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Use of recombinant epitopes to study the heterogeneous nature of the autoantibodies against thyroid peroxidase in autoimmune thyroid disease

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SUMMARY

Microsomal antigen is often recognized by the sera from patients with autoimmune thyroid disease (AITD). Human thyroid peroxidase (hTPO) is the main component of this antigen. In a previous study, we expressed hTPO cDNA as fusion proteins in prokaryotic vector; we thereby defined seven antigenic peptides by using two rabbit polyclonal anti-hTPO antibodies. In the present study we used the seven epitopes and three widened peptides to define the reactivity pattern of 61 sera from patients with AITD. Thirty-eight of them reacted against at least one of the seven hTPO-restricted epitopes; 14 were negative against the seven determinants but recognized one or two of the extended peptides. Thus, the antibody response against hTPO appeared to be highly heterogeneous in AITD patient sera. Moreover, we demonstrated that the immunodetection of the hTPO on Western blotting with deoxycholate solubilized microsomes can be perfectly correlated with the recognition of one of the epitopes in the region 554–735.

Keywords thyroid peroxidase recombinant proteins epitopes autoantibodies

INTRODUCTION

Human thyroid peroxidase (hTPO) is a membrane-bound haemoprotein which plays a key role in the biosynthesis of thyroid hormones, by catalysing both iodination and coupling of tyrosine residues in thyroglobulin (Tg) [1]. Its complete 933 amino acid sequence has been established [2,3] and hTPO mRNA species generated by alternate splicing have been described [2,4,5]. Autoimmune thyroid disease (AITD) is essentially divided into two distinct pathologies: Graves' disease (GD) and Hashimoto's thyroiditis (HT) [6]. Patients with AITD possess autoantibodies against thyroid constituents such as Tg, thyrotropin receptor and microsomal antigen (TMA) [7]. Biochemical and immunological investigations have provided evidence that the main component of TMA is hTPO [3,8,9].

Extensive studies have revealed the heterogeneous nature of the autoantibodies against TMA/hTPO [10–13]. By electrophoresis under denaturing and non-denaturing conditions, Hamada *et al.* [10] found three types of autoantibody against TMA. The same techniques in combination with the measurement of enzymatic activity inhibition by autoantibodies allowed Doble *et al.* [11] to exhibit six distinct determinants on hTPO. Conversely, criss-cross experiments showed only two antigenic domains on the molecule [13]. Analysis of the cross-reactivity of antibodies against hTPO with other peroxidases confirmed this heterogeneity [14]. But, in all these experiments, no localization of the different epitopes was established. By screening of a thyroid cDNA library, Libert *et al.* [3] defined a major immunogenic peptide, called C2, on the hTPO sequence 590–675 which was recognized by 65% of AITD patient sera [15].

Recently, by expression of hTPO peptides as bacterial fusion proteins and screening with two rabbit polyclonal anti-hTPO antibodies, we precisely defined seven antigenic determinants, named I to VII, on the hTPO primary sequence [16]. In the present study we used the seven peptides to study the antibody pattern of 61 sera from patients with AITD. Thirty-eight presented a reactivity against at least one of the seven restricted epitopes. The remaining 23 sera were negative against epitopes I to VII; but 14 of them recognized greater regions (hTPO sequences 1–242 and 554–735). Finally, we found that the determinants detected by Western blotting on the entire hTPO are contained in the region 554–735.

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MATERIALS AND METHODS

hTPO recombinant peptides

Seven antigenic determinants on hTPO, numbered I to VII, were previously defined by expression of hTPO fragments in a prokaryotic vector (pEX; Boehringer, Mannheim, Germany) [16] after serial subclonings and screenings with immunopurified rabbit polyclonal anti-hTPO antibodies produced as previously described [8]. Greater cDNA, named A, B and C, originally called A12, A45 and M5.553 respectively [16], were cloned in the same expression vector.

