CLINICAL STUDY

B-cell autoepitopes on the acetylcholinesterase-homologous region of human thyroglobulin: association with Graves' disease and thyroid eye disease

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Abstract

Objective: Thyroglobulin (Tg) is a large autoantigen involved in autoimmune thyroid diseases. Tg epitopes have, so far, been identified within large peptides. In the present study, we used small synthetic peptides to finely map serological epitopes on the highly immunogenic C-terminal region of Tg. Homology of this region to acetylcholinesterase (AChE) has been implicated in the pathogenesis of thyroid eye disease (TED) through cross-reactive antibodies.

Methods: We tested total IgG purified from four pilot Graves' disease (GD) sera reactive with both Tg and AChE and from three healthy controls, for reactivity against overlapping 20mer peptides (pin synthesis) covering the sequence 2171–2748 of human Tg. Antibody-reactive peptides were subsequently synthesized by a solid-phase technique for confirmation with a large number of sera: 99 GD, 32 Hashimoto's thyroiditis (HT) and 45 healthy controls.

Results: Peptides TgP15, TgP26 and TgP41 (amino acids 2339–2358, 2471–2490 and 2651–2670 respectively) were found to be targets of autoantibodies on intact Tg, recognized by a statistically significant proportion of GD sera (22.2%, 35.4% and 30.3% respectively), compared with HT (0%, 15.6% and 6.3% respectively) and healthy controls (0%, 4.4% and 4.4% respectively). The majority of GD sera (56.6%) were positive for at least one of the three peptides. In GD, TgP26 reactivity was found to be associated with TED (48.6% with TED versus 25.5% without TED, $P < 0.05$).

Conclusion: Some epitopes on the C-terminal region of Tg are associated with GD. A subset of Tg-reactive autoantibodies, directed to this region, is associated with TED and may be involved in the development of the disease.

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Introduction

Thyroglobulin (Tg), a homodimeric glycosylated iodoprotein (2×330 kDa) produced by the thyroid gland, serves as a precursor for the thyroid hormones thyroxine and 3,5,3'-triiodothyronine (1). Autoantibodies to Tg have been particularly useful for the diagnosis of autoimmune thyroid diseases, such as Graves' disease (GD) and Hashimoto's thyroiditis (HT) (2), but their pathogenic role is still debatable (3, 4). Several epitopes recognized by these autoantibodies or by Tg-induced antibodies in animals have been characterized throughout the Tg sequence (5–9). Either large recombinant peptides (5–8) or proteolytic fragments (9) have been used to map serological epitopes on Tg; small synthetic peptides have not been used so far due to the large size of the protein. The

C-terminal region of Tg is highly immunogenic and encompasses four of the five pathogenic T-cell epitopes known in animal studies (10). Moreover, this portion of Tg (2171–2748 of human Tg) shares significant sequence homology (28%) (11) and a common folding pattern (12, 13) with the catalytic subunit of acetylcholinesterase (AChE), an enzyme of the cholinergic synapses of the central and peripheral nervous systems (14, 15) (homology reaches 64% between segments 2314–2360 of human Tg and 147–197 of *Torpedo californica* AChE (16)). AChE has two molecular forms, the globular and the asymmetric, both containing catalytic subunits, while the asymmetric form also contains a filamentous tail (17).

Thyroid eye disease (TED) is commonly associated with autoimmune thyroid diseases, especially with GD, although the pathogenic mechanism remains

unknown. Several theories have been formulated about the pathogenesis of TED (18–21). Based on the structural homology between Tg and AChE, Ludgate *et al.* (22) suggested the pathogenic role of autoantibodies cross-reacting with Tg and orbital muscle AChE. Eye-specificity of these antibodies could be explained since orbital muscle AChE may be different from that of skeletal muscle (23, 24). In a previous study, we showed that immunoglobulin G (IgG) antibodies reacting with both Tg and AChE are present in a significant proportion of GD patients and are associated with TED (25). Furthermore, an 89-amino acid (a.a.)-long recombinant peptide of human Tg (2376–2464) was found to bear a Tg/AChE shared epitope, since it was recognized by rabbit anti-AChE antibodies (5). Antibodies binding to this epitope were present in a higher proportion of patients with GD than with HT, although their levels did not correlate closely with the presence of TED (24).

In the present study, we used 20mer overlapping synthetic peptides spanning the highly immunogenic and AChE-homologous C-terminal region of human Tg, to finely map Tg epitopes associated with autoimmune thyroid diseases and particularly with TED.

