CLINICAL STUDY

Restricted thyroglobulin antibody epitope specificities in subjects with type 1 diabetes mellitus

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Abstract

Objectives: Following iodisation in Sri Lanka we observed a high prevalence of thyroglobulin antibodies (TgAbs) in type 1 diabetic (T1DM) patients. The clinical significance of these TgAbs is uncertain. We sought to obtain a detailed epitope analysis of TgAbs in T1DM patients recruited from diabetes clinics and to compare these with TgAb epitope specificities in patients with autoimmune thyroid disease (AITD) and healthy individuals in that country.

Design and methods: We used a panel of 10 Tg-MABs in competitive ELISA reactions in a prospective study of subjects recruited from Colombo, to determine the epitopes recognised by TgAb-positive patients with T1DM ($n=58$, 34F:24M, median age 16 years), AITD patients ($n=42$, 33F:9M, median age 37 years) and healthy subjects ($n=50$, 39F:11M, median age 27 years). The outcomes were a comparison of reactivity with six Tg clusters (I–VI) in these subjects, and the relation of epitope specificity patterns with free thyroxine and TSH.

Results: Patients with T1DM and AITD but not healthy control subjects preferentially recognised the immunodominant clusters, I, III and IV. Patients with these narrow epitope specificities had higher median TSH levels (1.60 vs 1.06; $P=0.01$), and were more frequently positive for antibodies to thyroid peroxidase than those with broad specificities (52.3 vs 7.1%; $P=0.004$).

Conclusions: The TgAb epitope specificities in euthyroid Sri Lankans with T1DM are similar to AITD patients. TgAb epitope studies may potentially identify T1DM patients at risk of thyroid dysfunction.

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Introduction

Type 1 diabetes (T1DM) and autoimmune thyroid disease (AITD) are classic examples of organ-specific autoimmunity. Both conditions frequently coexist and up to one-third of patients with T1DM develop thyroid dysfunction during long-term follow-up (1). AITD is characterised by the presence of circulating antibodies to thyroid peroxidase (TPOAb) and thyroglobulin (TgAb) (2). These antibodies occur more frequently in patients with T1DM than in healthy individuals (3). However, thyroid antibodies are also present in about 10% of healthy euthyroid individuals (4) and have been reported in 30% of healthy persons in newly iodised populations (5, 6). In the wake of iodine prophylaxis in Sri Lanka, we observed a high prevalence of TgAbs in various population subgroups (6). These TgAbs were not associated with thyroid dysfunction and probably represented a non-pathological antibody response to iodination (6). Recently, we also reported a high frequency of TgAbs in Sri Lankan patients with T1DM (7). The clinical significance of TgAbs in these diabetic

patients is thus unclear and a point of interest is whether they indicate thyroiditis or whether they simply represent a non-pathological effect of iodination.

Studies on TgAb epitope reactivity have provided a potential means of distinguishing the TgAbs seen in health from those seen in disease (8, 9). Tg, a large molecular weight (660-kDa) glycoprotein, is the major colloid protein and serves as a prohormone and storage protein for the thyroid hormones, thyroxine (T_4) and triiodothyronine (10). Detailed panels of murine Tg-MABs have delineated several B-cell epitopes on Tg that are differentially recognised by sera from healthy individuals and AITD patients (11). These immunodominant Tg epitopes are located in the less conserved non-hormonogenic portions of the Tg molecule and are preferentially recognised by sera from the majority of AITD patients. On the other hand, healthy euthyroid individuals exhibit a broad or less-restricted Tg epitope reactivity pattern (8, 9, 12).

The TgAb epitope specificities in patients with T1DM are unknown, and to the best of our knowledge have not been previously tested in any population group.

Knowledge of these specificities will shed more light on the significance of thyroid antibodies in T1DM and could potentially identify the subsets of diabetic patients at risk of thyroid dysfunction. Our objective in the present study was thus to obtain a detailed epitope analysis of TgAbs in Sri Lankan T1DM patients using a well-characterised panel of Tg-MAbs. Furthermore, we compared these TgAb patterns with the typical patterns seen in health and in thyroiditis by evaluating TgAb specificities in healthy euthyroid individuals and in patients with established AITD.