Sera samples

Serum was obtained from 61 patients with AITD (30 HT and 31 GD). Diagnosis was based on clinical and laboratory criteria. All these sera presented autoantibodies against TMA as evaluated by indirect immunofluorescence (IIF) on thyroid section (titre ranging from 1:10 to 1:500) and passive haemagglutination (PHA) (Wellcome, Dartford, UK) (titre ranging from 1:400 to 1:409 600). They were used at 1:20 dilution in Tris buffer saline (TBS; 50 mM Tris pH 7·5, 150 mM NaCl), bovine serum albumin (BSA) 1%, 1% *Escherichia coli* wild-type pEX bacterial lysate (*E. coli* transformed with wild-type pEX plasmid).

SDS–PAGE and immunoblotting

Bacterial extracts containing fusion protein were produced as previously described [17]. Deoxycholate solubilized thyroid microsomes were prepared as recommended [18]. Bacterial extracts (20 μl; about 20 μg of fusion protein) or solubilized microsomes (150 μl; 675 μg) were dissolved in sample buffer (2% SDS, 0·1 M dithiothreitol, final concentration), boiled for 5 min, applied to a single sample lane of 6% SDS–PAGE, electrophoresed and transferred to nitrocellulose (BA85; Schleicher and Schüll, Dassel, Germany). For the immunoreactivity test, nitrocellulose sheets of the different preparations were cut into 5 mm wide strips. After saturation with 3% BSA in TBS, the strips were incubated for 2 h at room temperature with the diluted sera, washed in TBS, incubated for 2 h in peroxidase-labelled antibody solution (Calbiochem, Behring Diagnostics, La Jolla, CA), absorbed and diluted in the same conditions as the first antibody, and revealed in peroxidase assay with 4-chloro-1-naphthol as chromogen.

RESULTS

Overlapping cDNA covering the complete hTPO mRNA sequence were inserted in the 3′-end of a modified β-galactosidase gene (*cro-lac* Z) in the bacterial expression pEX plasmid [16]. The corresponding hTPO peptides were thereby produced as hybrid proteins (*cro-lac* Z + foreign peptide). A high level of fusion protein synthesis in this system allowed easy visualization on SDS–PAGE; electrophoresis migration of fusion proteins was, therefore, directly related to the size of cDNA inserts (Fig. 1a). Immunoblotting was performed on these bacterial extracts with two rabbit polyclonal anti-hTPO antibodies. The positive clones were then reduced by several subclonings using restriction enzymes and re-screenings with the two rabbit polyclonal anti-hTPO antibodies. Thus, using these two heterologous sera, we defined seven antigenic hTPO peptides between amino acids 21 and 49 (numbered I to VII). Epitope III was defined as a 45 amino acid peptide (hTPO sequence 233–275), but this shorter peptide was less immunoreactive than the original A3 region on colony screening, and undetected on immunoblotting [16]. In the present study, epitope III was considered as being constituted of the A3 region (99 amino acids, hTPO sequence 233–331). The localization of epitopes I to VII on the hTPO primary sequence is shown in Fig. 2.

The hybrid proteins expressing hTPO epitopes I to VII and the wild-type *cro-lac* Z protein were separated on SDS–PAGE (Fig. 1a). After transfer on nitrocellulose, the reactivity of 61 sera from patients with AITD (30 HT and 31 GD) was investigated on these antigenic determinants (Fig. 1b). Thirty-

