0021-972X/04/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 89(10):5116–5123

doi: 10.1210/jc.2003-032173

Human Monoclonal Thyroglobulin Autoantibodies: Epitopes and Immunoglobulin Genes

FRANCESCO LATROFA, MARTIN PHILLIPS, BASIL RAPOPORT, AND SANDRA M. MCLACHLAN

Autoimmune Disease Unit (F.L., B.R., S.M.M.), Cedars-Sinai Research Institute and the University of California, Los Angeles School of Medicine, Los Angeles, California 90048; and Department of Chemistry and Biochemistry (M.P.), University of California, Los Angeles, California 90095

Autoantibodies to thyroglobulin (TgAbs) are common markers of thyroid autoimmunity, but relatively few human monoclonal TgAbs have been described. From a panel of 64 human monoclonal TgAbs (isolated from a thyroid-disease derived combinatorial Ig gene library), we selected seven with unique genetic features for detailed characterization. These TgAbs preferentially recognize native (not denatured) Tg, like serum autoantibodies. Most have high affinities for Tg (dissociation constant 10^{-10} to 10^{-9} M). Their light (L) chain Ig genes are not unusual, but four of the five heavy (H) chain genes are new. Moreover, one H chain belongs to the small VH2 family, not previously reported for autoantibodies to Tg or thyroid peroxidase. The TgAbs inhibit the binding to Tg of the thyroid donor's serum autoantibodies, indicating epitopic overlap. Competition analysis (surface plasmon resonance) shows that the TgAbs recognize overlapping epitopes in an immunodominant region on the Tg dimer (~660 kDa). Two major and several minor epitopic regions were defined, each associated with a particular H + L chain combination. In conclusion, our TgAb panel provides novel information regarding the repertoire of H chain genes encoding human TgAbs as well as the relationship between the H chains and the epitopes recognized on this major thyroid autoantigen. (*J Clin Endocrinol Metab* 89: 5116–5123, 2004)

THYROGLOBULIN AUTOANTIBODIES (TgAbs) are characteristic of autoimmune thyroid disease and may be present at high concentrations (milligrams per milliliter) in serum (1). These autoantibodies are also found in some clinically euthyroid individuals (*e.g.* Ref. 2), usually at lower concentrations than in patients with clinical disease. The pathogenetic role of TgAbs is unknown. On the one hand, TgAbs may be epiphenomena because, unlike autoantibodies to thyroid peroxidase (TPO), they do not fix complement (3). On the other hand, TgAbs could play a role in antibody-dependent cell-mediated cytotoxicity. An issue on which most clinical thyroidologists agree is that TgAbs interfere in the assay for thyroglobulin (Tg) a marker for metastasis in thyroid carcinoma. However, increasing evidence indicates that TgAbs may be used as surrogates for Tg (4, 5).

Human monoclonal TgAbs have been isolated from patients with autoimmune thyroid disease by a variety of approaches including Epstein-Barr virus immortalization (6) and cell fusion (7) as well as from combinatorial Ig gene libraries (8–10). Importantly, the monoclonal TgAbs resemble patient's serum TgAb in terms of affinities and epitopic recognition (6, 7, 10, 11). However, only a small number of IgG class human monoclonal TgAbs had been isolated and characterized, and their heavy (H) chains were derived from a limited number of Ig genes (reviewed in Ref. 12).

Recently we addressed the possibility of cloning bispecific autoantibodies that cross-react with Tg and TPO. Evidence for this intriguing idea had been obtained from serum studies (*e.g.* Ref. 13–15). As observed for the serum studies, we found enrichment for Tg-PO binding phage after alternating panning from Tg to TPO of a combinatorial Ig gene library. However, analyzing individual clones, the bispecific phage pool comprised phage specific for Tg and TPO as well as phage that bound nonspecifically to numerous unrelated antigens (16). As part of this investigation, we isolated a panel of monoclonal TgAbs. In the present study, we focused on these new TgAbs and now provide a detailed characterization of their diverse Ig genes, their immunological properties, and the epitopes they recognize on Tg.