Subjects and methods

Patients

Sera from 99 patients with GD (mean age 48 years, range 17–84 years, female/male 74/25) and 32 patients with HT (mean age 43 years, range 15–78 years, female/male 28/4) were used. The diagnosis of patients was based on conventional clinical criteria and laboratory findings. Patients were subjected to detailed clinical examination regarding TED, including measurement of exophthalmos. In those patients where ophthalmopathy was not clinically evident, orbital echography was performed in order to ascertain the orbital muscle involvement. Among GD patients, 37 presented TED of grade II–IV, according to Werner's modification of the American Thyroid Association classification (26, 27), 55 did not present TED, while for 7, data were not available. Seventy-eight out of ninety-nine GD patients (78.8%) were thyroid-binding inhibiting immunoglobulins (TBII) positive as determined by a radio-receptor assay (Biocode Biotechnology, Sclessin-Liege, Belgium). No correlation was observed between TED and TBII positivity. Moreover, 45 sera from healthy controls (mean age 45 years, range 20–75 years, female/male 35/10), without thyroid disorder were used. All sera were stored in aliquots at -70°C until use.

Reagents

Human Tg was purified from thyroid glands, obtained at operations, applying a well-established methodology

(28). AChE from *Electrophorus electricus* (approx. 1000 U/mg) was purchased from Boehringer (Mannheim, Germany). Both antigen preparations were checked for purity by SDS-PAGE, under reducing and non-reducing conditions (29). Anti-human γ chain antibodies coupled to peroxidase or alkaline phosphatase were purchased from Sigma (St Louis, MO, USA).

Synthetic peptides

Pin-bound peptides Forty-eight sequential 20mer peptides (TgP1–48) overlapping by eight residues and covering the sequence 2171–2748 of human Tg (numbering of mature protein, not including the signal peptide) were prepared in duplicate according to the method of Geysen *et al.* (30), on prederivatized polyethylene pins (Chiron, Clayton, Australia). The synthesis protocol was based on the principles of solid-phase peptide synthesis (31) and the 9-fluorenylmethoxycarbonyl (Fmoc) protecting group strategy was followed.

Solid-phase synthesized peptides The peptides TgP15, TgP26 and TgP41, in free form, were synthesized according to standard solid-phase methods by Genosys Biotechnologies (Cambridge, UK). Peptide purity was assessed by HPLC and mass spectroscopic analysis.

Isolation of total serum IgG

Total IgG was purified on a protein G-Sepharose column (Pharmacia, Uppsala, Sweden) from four GD and three healthy control sera according to established methodology. Briefly, the serum passed through the column for 2 h, then the column was washed with phosphate-buffered saline 10 mmol/l, pH 7.4, containing 150 mmol/l NaCl (PBS). Bound IgG was eluted with 0.1 mol/l HCl-glycine pH 2.2, at 4°C and neutralized adding 2 mol/l Tris. Fractions containing IgG (absorbance measured at 280 nm) were concentrated and dialyzed against PBS. The IgG was stored in 50% glycerol, at -20°C .

Anti-Tg depletion and isolation of specific antibodies

Anti-Tg antibodies were depleted from the total IgG fraction on a Tg immunoadsorbent (4 mg Tg per ml beads); purified human Tg was coupled to glutaraldehyde-activated polyacrylamide-agarose beads, Ultrogel AcA22 (IBF biotechnics, Villeneuve-la-Garenne, France), as described previously (32). The efficiency of anti-Tg depletion was confirmed by ELISA. Anti-Tg antibodies were subsequently eluted from the immunoadsorbent with 0.2 mol/l HCl-glycine pH 2.2, at 4°C . After neutralization, concentration and dialysis

against PBS, the anti-Tg fraction was stored in 50% glycerol, at -20°C .