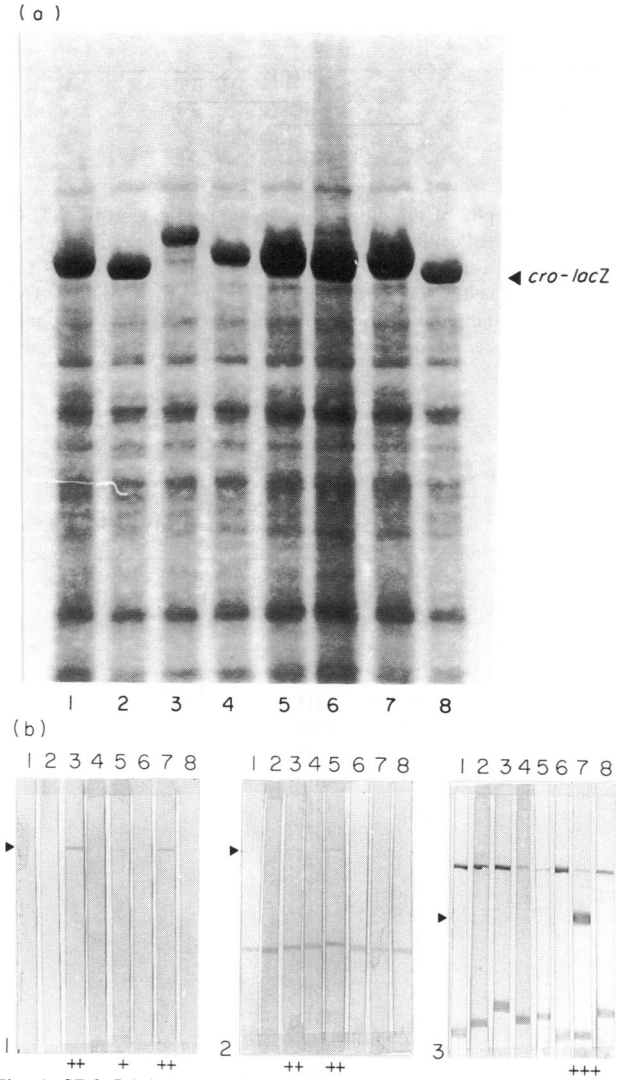

Fig. 1. SDS–PAGE analysis of hybrid proteins expressing the seven hTPO antigenic domains (a) and three examples of immunoblotting with pathologic sera (b). (a) Samples were set down as follows: 1: I, 2: II, 3: III, 4: IV, 5: V, 6: VI, 7: VII, 8: Wild-type pEX. The arrow gives the position of the wild-type *cro-lac* Z protein. After electrophoresis and transfer on nitrocellulose, immunorevelation with immune sera was performed as described in Materials and Methods. (b) For sera 1, 2 and 3, lanes 1–8 correspond to the eight fusion proteins in the same order as on SDS–PAGE (a). The position of the fusion proteins is indicated by an arrow. +, + + and + + + correspond respectively to weakly positive, positive and highly positive detection.

eight sera presented a reactivity against at least one of the recombinant epitopes. Their antibody patterns against epitopes I to VII are shown on Table 1. The fact that none of the sera recognized the wild-type *cro-lac* Z protein proved the specificity of immunoblottings. In spite of the addition of wild-type pEX lysate in the antibody dilution buffer, bacterial components were revealed by the human sera (sera 2 and 3, Fig. 1b). Many different antibody patterns can be noted in Table 1. Some sera recognized several epitopes, but only serum 2 showed a positive signal for the seven hTPO peptides. However, a majority of sera (20 out of 38) reacted against only one epitope: 11 against epitope III, four against epitope V, three against epitope VII,

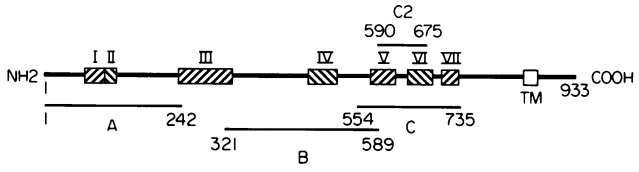

Fig. 2. Cartography of the different antigenic peptides on the hTPO primary sequence. Position of the seven restricted peptides is given by hatched boxes. Position of extended peptides A, B and C and the C2 determinant defined by Libert *et al.* [3] is indicated by solid lines. TM corresponds to the putative transmembrane domain of the molecule. Amino acid numbering corresponds to the hTPO sequence including the leader peptide.

one against epitope I and one against epitope II. Twenty-three sera out of 61 (38%) recognized none of the seven peptides (nine out of 30 HT patient sera and 14 of 31 GD patient ones). This negativity cannot be explained by a low presence of autoantibodies against TMA; some of these sera had a titre of autoantibodies against TMA of 1:200 (IIF) and/or 1:409 600 (PHA).