Materials and Methods

Cloning and expressing TgAbs from an Ig gene library

Construction, screening, and isolation of a panel of TgAbs from a thyroid-derived Ig gene library was described previously (16). In brief, thyroid tissue from a patient (coded AF) with high TgAb titers (>1: 30,000 by ELISA; see below) was used to construct an IgG/κ library in the pComb3H phage display vector (17) (provided by Dr. Carlos Barbas, Scripps Research Institute, San Diego, CA). We performed three rounds of library panning on human Tg. The Tg was prepared from Graves' glands by ammonium sulfate (30% final concentration) precipitation of the tissue homogenate 100,000 × *g* supernatant, followed by diethyl aminoethyl-Sephadex chromatography and Sephacryl S-200 gel filtration (18). In later experiments, human Tg was purchased from Calbiochem (San Diego, CA). Different colonies (264) from the final round were tested to identify Tg-binding clones by ELISA. From 64 colonies, 15 clones with different *Bst*NI restriction patterns were selected for automated nucleotide sequencing of double-stranded plasmid (Cedars-Sinai Research Institute core facility). Ig H and light (L) chain genes were classified according to the V Base sequence directory (19).

Human monoclonal TgAbs were expressed as soluble proteins [fragment antigen binding (Fab)] without removal of the cpIII gene from the pComb3H plasmid, as previously described (20): plasmid-bearing XL1 Blue cells were cultured (37 C, 6–7 h), and protein synthesis was induced

Abbreviations: Fab, Fragment antigen binding; H, heavy chain; L, light chain; RU, resonance unit; Tg, thyroglobulin; TgAb, Tg autoantibody; TPO, thyroid peroxidase.

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

by overnight incubation (30 C) with isopropyl-thio-galacto-pyranoside (1 mM; Calbiochem). TgAb activity was assayed in the supernatants.

Thyroid tissue and sera in this study were obtained and studied in accordance with institutional guidelines.

ELISA for TgAbs

Binding of serum TgAbs or TgAb Fab was performed using wells coated with Tg (400 ng/ml; Calbiochem) and detection with murine monoclonal antihuman kappa (QE11, Recognition Sciences, Birmingham, UK) followed by affinity-purified antimouse IgG-conjugated to horseradish peroxidase (Sigma Chemical Co., St. Louis, MO). The substrate was o-phenylene diamine + H_2O_2 and ODs were read at 490 nm. Where indicated, Tg was denatured using dithiothreitol followed by iodoacetamide (Sigma) as reported previously for TPO (21). TgAb affinities were estimated by incubating TgAb-containing supernatants on Tg-coated ELISA wells in the absence or presence of soluble Tg (10^{-7} to 10^{-12} M). In these affinity studies, TgAbs were diluted to obtain ODs of approximately 0.80 in the absence of soluble Tg. As a negative control, we used TPO Fab 55 that does not bind Tg (16).

TgAb inhibition of serum autoantibody binding to Tg

Serum autoantibody binding of Tg was determined in the absence and presence of TgAb Fab by ELISA. Sera (diluted to provide OD ~1.0) were incubated alone and together with an equal volume (50 μl/well) of TgAb Fab. Horseradish peroxidase-conjugated antihuman IgG-Fc (Sigma), an antibody that does not bind to Fab, was used to detect serum autoantibodies binding. The inhibition by TgAbs was calculated as the percentage of serum binding plus TgAb/serum binding without TgAbs. Nonspecific Tg binding of a TgAb-negative serum (~15%) was subtracted in calculating percent inhibition.

Surface plasmon resonance evaluation of Tg epitopes recognized by TgAb

Measurements were made using a Biacore X (Piscataway, NJ). Carboxy-methylated dextran sensor chips (CM5, Biacore) were covalently coupled with human Tg (Calbiochem) as follows: after activation with 50% 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (amine-coupling kit, Biacore) and 50% *N*-hydroxysuccinimide (amine-coupling kit, Biacore), Tg was injected [35 μl, 5 μg/ml in 10 mM Na acetate (pH 4.8), Biacore coupling buffer 3] and the reaction stopped with ethanolamine HCl (amine-coupling kit, Biacore). At this stage, an increase of 2000 resonance units (RUs) was detected, compared with baseline. Specific TgAb binding to Tg was calculated by subtracting the nonspecific signal obtained in the reference flow cell from the signal in the flow cell cross-linked with Tg.