ELISA

Non-competitive ELISA with pin-bound peptides

Peptides covalently attached to polyethylene supports were tested for antibody binding by ELISA in 96-well microtitration plates. Purified IgG (instead of whole sera) was used to test reactivity against pin-bound peptides in order (a) to reduce the non-specific binding observed with the use of whole serum and (b) to avoid possible peptide damage by serum proteases. Pins were immersed in PBS containing 0.3% Tween-20, 1% bovine serum albumin and 1% ovalbumin, for 1 h at room temperature, to block free binding sites. Serum IgG, diluted in the above buffer, was added to the wells and incubated overnight at 4°C . In optimization experiments, the screening concentration of $50\text{ }\mu\text{g/ml}$ IgG was deemed to maximize the differences between positive and negative wells and it was therefore used throughout the study (affinity-purified Tg-specific antibodies were used at a screening concentration of $5\text{ }\mu\text{g/ml}$). After washing with PBS containing 0.5% Tween-20, anti-human γ -peroxidase conjugate ($1\text{ }\mu\text{g/ml}$ in blocking buffer) was added and incubated for 1 h at room temperature. The pins were again washed and the binding of antibodies was detected by the addition of enzyme substrate ($\text{H}_2\text{O}_2 + 2,2'\text{-azino-bis-3-ethylbenzothiazoline sulfonic acid: ABTS}$). The absorbance of the color was measured at 405 nm ($A_{405\text{ nm}}$). Bound antibodies were removed from the pins by sonication in $0.1\text{ mol/l NaH}_2\text{PO}_4$, 1% SDS and 0.1% 2-mercaptoethanol, for 30 min at 60°C . In order to determine whether bound antibodies were completely removed, pins were incubated with anti-human γ -peroxidase conjugate followed by the addition of substrate solution ($\text{H}_2\text{O}_2 + \text{ABTS}$). In all cases the final $A_{405\text{ nm}}$ was equal to the background. The cut-off point of positivity was calculated for each peptide as the mean $A_{405\text{ nm}}$ values + 3 S.D. of serum IgG from the healthy controls. With this method only the serum IgG of one patient can be tested (in duplicate) each time for reactivity against the panel of 48 pin-bound peptides. Therefore, the method should be considered only as a screening method for the detection of target peptides.

Non-competitive ELISA with immobilized antigens

ELISA plates, Maxisorb (Nunc, Roskilde, Denmark), were coated overnight at 4°C with Tg or AChE, or each of the peptides ($10\text{ }\mu\text{g/ml}$, $100\text{ }\mu\text{l}$ per well), in 0.1 mol/l carbonate-bicarbonate buffer pH 9.6. Free binding sites were blocked with PBS containing 5% bovine serum (PBS-BS) for 45 min at 37°C . Sera, diluted (1:50) in PBS-BS containing 0.1% Tween-20 (PBS-BS-T), were added to the wells in duplicates and incubated

with immobilized antigen. In ELISA with immobilized Tg or AChE, this incubation lasted 90 min at 37°C , while for the immobilized peptides the incubation was performed overnight at 4°C . The plates were then incubated with anti-human γ -alkaline phosphatase conjugate ($0.5\text{ }\mu\text{g/ml}$ in PBS-BS-T) for 1 h at 37°C . After each step the plates were extensively washed with PBS containing 0.1% Tween-20. The assay was completed by the addition of the enzyme substrate (*p*-nitrophenyl phosphate). The absorbance of the produced color was read spectrophotometrically at 405 nm ($A_{405\text{ nm}}$). Absorbance of experimental wells was calculated after subtraction of the background determined in the absence of coated antigen. The cut-off point of positivity was calculated as the mean $A_{405\text{ nm}}$ values of the healthy control sera + 2.5 S.D. Serum antibody activity was expressed in units (cut-off point = 100 units). Sera with a reactivity equal to the cut-off point were included in each assay to compensate for interassay variation.

Competitive ELISA Binding of sera to immobilized AChE was checked for inhibition by soluble peptides TgP26 and TgP41. The same procedure was followed as for the non-competitive ELISA with the difference that the serum (at a dilution corresponding to 50% of maximum binding) had been preincubated with soluble peptide (TgP26 or TgP41, at $1.6\text{--}50\text{ nmol/ml}$) for 90 min at 37°C , before being placed into the AChE-coated wells. The results were expressed as inhibition percent (%) plotted versus concentration of inhibitor (nmol/ml).

Statistical analysis

The proportions of positive sera in the patients' groups were compared with each other as well as with that in the healthy control group, using a χ^2 test of homogeneity. The same test was used to compare the two subgroups of GD patients (with and without TED). A *P* value less than 0.05 was considered statistically significant.

Results

Epitope mapping on the AChE-homologous region of Tg

Forty-eight 20mer peptides overlapping by eight residues and covering the C-terminal portion of Tg (sequence 2171–2748 of human Tg) were prepared in duplicate on polyethylene pins. Total IgG was purified from sera of four pilot GD patients, which were previously found to react with both Tg and AChE (25). Total serum IgG was also purified from three healthy donors used as a control. IgG ($50\text{ }\mu\text{g/ml}$) was tested by ELISA for reactivity against the synthetic

Figure 1 Number of positive IgG preparations (50 μ g/ml) from GD patients for each of the pin-bound peptides. The cut-off point of positivity was calculated for each peptide as the mean $A_{405\text{ nm}}$ values + 3 S.D. of serum IgG from the three healthy controls.

peptides. Seventeen out of the forty-eight peptides were recognized by all four GD IgG preparations, nine of the peptides were recognized by three of them, nine of the peptides were recognized by two of them and eleven of the peptides were recognized by one of them (Fig. 1). None of the total serum IgG was positive for six of the peptides tested. Interestingly, no immunodominant region was identified and the peptides recognized by a high proportion of GD IgG preparations were spread throughout the examined portion of Tg.