In order to explain the non-reactivity of 23 pathologic sera, we performed the same experiment with extended recombinant hTPO peptides. Peptide A covers the first 242 hTPO amino acids and contains the epitopes I and II; peptide B covers the region 321–589 and contains the epitope IV, and peptide C covers the region 554–735 and contains the epitopes V, VI and VII (Fig. 2). The results are shown in Table 2. The use of a longer peptide corresponding to the -COOH extremity of the hTPO (sequence 554–933) instead of peptide C gave the same results. The 61

Table 1. Immunoreactivity of 38 human pathologic sera on the seven antigenic peptides

No. of patients	Pathologies	I (68–105)	II (106–126)	III (233–331)	IV (467–515)	V (574–621)	VI (641–685)	VII (701–730)
1	GD	–	–	+	–	+++	–	–
2	GD	+	+++	++	+	++	+	++
3	GD	–	–	++	–	++	–	–
4	GD	–	–	+	–	–	–	–
5	GD	–	–	–	–	+++	–	–
6	GD	–	–	++	–	+	–	+++
7	GD	–	–	+	+	–	–	–
8	GD	–	–	++	–	–	–	–
9	GD	–	–	++	–	–	–	–
10	GD	–	–	++	–	–	–	–
11	GD	–	–	++	–	–	–	–
12	GD	–	–	+	–	–	–	+
13	GD	–	–	+	–	–	–	–
14	GD	–	–	+	–	–	–	–
15	GD	–	–	+	–	–	–	–
16	GD	–	–	+	–	–	–	–
17	GD	–	++	–	–	–	–	–
18	HT	–	–	–	–	+	+	+++
19	HT	–	–	+	+	+	–	–
20	HT	–	+++	–	–	+++	–	–
21	HT	–	–	–	–	+++	–	–
22	HT	+	–	++	+	–	+	–
23	HT	+	–	++	+	++	+	++
24	HT	+	–	++	+	++	–	–
25	HT	+	–	+++	–	+	–	–
26	HT	–	–	++	–	+	+	–
27	HT	–	–	++	+	+	–	–
28	HT	–	–	+	+	+	–	–
29	HT	–	–	–	–	+	–	–
30	HT	–	–	–	–	+	–	–
31	HT	–	–	–	–	+	–	+
32	HT	–	–	–	–	–	–	+++
33	HT	–	–	–	–	–	–	+++
34	HT	–	–	–	–	–	–	+++
35	HT	+	–	++	+	–	–	–
36	HT	–	–	+	–	–	–	–
37	HT	–	–	+	–	–	–	–
38	HT	+	–	–	–	–	–	–

–, +, ++ and +++ correspond respectively to negative, weakly positive, positive and highly positive detection on immunoblotting.

Table 2. Immunoreactivity of 61 human pathologic sera on hTPO peptides and solubilized microsomes