Epitopic competition by individual TgAbs was determined by saturating the Tg binding sites with the inhibitor TgAb-1 and later injecting a second TgAb-2, the inhibited autoantibody. For these assays, the inhibitor (TgAb-1) was diluted in Buffer HBS-EP [0.01 M HEPES (pH 7.4), 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P200; Biacore; 1:2 or 1:4, 35 μl final volume] and injected (at 5 μl/min) two or more times until no further binding was detectable. Subsequently the inhibited TgAb (diluted as reported above) was injected once to determine the increase in RUs. The effect of the inhibitor TgAb was calculated as follows:

$$\frac{\text{RU by TgAb-2 in the presence of TgAb-1}}{\text{RU by TgAb-2 in the absence of TgAb-1}} \times 100$$

After each cycle of binding, Tg on the chip was regenerated by injecting 20 μl of 10 mM glycine HCl (pH 2) (regeneration solution, Biacore) containing 200 mM NaCl.

Results

Ig genes encoding TgAb

We isolated a large number (119) of human monoclonal autoantibodies to Tg. However, based on the experience of others for Tg antibodies isolated from phage display libraries (10), many were likely to have very similar H and L chain genes and Tg binding characteristics. To avoid characterizing replicate autoantibodies, we screened 64 TgAbs by restriction digestion (see *Materials and Methods*) of their Ig genes. Fifteen TgAbs had unique *Bst*N1 restriction enzyme profiles and their nucleotide sequences were determined. The derived amino acid sequences of the H and/or L chain variable regions of some members of the TgAb panel were similar, although nonidentical. However, among the 15 Tg-binding clones, seven were encoded by distinct combinations of the variable regions of their H and/or L chain genes. Incidentally, some abbreviated information was previously provided for comparison with the encoding TPOAb and potential TgPOAb (16).

For the purpose of grouping the TgAbs, we provide information on the germline VH and VL genes to which the new TgAb panel are related. The H chains are derived from five different genes (Table 1): a VH1 gene [locus 1–03; V1–3b/DP-25; (22)]; a VH2 gene [locus 2–05; VII-5b+ (22)]; and three VH3 genes [locus 3–23, VH26/DP-47 (23); 9–1+/DP38 (24, 25) and locus 3–30, BHGH1 (26)]. Three related H chains are encoded by the gene BHGH1, whereas each of the other genes encoded a single H chain. The CDR3 region varies in length from 7 to 18 amino acids and the joining regions include JH3, JH4, and JH6. Incidentally, the VH2 clone (TgAb 18) lacked the entire FR1, CDR1,and part of the FR2 regions. Sequencing with internal primers showed that the H chain was cloned into the vector by a natural *Xho*I restriction site within the FR2 region. Two L chains (VKIII family) are paired with the H chains: humkv328h5+/DPK21 (27, 28) and A27/humkv325/DPK22 (28–30). The L chain-joining regions include Jk1, Jk4, and Jk5. The V regions of the TgAb, particularly the H chains, show considerable mutation from their germline counterparts, consistent with affinity maturation. For the H chains, amino acid homologies with their germline sequences ranged from 78 to 90% and for the L chains, from

TABLE 1. Ig H and L chain genes encoding the panel of TgAb used in the present study

Family	VH gene	%[a]	VH-CDR3	JH	Family	Vk gene	%[b]	Jk	Clone (no.)
VH1	V1-3b+/DP25	87	EGYGGFE	4	VKIII	A27/humkv325	90	2	37
VH2	VII-5b+	85	AGYHRGAFDV	3	VKIII	A27/humkv325	83	1	18
VH3	VH26/DP47	90	HHAPYGDYPPDFDS	4	VKIII	humkv328h5+	95	4	6
VH3	BHGH1	86	VNAPYYSGQGLPFDV	3	VKIII	A27/humkv325	84	1	30
VH3	BHGH1	85	VNAPYYSGQGLPFDV	3	VKIII	A27/humkv325	90	5	26
VH3	BHGH1	84	NHVPYGSARFASDY	4	VKIII	humkv328h5+	96	5	15
VH3	9-1+/DP38	78	APVREVRLGAHSYYIMDV	6	VKIII	humkv328h5+	91	2	32

[a] Percentage amino acid homology with H chain germline gene.
[b] Percentage amino acid homology with L chain germline gene.

