus wild type within positive sample population sera. The solid line represents equivalent binding with or without tTG treatment.

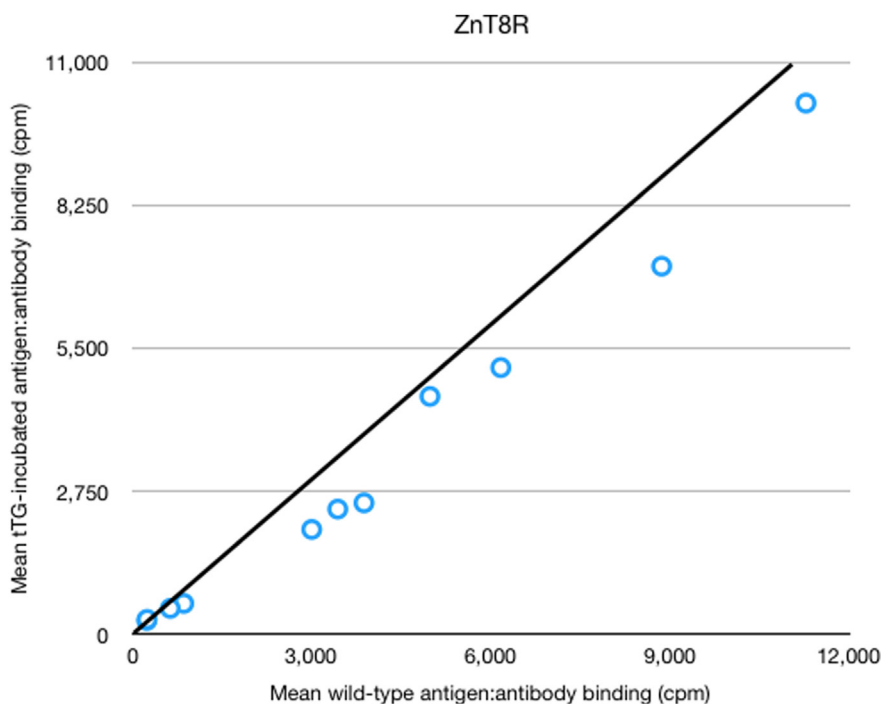


Fig. 2. Binding (cpm) of BOX sera to ZnT8R treated with tTG versus wild type in positive sample population sera. The solid line represents equivalent binding with or without tTG treatment.

3.1.2. GAD65

18 patients were positive for GAD65 auto-antibodies. Treatment of GAD65 with tTG caused an overall increase in GAD65-binding, with a Med. PR of -9% (range -25-7%; $P < 0.05$) (See Fig. 3).

3.1.3. IA-2ic

16 patients were sera-positive for IA2-ic. Binding of tTG treated-IA-2ic (see Fig. 4) had minimal effects on patient IA-2 antibody binding, showing a median binding reduction of 2% (range -33-24%). The p-value for this assay reflects the similarity in binding between tTG-treated and WT IA-2ic at $P > 0.05$, and so is not