No. of patients	Pathologies	A	III	B	C	Mic	No. of patients	Pathologies	A	III	B	C	Mic
1	GD	−	+	−	+++	+++	32	HT	−	−	−	+++	+++
2	GD	+++	++	−	++	++	33	HT	+	+	−	−	−
3	GD	−	++	−	+	+	34	HT	+++	−	−	+++	+++
4	GD	−	+	−	−	−	35	HT	−	−	−	+++	+++
5	GD	−	−	−	+++	+++	36	HT	+	++	−	−	−
6	GD	−	++	−	+++	+++	37	HT	+	++	−	++	++
7	GD	−	+	−	−	−	38	HT	+	++	−	+	+
8	GD	−	++	−	−	−	39	HT	+	+++	−	+	+
9	GD	−	++	−	−	−	40	HT	−	++	−	+	++
10	GD	−	++	−	−	−	41	HT	−	++	−	+	+
11	GD	−	++	−	−	−	42	HT	+	+	−	+	+
12	GD	−	+	−	+	+	43	HT	−	−	−	+	+
13	GD	−	+	−	−	−	44	HT	+	−	−	+	+
14	GD	−	+	−	−	−	45	HT	−	−	−	+	−
15	GD	−	+	−	−	−	46	HT	−	−	−	+++	+++
16	GD	−	+	−	−	−	47	HT	−	−	−	+++	+++
17	GD	++	−	−	+	+	48	HT	−	−	−	+++	+++
18	GD	−	−	−	++	++	49	HT	+	++	−	−	−
19	GD	+	−	−	+	+	50	HT	−	+	−	+	−
20	GD	+	−	−	−	−	51	HT	−	+	−	−	−
21	GD	+	−	−	−	−	52	HT	++	−	−	+	+
22	GD	−	−	−	−	−	53	HT	+++	−	−	−	+
23	GD	−	−	−	++	++	54	HT	+++	−	−	+	+
24	GD	−	−	−	−	−	55	HT	−	−	−	++	++
25	GD	−	−	−	+	+	56	HT	−	−	−	−	−
26	GD	−	−	−	+	+	57	HT	−	−	−	−	−
27	GD	+	−	−	−	−	58	HT	−	−	−	−	−
28	GD	−	−	−	−	−	59	HT	−	−	−	−	−
29	GD	−	−	−	+	+	60	HT	−	−	−	−	−
30	GD	−	−	−	−	−	61	HT	−	−	−	+	+
31	GD	+	−	−	+	+							

−, +, ++ and +++ correspond respectively to negative, weakly positive, positive and highly positive detection on immunoblotting. Sera 1–17 and 32–52 correspond to sera 1–38 in Table 1 in the same order.

human sera were tested; 52 presented a reactivity against at least one hTPO region. A detection of one of the hTPO epitopes I, II, V, VI or VII in the first experiment was confirmed by an immunoreactivity against the widened region. For example, sera 1, 5, 20 and 21 highly recognized epitope V (Table 1) and peptide C (corresponding sera 1, 5, 34 and 35 in Table 2). Conversely, the slight detection of epitope IV by sera 2, 7, 19, 22, 23, 24, 26, 27 and 35 (Table 1) was never confirmed on peptide B (Table 2). Furthermore, none of the 61 sera showed an antibody response against peptide IV.

Fourteen sera (10 GD and 4 HT) which were negative against the seven restricted epitopes presented a reactivity against greater peptides A or C (seven against peptide A and 10 against peptide C). Moreover, some of the 38 sera which were positive against a restricted epitope appeared to recognize another region (e.g. serum 33 on peptide A or serum 50 on peptide C, Table 2). Another pointer was the strong detection of peptide C by sera 18 and 55 or the stonger detection of peptide A by sera 52, 53 and 54 (Table 2) which did not correspond to the recognition of one of the restricted epitopes (I to VII) they contained. In spite of the use of greater peptides, nine sera recognized none of the different hTPO peptides.

In a further experiment, we compared the recognition of one of the four hTPO regions, A, III, B and C which cover the hTPO sequence 1–735 with the result on Western blotting with deoxycholate solubilized microsomes (Table 2). Out of 61 sera, 27 presented no reactivity against the 100-kD hTPO band. Nevertheless, we observed a perfect correlation between the detection of the region C and/or one of its restricted epitopes V or VII and the recognition of the TPO in the microsome preparation. Conversely, the strong recognition of peptide A by sera 53, 54 or peptide III by serum 39 did not correlate with an immunodetection of the entire hTPO on Western blotting (Table 2). Finally, nine sera reacted against neither the different hTPO peptides nor the hTPO on solubilized microsomes.