To ascertain which peptides encompass epitopes on intact Tg, we proceeded to deplete anti-Tg antibodies from total IgG of two GD patients, by immunoadsorption on a Tg-affinity column. The anti-peptide reactivity of total IgG was compared with that of anti-Tg-depleted IgG (Fig. 2). The greatest decrease of reactivity after depletion of Tg-specific IgG, common for both GD patients, was observed for peptides TgP15, TgP26 and TgP41. The segments of human Tg corresponding to the above peptides are 2339–2358, 2471–2490 and 2651–2670, while their respective amino acid sequences are QVAALTWVQTHIRGFGGDPR, PPAR-ALKRSLWVEVDLLIGS and PYEFSRKVPTEATPWPDPFVP. These data suggested that these peptides are likely epitopes on intact Tg recognized by autoantibodies.

To confirm this finding, Tg-specific IgG was purified by immunoaffinity from serum IgG fractions of two GD patients and further tested (at 5 μ g/ml) for reactivity against five pin-bound peptides, selected according to the previous test (Fig. 2): TgP15, TgP26 and TgP41 (for which the reactivity was expected to be high) and

TgP21 and TgP44 (for which a very low reactivity was expected). As shown in Fig. 3, the expected anti-peptide reactivity was confirmed.

Reactivity of sera against TgP15, TgP26 and TgP41

The peptides TgP15, TgP26 and TgP41, synthesized in free form by a solid-phase technique, were used in ELISA for screening of a large number of sera (99 GD, 32 HT and 45 healthy controls). The anti-peptide reactivity of individual GD, HT and healthy control sera are shown in Fig. 4. Among 99 GD sera tested, 22 were positive for TgP15 (22.2%), 35 for TgP26 (35.4%) and 30 for TgP41 (30.3%). Among patients with HT, none of the 32 sera was positive for TgP15 (0%), five sera were positive for TgP26 (15.6%) and only two for TgP41 (6.3%). None of the 45 healthy controls was positive for TgP15 (0%), while a positive reaction was exhibited by two healthy controls for TgP26 and two for TgP41 (4.4%) (Table 1). Overall, 56.6% of GD sera were positive for at least one of the three peptides, in contrast to 21.9% and 8.9% in HT and healthy controls respectively. Statistically significant differences were found between GD and HT ($P < 0.001$) as well as between GD and healthy controls ($P < 0.001$), but not between HT and healthy controls (Table 1).

Figure 2 Differences in $A_{405\text{ nm}}$ values before and after depletion of anti-Tg antibodies from the total IgG, for two patients with GD (1 and 2). IgG before and after anti-Tg depletion was tested at 50 $\mu\text{g/ml}$ against the pin-bound peptides shown.

Correlation of serum anti-peptide reactivity with TED

Within the GD group, 37 patients also presented TED and 55 showed no evident TED. When the above data were analyzed according to the presence of TED, it was found that 17 of the 37 sera (48.6%) from GD patients with TED reacted with TgP26, whereas only 14 of the 55 sera (25.5%) from GD patients without TED reacted with the same peptide (Table 1). This is a statistically significant difference ($P < 0.05$), revealing an association of anti-TgP26 reactivity with TED. Reactivity

against TgP15 and TgP41 was not associated with TED (Table 1).

Inhibition of anti-AChE reactivity by soluble peptide TgP26

Ten GD sera, reactive with TgP26, were chosen to check the inhibition of their reactivity to immobilized AChE by the soluble peptide TgP26. Antibody binding in two of these sera was inhibited by up to 45% for one of them and 50% for the other, at peptide

Figure 3 Reactivity of affinity purified anti-Tg antibodies (5 µg/ml) from two patients with GD (1 and 2) against selected peptides of human Tg.

concentrations of 50 nmol/ml (Fig. 5), whereas in the other eight sera no inhibition was observed at concentrations up to 50 nmol/ml. Soluble TgP41 peptide was used as a negative control. Antibody-binding to AChE was not inhibited by TgP41 in any of the sera tested.