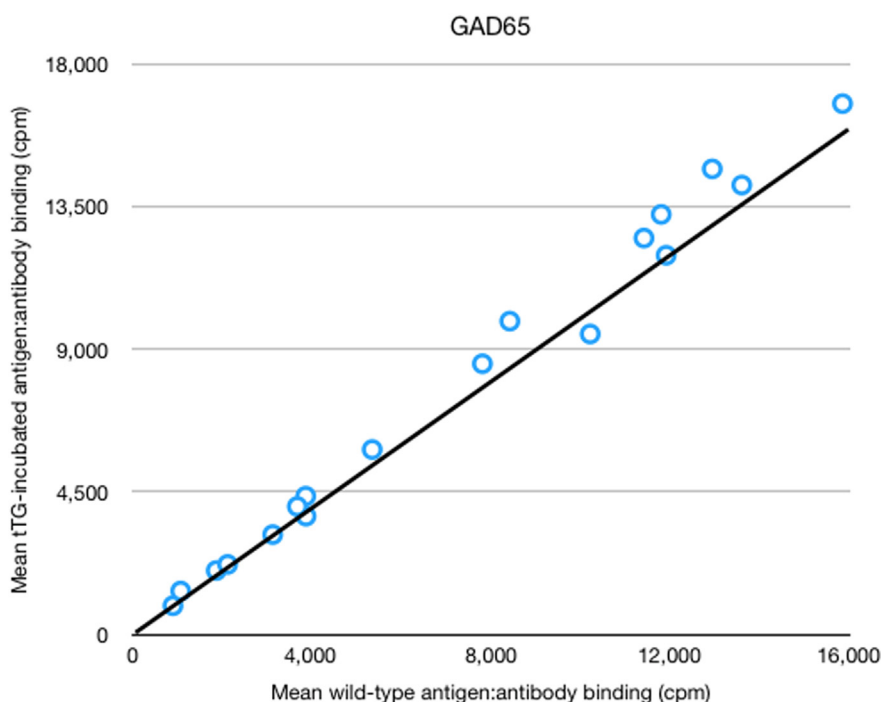


Fig. 3. Binding (cpm) of BOX sera to GAD65 treated with tTG versus wild type in positive sample population sera. The solid line represents equivalent binding with or without tTG treatment.

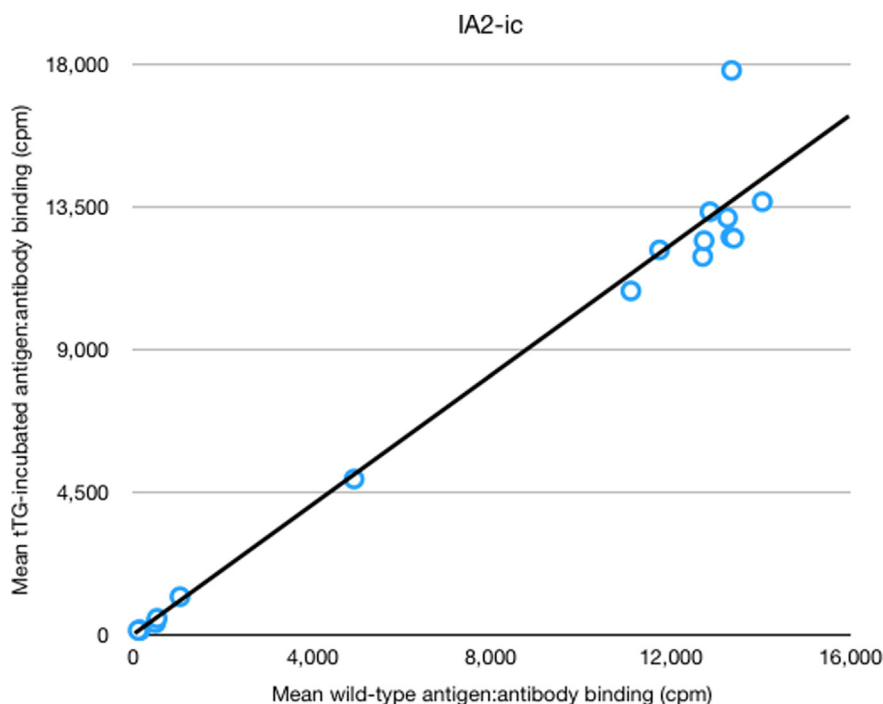


Fig. 4. Binding (cpm) of BOX sera to IA2-ic treated with tTG versus wild type in positive sample population sera. The solid line represents equivalent binding with or without tTG treatment.