The results of the experiments with recombinant peptides are summarized in Table 3. It emerges from this assessment that the epitopes III (hTPO sequence 233–331) and V (sequence 574–621) are the ones most often recognized by the sera from patients with AITD (33 out of 61, 54%) and that the region 554–735 (peptide C) constitutes an immunodominant region recognized by 56% of the AITD patients. Nevertheless, some sera can present a high antibody response against other epitopes such as epitope II. Otherwise, the present data show that no clear

Table 3. Reactivity of the human sera on the hTPO peptides, assessment of Tables 1 and 2

	Antigenic peptides*	Total sera ($n=61$)	GD ($n=31$)	HT ($n=30$)
A	(1–242)	19 (31)†	7 (23)	12 (40)
I	(68–105)	7 (12)	1 (3)	6 (20)
II	(106–126)	3 (5)	2 (7)	1 (3)
III	(233–331)	26 (43)	15 (48)	11 (37)
B	(321–589)	0 (0)	0 (0)	0 (0)
IV	(467–515)	9 (15)	2 (6)	7 (23)
C	(554–735)	34 (56)	14 (47)	20 (67)
V	(574–621)	18 (30)	5 (16)	13 (43)
VI	(641–685)	5 (8)	1 (3)	4 (13)
VII	(701–730)	9 (15)	3 (10)	6 (20)

* The corresponding hTPO sequence of each peptide is indicated in parenthesis. The percentages are calculated with the number of total sera indicated at the top of each column.

† Numbers in parenthesis are percentages.

distinction can be established between HT and GD patients based on different recognition of hTPO epitopes expressed in a prokaryotic system.

DISCUSSION

We used hTPO epitopes expressed as fusion proteins in a prokaryotic vector in order to investigate the specificity of antibodies against hTPO of 61 immune sera from patients with HT or GD. We first tested the antibody response against seven antigenic peptides previously defined [16], numbered I to VII. Thirty-eight of the sera recognized at least one of the seven epitopes, mostly epitopes III and V (Table 3); but 23 sera (38%) reacted against none of the hTPO cloned antigenic peptides. To explain the non-recognition of the seven determinants by 23 sera, we performed Western blotting with extended hTPO peptides, named A, B and C, produced in the same prokaryotic system. The 61 human sera were tested against these widened peptides. The sera which were positive on the epitopes I and II or V, VI and VII confirmed their antibody response against greater peptides A and C, respectively. Fourteen of the 23 sera which presented no reactivity against determinants I to VII showed an antibody response against hTPO region A (sequence 1–242) or C (sequence 554–735).

The recognition of the recombinant peptides by 38 patient sera demonstrated that the native conformational structure was respected at least partially. However, 14 sera recognized only greater peptides. This shows that some epitopes are more conformational or discontinuous and are thus destroyed by excessive restriction. Nevertheless, these determinants can be restored in more extended peptides showing that peptides produced in a prokaryotic system can mimic the three-dimensional structure of complex epitopes. This result increases the heterogeneity of the antibody response and argues for the presence of at least two other epitopes, one in the sequence 1–242 and one in the sequence 554–735. However, the antigenic structure of peptide III was dramatically disrupted when we tried to reduce it. This may be linked to the presence of five cysteine and five proline residues on this 99 amino acid sequence. The presence of intrachain disulphide bonds within hTPO has been proposed [19] and one of them has been clearly established on human TMA [20] and porcine TPO [21]; another one may be present in the region III. Conversely, peptide B was never detected by the 61 human sera, even the nine sera positive against epitope IV. The explanation for this apparent contradiction is probably in the conformational nature of this region. Moreover, no sera recognized the epitope IV alone. These different points argued for a low antigenic nature of the sequence 321–589 of the hTPO.