84 to 96% (Table 1). Nucleotide sequences for the VDJ and VJ regions are provided in GenBank (accession no. for TgAb 6, 15, 18, 26, 30, 32, 37 are AY365327–365333 for the H chains and AY365334–365340 for the L chains).

TgAb binding to Tg in ELISA

TgAb bound preferentially to native rather than denatured Tg, like the serum of the patient (AF) from whom the library was prepared (Fig. 1A). Moreover, binding of TgAbs was inhibited by preincubation with low concentrations of Tg for most TgAbs (Fig. 1B). Half maximal inhibition of TgAb binding was attained with 5×10^{-10} and 3×10^{-9} M Tg for TgAbs (18, 26, 30, 37, and 15). The estimated affinity was higher (dissociation constant Kd $\sim 10^{-11}$ M) for TgAb 6 and lower for TgAb 32, the latter requiring approximately 10^{-8} M Tg for 50% inhibition. Of interest, despite lacking the amino-terminal 46 amino acid residues in its H chain (see above), the antibody secreted by clone 18 bound Tg with high affinity (dissociation constant $\sim 10^{-10}$ M).

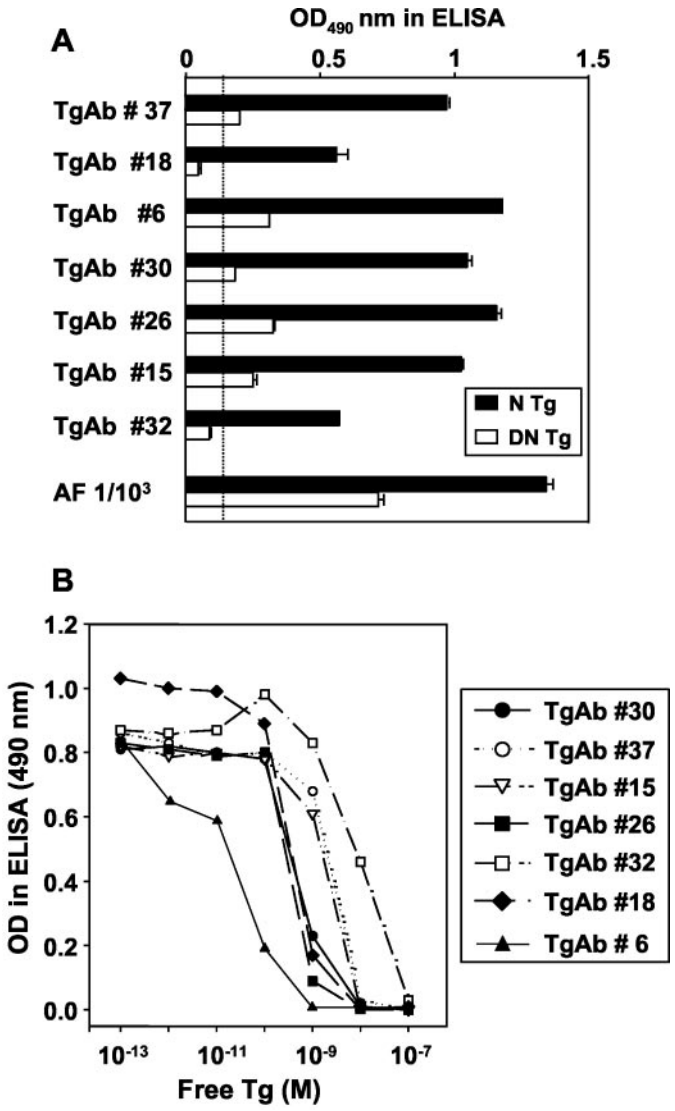

FIG. 1. Binding characteristics of the new panel of TgAb. A, TgAb recognize native Tg better than denatured Tg. TgAb binding was compared using ELISA wells coated with native (N) Tg or denatured (DN) Tg. Also included are the data for serum for patient (AF) whose thyroid tissue was used to construct the Ig gene library. Data are presented as the mean of duplicate OD readings in ELISA; *error bars* represent SEM. The *dotted line* represents the mean + 2 SD for a control antibody to TPO that does not bind to Tg. B, Soluble Tg inhibits binding to Tg-coated ELISA wells by the new panel of TgAb. Binding was measured in the absence and presence of Tg (up to 10^{-7} M). Data presented are the mean of duplicate OD readings in ELISA.