Methods

Subjects

We tested serum for TgAbs in 217 patients with T1DM attending clinics in Colombo, Sri Lanka, and selected all 58 TgAb-positive patients for Tg epitope analysis (34F:24M, median age 16, age range 5–35 years, median duration of diabetes 9.38 months, range 0.4–57.08 months). Details of the prevalence of other disease-associated antibodies in the group have been described elsewhere (7). T1DM was diagnosed according to WHO criteria (13). The AITD control group comprised 42 TgAb-positive Sri Lankan patients with Hashimoto's thyroiditis (HT) attending a thyroid clinic in Colombo (33F:9M; median age 37 years, age range 16–41 years). HT was diagnosed based on the presence of goitre, TgAbs and/or TPOAbs, and in some cases, thyroid-histological and/or ultrasound features of autoimmune thyroiditis. The healthy control group consisted of 50 TgAb-positive healthy blood donors (belonging to the ethnic Sinhala group) without thyroid disease or diabetes (39F:11M; median age 27 years, age range 18–39 years). Ethical permission for the project was obtained from the local Ethics Committee and informed consent was obtained from the subjects or their parents as required.

Thyroid function tests

We measured free T₄ (FT₄; normal 9.8–23 pmol/l) with a competitive labelled antibody assay and TSH (normal 0.35–5.2 mU/l) by a two-site immunochemiluminometric assay (Bayer Plc Diagnostics Division). These were analysed on an automated immunoassay analyser, the ACS-180 Plus (Chiron Diagnostics Ltd, Halstead, Essex, UK). The interassay coefficient of variation for FT₄ was 4% (at a mean of 13.6 pmol/l) and the variation for TSH was 7.56% (at a mean of 4.89 mU/l).

Autoantibody estimations

We measured TgAb (normal <98 kIU/l) and TPOAb (normal <19.4 kIU/l) by an ELISA technique standardised against National Institute for Biological Standards

and Control reference standards (14). Intra-assay variations were 4.1% for TPOAb (at a mean of 150 kIU/l) and 4.4% for TgAb (at a mean of 1420 kIU/l). The interassay variations were 7.2% for TPOAb (at a mean of 138 kIU/l) and 6.7% for TgAb (at a mean of 1350 kIU/l).

Competitive ELISA studies

Tg-MAbs were produced and characterised at the INSERM U555, Faculté de Médecine, Marseille, France (11). These Tg-MAbs (MAb1–3, 5–11) recognise six antigenic clusters on Tg (I–VI) of which clusters I, III and IV define the immunodominant region typically recognised by AITD sera (15). TgAb epitope recognition was determined in competitive ELISA reactions between alkaline-phosphatase-labelled Tg-MAbs and TgAb in test serum as previously described (15). Briefly, 96-well microtitre plates were coated overnight with 100 µl of a 10 µg/ml solution of Tg in carbonate-bicarbonate buffer. The plates were then washed with PBS–Tween after which 100 µl test serum at various dilutions (1/10, 1/100, 1/1000) was added to the wells and incubated for 2 h in a humid box at 37 °C. Plates were washed again prior to incubation with 100 µl labelled Tg-MAb for 2 h in a humid box at 37 °C. After further washing, 4-nitrophenyl phosphate was added to the wells as substrate. Percentage inhibition of MAb binding to Tg by serum samples was calculated based on the optical densities (OD) at 405 nm according to the formula:

$$\frac{\text{OD in the absence of serum} - \text{OD in the presence of serum}}{\text{OD in the absence of serum}} \times 100.$$

Inhibition curves for various MABs were obtained using dilutions of pooled TgAb-positive AITD sera. More than 70% inhibition was taken as complete inhibition and 35–70% was interpreted as partial inhibition. OD obtained at 1:100 dilutions was used for analysis.