Libert *et al.* [3] have described an hTPO major antigenic region, called C2, which was detected by sera from 65% of AITD patients [15]. Furthermore, Elisei *et al.* [22] showed that the C2 peptide was composed of two distinct epitopes. In our study we found that epitope V was detected by 30% of AITD patient sera and was one of the most recognized peptides with determinant III (Table 3). The difference in percentage is probably explained by the methodologies used, ELISA for Ludgate *et al.* [15], Western blotting in our case. By performing sequence comparisons (Fig. 2) one may conclude that peptides C2 and V harbour a common epitope (region 590–621 on hTPO sequence) which is often recognized by the autoantibodies and that the epitope shared by TPO and H^+ K^+ ATPase corresponds to the determinant VI in our study. However, we demonstrated that no particular epitopes, even peptides V or C2, attested to antibody response against hTPO in any pathologic sera.

In another part of our investigation we have compared the recognition of the different hTPO recombinant epitopes with the detection of entire hTPO in Western blotting. Interestingly, we found a perfect correlation between the recognition of peptide C or epitopes V and/or VII and the immunodetection of the 100-kD band on solubilized microsomes. We may conclude that the detection of hTPO in Western blotting can be strictly attributed to the antibody response against an hTPO epitope in the region 554–735. This is the first argument to strongly suggest that the epitopes recognized on the entire hTPO on Western blotting are in the sequence 554–735.

Thirty-four sera out of 61 (56%) presented a reactivity against hTPO on Western blotting. This result disagrees with that of Hamada *et al.* [10] and Doble *et al.* [11], showing that autoantibodies did not recognize SDS-denatured TPO in the majority of sera. On the other hand, it agrees with the study of Banga *et al.* [14] who showed that 10 out of 20 sera recognize denaturated TMA. Yokoyama *et al.* [23] observed a good correlation between the autoantibody titre against TMA and the recognition of purified hTPO by autoantibodies on immunoblotting under reducing conditions and concluded that many hTPO epitopes retain their ability to be recognized by autoantibodies even if the protein is highly unfolded. From our experiments, we suppose that the sera they used contained antibody against region C.

To avoid false positive detections generated by antibodies against bacterial constituents, we used immunoblotting to study the heterogeneity of antibody against hTPO. Such techniques rule out all ambiguity regarding the recognized antigen but may dramatically disrupt the conformation of some determinants. Some of the hTPO epitopes may be discontinuous and/or stabilized by disulphide bridge(s) (as may be the case for determinant III) or may be dependent on post-translational

modifications such as glycosylation, although Foti & Rapoport [24] showed that carbohydrate moieties did not play an important role in the recognition of TPO by autoantibodies. Moreover, the nine sera which presented no reactivity against all the peptides were also negative against the entire hTPO on Western blotting, in spite of their important autoantibody titre against TMA evaluated by IIF and PHA. These sera did not recognize other bands on Western blotting, but Tg. They may present an antibody reactivity against a discontinuous hTPO epitope, not accessible by our approach or they may correspond to false positive in the titration tests.

The autoantibody patterns reported in the present study confirm, by a different approach, the 'considerable heterogeneity among microsomal antibodies from different patients' described by Yokoyama *et al.* [12]. Moreover, this study demonstrates that no clear distinction can be established between HT and GD based on the detection of some hTPO epitopes by the autoantibodies. It also argues against titration of antibodies against hTPO based on competitive assay with MoAb [25]. Nevertheless, the hTPO recombinant epitopes offer new tools to clarify the physiopathologic role of antibodies against hTPO in AITD. Hamada *et al.* [10] suggested that antibody against TMA against denatured or denatured and reduced determinants may be related to destruction of the gland; these kinds of epitopes are typically accessible by our methodology. Kohno *et al.* [26] defined four types of hTPO determinants on the basis of the antibody interaction with enzymatic activities. Similarly, Okamoto *et al.* [27] reported that the inhibition of TPO activity by antibodies against hTPO was more effective in patients with HT than in those with GD. Moreover, antibodies against hTPO are classically implicated in complement-mediated cytotoxicity [28]. Investigations are now necessary to know if the hTPO peptides used in the present study will be particularly efficient in correlating autoantibody binding on some epitopes with inhibition of the TPO enzymatic activity or thyroid cellular damage.

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