Discussion

Due to the large size of Tg, the identification of antigenic sequences on Tg, recognized by disease-associated or Tg-induced antibodies in animals, has so far been accomplished using either large recombinant peptides (5–8) or proteolytic fragments (9). In the present study, we used small synthetic peptides spanning the AChE-homologous C-terminal portion of human Tg (a.a. 2171–2748) to identify serological epitopes targeted by IgG, isolated from Tg/AChE-reacting GD sera. No immunodominant region was identified within the examined portion of Tg, since the peptides (defining linear epitopes) recognized by all four

GD IgG preparations tested were spread throughout the sequence. Using proteolytic fragments or denatured Tg, some researchers concluded that the main Tg auto-epitopes are mostly conformational, since only native Tg or large Tg fragments are recognized by autoantibodies (33–35). This discrepancy could be explained by the non-immunodominant nature of linear epitopes and because these epitopes may be masked or destroyed when Tg is denatured or digested with enzymes.

We identified the peptides TgP15, TgP26 and TgP41 as the most likely epitopes recognized on intact Tg. In their free form, these peptides were recognized by a significant proportion (22–35%) but not by the majority of GD sera, although more than half of GD sera (56.6%) were positive for at least one of the three peptides. Our results confirm those of other researchers (36) showing that Tg-immunodominant epitopes are conformational.

Increased serum anti-TgP26 reactivity of GD patients was statistically associated with the presence of TED. Previous studies had demonstrated the presence of

Figure 4 Distribution of reactivity values (ELISA units) against (A) TgP15, (B) TgP26 and (C) TgP41 of the sera (at 1/50 dilution) from the three groups tested: Graves' disease (GD), Hashimoto's thyroiditis (HT) and healthy controls (HC). The cut-off point (calculated as the mean $A_{405\text{ nm}}$ values of the healthy control sera + 2.5 s.d.) is indicated by horizontal lines.

Table 1 Incidence of peptide-positive sera from Graves' disease (GD), Hashimoto's thyroiditis (HT) and healthy controls (HC).

Peptide	Amino acid sequence	GD (%) (n = 99)	HT (%) (n = 32)	HC (%) (n = 45)	GD with TED (%) (n = 37)	GD without TED (%) (n = 55)	P* GD-HT	P GD-HC	P HT-HC	P GD with/without TED
TgP15	2339–2358	22.2	0	0	16.2	23.6	<0.005	<0.001	NS	NS
TgP26	2471–2490	35.4	15.6	4.4	48.6	25.5	<0.05	<0.001	NS	<0.05
TgP41	2651–2670	30.3	6.3	4.4	21.6	36.4	<0.01	<0.001	NS	NS
At least one peptide**		56.6	21.9	8.9	—	—	<0.001	<0.001	NS	—

*Statistical comparison among GD, HT and HC sera, as well as between GD sera with and without thyroid eye disease (TED). P values were deduced from the χ^2 test. NS, differences not statistically significant. **TgP15, 26 or 41.

Figure 5 Inhibition of binding to immobilized AChE of two TgP26-reactive GD sera (1 and 2), by soluble peptides TgP26 (▲ and ● for serum 1 and 2 respectively) and TgP41 (△ and ○ for serum 1 and 2 respectively). The graph indicates percentage of inhibition plotted versus concentration of inhibitor.

common epitope(s) between Tg and AChE (5, 24, 25), which may be involved in the pathogenesis of TED. Within the 20 a.a. sequence of TgP26, three amino acids are shared and five are conservative substitutions in the homologous sequence of human AChE (37). Binding to AChE was inhibited by soluble TgP26 in the case of two out of the ten tested TgP26-reactive GD sera. This result suggests that TgP26 comprises a Tg/AChE common epitope. The low incidence found can be explained, since (a) blockade of a subgroup of AChE-reactive autoantibodies (those directed against the TgP26-homologous site on AChE) will not significantly influence anti-AChE reactivity, and (b) the affinity of the autoantibodies is possibly higher for the protein than for the 20mer peptide. The epitopes defined by the peptides TgP15 and TgP26 do not overlap with previously characterized autoepitopes or heteroepitopes on Tg (5–9). The sequence of TgP41 is encompassed in the segment 2644–2730 of human Tg, bearing a heteroepitope (6), and comprises an overlap of 14 residues with the sequence 2657–2748 of human Tg, recognized by a few HT sera (9).

In conclusion, we identified three epitopes on the C-terminal region of Tg serologically differentiating GD

from HT. One of these epitopes was found to be associated with TED. Studies are in progress in order to assess the pathogenicity, if any, of this epitope.

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