Statistical analysis

Data is presented as means (s.d.) except where otherwise indicated. All statistical analysis was performed using SPSS for Windows, version 16.0 (SPSS Inc., Chicago, IL, USA). Continuous data in various groups were compared using the Student's *t*-test or the Mann–Whitney *U* test as appropriate. Recognition of various Tg clusters was compared between subject groups using the χ^2 test with the Bonferroni correction applied for multiple-group comparisons. The level of statistical significance at which the null hypothesis was rejected was chosen as 0.05.

Figure 1 Reactivity with Tg epitope clusters in T1DM, AITD and healthy control subjects. There was a significantly higher preference for immunodominant clusters I, III, IV among subjects with T1DM and AITD compared to healthy controls. Antigenic clusters are labelled I–VI. Cluster I is defined by MAb 6 (J7C73.7), MAb 8 (J8A53.9), MAb 10 (J8A32.13), and MAb 11 (J7B49.15); cluster II is defined by MAb 2 (J8B6.12) and MAb 9 (J8B45.5); cluster III is defined by MAb 3 (J8B89.5) and MAb 5 (J7C44.6); cluster IV is defined by MAb 6 (J7C73.7), MAb 8 (J8A53.9), MAb 10 (J8A32.13), MAb 11 (J7B49.15) and MAb 5 (J7C44.6); cluster V is defined by MAb 1 (J7C9.3); cluster VI is defined by MAb 7 (J7C76.20). Original Tg-MAb nomenclature as characterised by Ruf *et al.* (11) is in parenthesis. *Healthy controls versus other subject groups.

Results

Epitope reactivity patterns

Figure 1 shows the percentage of subjects in each group recognising individual Tg clusters (I–VI). A subject was said to recognise a Tg cluster if there was partial (35–70%) or complete (>70%) inhibition of one or more Tg-MABs within the cluster. There is preferential recognition of the immunodominant clusters I, III and IV by the majority of patients with AITD. On the other hand, only a small percentage of healthy control subjects reacted with these clusters. Among patients with T1DM, the proportion of patients reacting with immunodominant Tg clusters was not different from AITD patients but was significantly different from that of healthy individuals. The median TgAb activity in

patients with T1DM was significantly lower than in the AITD group (720 vs 1400 IU/l; $P < 0.05$) but was not different from the healthy control groups (720 vs 620 IU/l; $P > 0.05$).

Clinical characteristics of patients according to epitope reactivity patterns

Table 1 shows the clinical characteristics of patients according to the epitope reactivity patterns. Patients who showed preferential recognition of one or more Tg-MABs within the immunodominant clusters were defined as restricted reactivity ($n = 44$), while patients who did not specifically recognise immunodominant clusters were defined as broad reactivity ($n = 14$). There were no differences in age, gender or duration of diabetes in the two groups of patients. Patients with restricted epitope specificities had a higher median TSH and a greater proportion of TPOAb positivity than patients with broad specificities. There was a higher proportion of patients with TSH > 3.0 mU/l in patients with restricted epitope specificities, although this difference was not statistically significant.

Of the 42 TgAb-positive patients with HT, 38 (90.5%) showed a restricted epitope recognition pattern, while 4 (9.5%) showed broad epitope reactivity. All HT patients were TPOAb positive, and median TPOAb concentrations were not significantly different in patients with restricted and broad Tg reactivity (680 vs 770 kIU/l; $P > 0.05$).

Discussion

In this study, we have demonstrated that the majority of TgAb-positive Sri Lankan patients with T1DM preferentially recognise immunodominant Tg clusters in the same manner as AITD patients. The link between T1DM and thyroid autoimmunity is well established. Both conditions may coexist with or without other autoimmune disorders, and may share common susceptibility genes (16). The similarity in epitope specificities between AITD and T1DM demonstrated in

Table 1 Clinical characteristics of thyroglobulin antibody (TgAb)-positive subjects with type 1 diabetes according to epitope reactivity.