Relationship between patient AF monoclonal and serum TgAbs

Before embarking on a detailed epitopic analysis of the monoclonal TgAbs, we wished to determine whether their epitopes corresponded to autoantibodies in the serum of patient AF, whose thyroid tissue was used to prepare the Ig gene library. In addition, we included two randomly selected TgAb-positive sera. We followed an approach, first used for TPO autoantibodies (31) and later adapted for TgAbs (32), in which serum autoantibody binding to Tg-coated ELISA wells is compared in the absence and presence of TgAbs, and the results are expressed as percent inhibition.

TgAbs encoded by three H chain types, V1–3b, BHGH1, and VH26, inhibited serum binding by approximately 80% for serum X and approximately 50% for sera Y and AF (Fig. 2). For TgAbs encoded by the other two H chains, inhibition was lower and more variable: approximately 10% for serum AF, 30–40% for serum Y and either 20% (9–1+ H chain) or 60% (VII5b+ H chain) for serum X. Overall, the epitopes of five TgAbs (37, 30, 26, 15, and 6), all with high or moderate affinities for Tg (see above), overlapped to a considerable degree with serum autoantibodies in all three patients, whereas the epitopes of two TgAbs (18 and 32) appear to be less common, at least for the three patients studied. The lower affinity of TgAb 32 (but not of the high affinity TgAb 18) could be involved in its reduced ability to inhibit the binding of patients' autoantibodies to Tg.

Surface plasmon resonance analysis of TgAb epitopes

We first established that TgAbs bound to Tg cross-linked to Biacore chips. Representative examples are provided for TgAb 37 and 30 (Fig. 3, A and B) and the lack of binding for a TPO-specific Ab (Fig. 3C). The increase in resonance units after a single injection ranged from 210 to 30 RU (TgAb 37 and 18, respectively).

To map the epitopes of individual TgAbs, the Tg binding sites for an inhibitor TgAb were saturated and subsequently the binding of a second TgAb (the inhibited TgAb) was evaluated. This approach is similar to that described for epitopic mapping of TPOAb (33, 34). Although between two injections (*e.g.* TgAb 6 and 26) and six injections (TgAb 32) were required to attain saturation, the outcome was independent of TgAb concentration. The ability of TgAb 15 and 26 to compete for binding is illustrated in Fig. 3: after saturation with two injections of TgAb 15, the increase in resonance units produced by injecting TgAb 26 was very small, only 3 RU (Fig. 3D), compared with 109 RU when injected alone (Fig. 3E). Consequently, TgAb 15 inhibited the binding of TgAb 26 by 97%. Likewise, the binding of TgAb 15 alone produced 76 RU (Fig. 3D), and the signal was barely in-

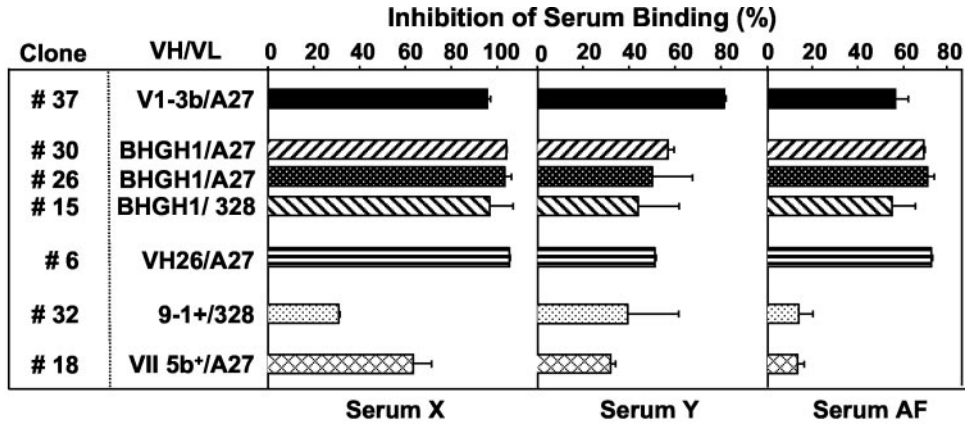

FIG. 2. Inhibition of serum autoantibody binding to Tg by the panel of TgAb. TgAbs are grouped according to the Ig genes encoding their H chains. Data are shown as the percent inhibition of serum autoantibody binding to Tg-coated ELISA wells in the presence of each TgAb. Inhibition was studied using serum from patient AF (from whose thyroid the library was prepared) and for sera from two other patients with autoimmune thyroid disease (X and Y).