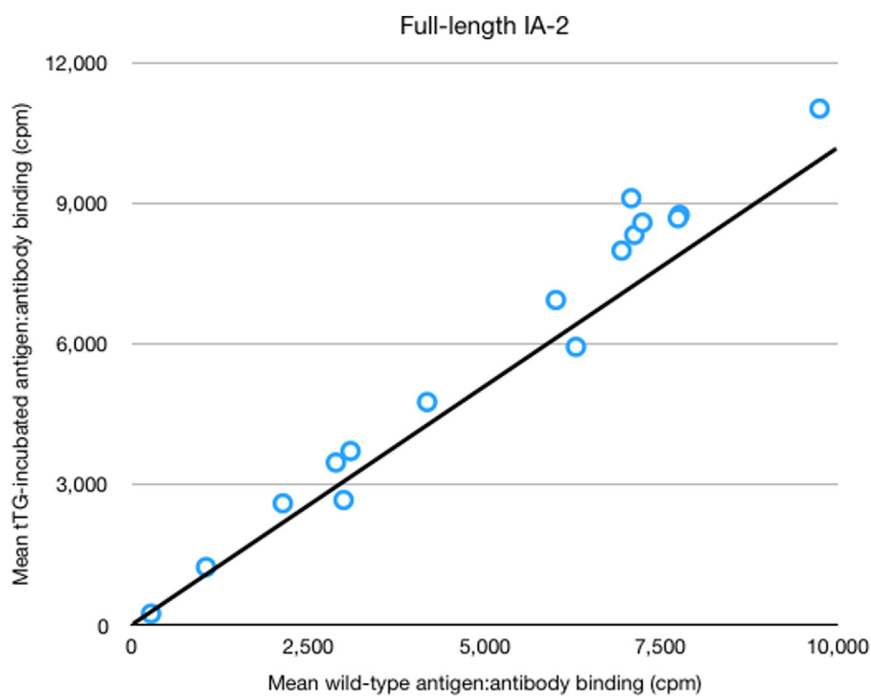


Fig. 5. Binding (cpm) of BOX sera to full length IA2 treated with tTG versus wild type in positive sample population sera. The solid line represents equivalent binding with or without tTG treatment.

statistically significant.

3.1.4. Full length IA-2

Similarly, full length IA-2 (Fig. 5) treated with tTG exhibited increased binding with IA-2 antibody, showing a Med. PR in binding of -15.5% (range -29-6%; $p < 0.05$) in the 16 sera-positive

samples.

4. Discussion

This study has demonstrated that tTG PTM of antigens has varying effects on antibody: antigen binding in many T1D auto-