	Restricted epitope reactivity	Broad epitope reactivity	P value
Number of patients	44	14	–
Age at study (years; median (range))	16 (9–32)	16 (5–35)	NS
Gender (M/F)	19/25	5/9	NS
Diabetes duration (months; median (range))	9.17 (0.4–57.1)	11.25 (0.4–47.3)	NS
FT ₄ (pmol/l)	17.6 ± 2.4	17.7 ± 2.7	NS
TSH (mU/l; median (range))	1.60 (0.12–3.67)	1.06 (0.65–2.04)	0.01
TSH > 3.0 mU/l (n (%))	11 (25%)	1 (7.1%)	NS
TSH > 5.2 mU/l (n (%))	0 (0%)	0 (0%)	NS
TPOAb positive (n (%))	23 (52.3%)	1 (7.1%)	0.004
TgAb level (kIU/l; median (range))	810 (30–4770)	670 (40–3690)	NS

A higher median TSH and prevalence of TPOAb was seen in subjects with 'restricted reactivity' (preferential recognition of one or more Tg-MABs within the immunodominant clusters) compared to subjects with 'broad reactivity' (did not preferentially recognise immunodominant clusters). There was no difference in age, gender or duration of diabetes in the two groups of patients.

this study may suggest identical immune response pathways to exposed immunodominant epitopes. However, it is likely that expression of epitope-specific TgAbs signifies co-existent autoimmune thyroiditis in these patients with T1DM.

T1DM patients with narrow epitope specificities had higher TSH levels and were more frequently positive for TPOAbs than those with broad specificities. Longitudinal studies have shown that positive TPOAb status at the onset of diabetes is a strong determinant of future thyroid dysfunction (1). Also, a TSH level in the upper range of normal has been reported to predict thyroid dysfunction in diabetic patients (17). Thus, it is likely that our panel of Tg-MAbs identified those TgAb-positive diabetic patients at risk of thyroid dysfunction. From the clinical perspective, patients with epitope-restricted TgAbs will benefit from close monitoring and prompt correction of thyroid dysfunction. It is recognised that undiagnosed thyroid dysfunction in diabetic patients could impair glycaemic control and increase cardiovascular risk through adverse effects on body weight and lipid metabolism (18).

Our findings here add to our previous observations in this population. Following iodisation in Sri Lanka, we detected an unusually high prevalence of TgAbs in healthy individuals (6). Most of these TgAbs exhibited broad epitope specificities and disappeared over time, suggesting a non-pathological antibody response to iodisation. On the other hand, TgAb-positive individuals with narrow epitope specificities had persistent autoimmune markers, confirming epitope restriction as a true reflection of thyroid autoimmunity (19). The predominance of epitope-restricted TgAbs in T1DM in this study further supports this distinction between disease-associated and non-specific antibodies.

We acknowledge that our findings in this Sri Lankan population may not necessarily apply to patients with T1DM elsewhere. Studies in stable iodine-replete populations will be instructive in this regard. Also, the relationship between epitope specificities and thyroid function will need to be clarified in longitudinal studies since our study sample included mostly young, newly diagnosed patients who were euthyroid at the time of study. Finally, the overall clinical utility of TgAb epitope reactivity studies in diagnosing AITD remains limited by its lack of applicability to TgAb-negative patients, some of whom may have AITD. Thus, TPOAb will no doubt remain the important prognostic indicator of AITD. But as we show in this study, Tg epitope specificities may serve a particular role in distinguishing disease-associated antibodies from non-specific antibodies in populations with a high background prevalence of TgAbs.

We conclude that the TgAb epitope specificities in Sri Lankans with T1DM are similar to the pattern seen in autoimmune thyroiditis. These TgAbs are likely to represent disease-associated antibodies and may potentially identify diabetic patients at risk of thyroid dysfunction.

Declaration of interest

All authors confirm that there are no conflicts of interest and that there are no financial disclosures to make.

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