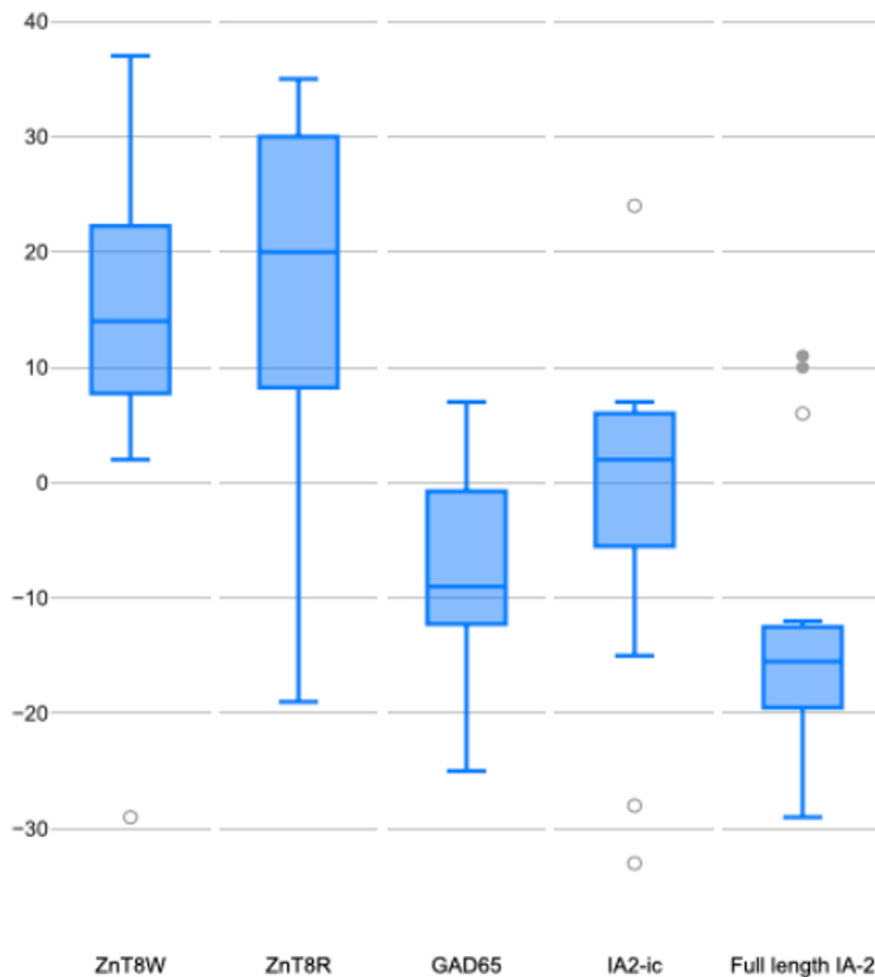


Fig. 6. Percentage binding of auto-antibody positive BOX sera to tTG-incubated antigen versus wild type.

antigens. Tissue TG treatment of ZnT8W, ZnT8R and IA-2ic showed no significant difference in binding with their corresponding antibodies. Increases in binding of autoantibodies were observed with tTG-treated full length IA-2 and GAD65, indicating that tTG modification enhanced the strength of antibody: antigen binding.

Previous studies can glean light on positive results from this study [13]. Regarding GAD65, other studies suggest that the majority of antibody binding occurs in an epitope cluster region within the middle of the protein (aa 245-450), or at two epitope cluster regions at the C-terminus of the protein (aa 450-585). Tissue TG deamidation sites are found at residues 62 and 77 in GAD65. Therefore, tTG treatment does not theoretically affect linear binding of the GADA paratope to its corresponding epitope, but may attract antibodies into close proximity of the antigen by electrostatic attractions or direct binding of GAD65 to regions of the autoantibody other than the paratope.

Published literature concerning IA-2 suggests that the cytoplasmic domain, IA-2ic (aa 606-979), is most crucial for antibody binding, with many humoral and T cell epitope regions clustered at residues 794-889 [13]. Consequently, tTG deamidation at Q785 and 949 has the capacity to directly affect antibody: antigen binding, causing the reduction in antibody:antigen binding with tTG-treated IA-2ic. However, the increase in binding with tTG-treated full length IA-2 may suggest that antibodies are also attracted by electrostatic attractions.

Strengths of our study include the well-characterized population of serum samples recently diagnosed (<3 months) with T1D. Moreover, a standardized protocol, the radiobinding assay, was adopted to measure antigen: autoantibody binding.

A weakness of this study was the small sample population. Ideally, the sample population would have been at least thrice as large to pick up rare antibody: epitope specificities. Additionally, we could not sequence proteins post-tTG incubation to assess whether or how proteins had been post-translationally modified. Moreover, while the radiobinding assay has undergone much standardization and is still considered one of the most accurate methods of detecting antigen: autoantibody binding, it is a far from perfect protocol; readings can be inaccurate, especially if pipetting is not accurate.

Future studies should focus on tTG-modification with GAD65, and full length and intracellular IA-2; with antigens sequenced post-tTG incubation to confirm which tTG modification event has occurred. Studies should also look at the effect of tTG-modification of T1D auto antigens on cell-mediated immunity.

Disclosures

The authors declare no financial interests or private funding for this research.

CRediT authorship contribution statement

Catherine Donnelly: Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Alistair Williams:** Conceptualization, Methodology, Resources, Supervision, Project administration, Funding acquisition.

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