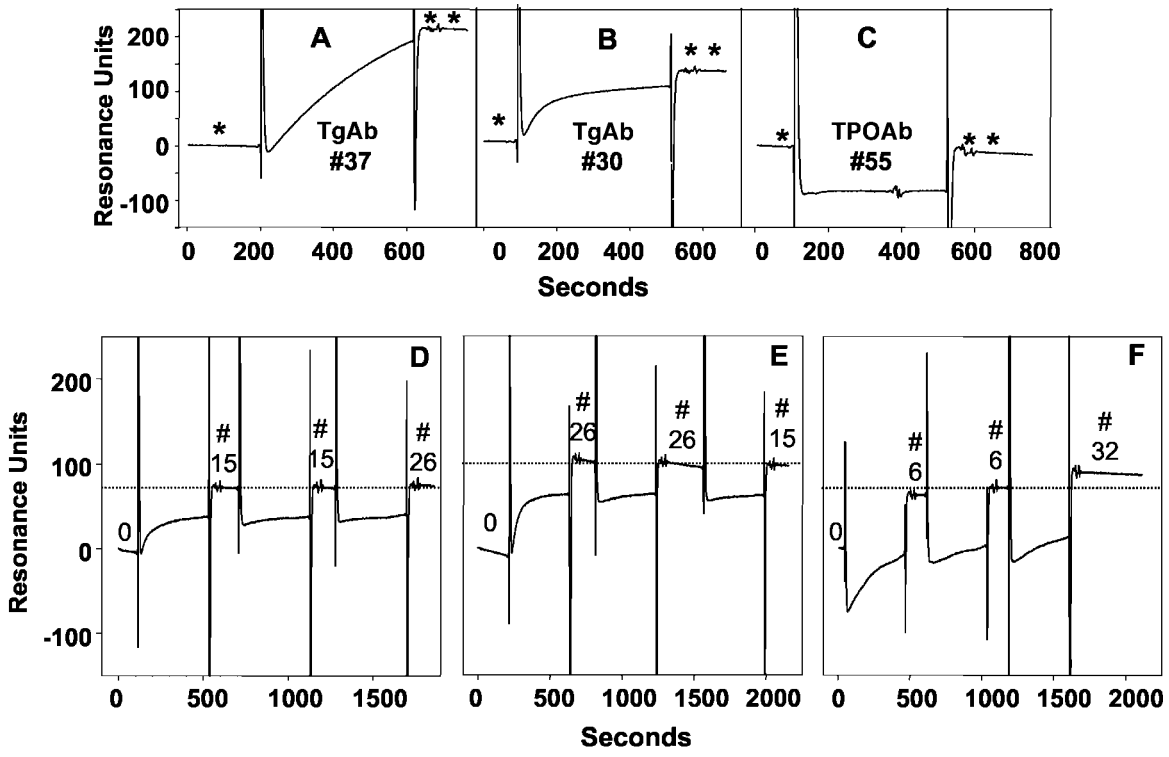

FIG. 3. Analysis of TgAb interactions with Tg evaluated by surface plasmon resonance. A–C, *upper panels*, TgAb 37 and 30, but not TPOAb 55, bind Tg cross-linked on a Biacore chip. *Asterisks* indicate the start (*) and end (**) of the injections. D–F, *lower panels*, TgAb 15 inhibits the binding of TgAb 26 to Tg, but TgAb 6 does not compete for the binding of TgAb 32. Two injections of TgAb 15 saturate its binding sites on Tg, and a subsequent injection of TgAb 26 produces an increase of only 3 RU (D). This value is much lower than that obtained when TgAb 26 is injected first (E). Similarly, after saturation with TgAb 26, injection of TgAb 15 induces an increase of only 3 RU, compared with 76 RU induced by TgAb 15 alone (E *vs.* D). In contrast, two injections of TgAb 6 saturated its binding sites on Tg, but subsequent injection of TgAb 32 increased the signal of 24 RU (F). TgAb 32 alone induced a signal of 25 RU (not shown). The *horizontal dotted line* indicates the signal at saturation achieved using the first TgAb.

creased (3 RU) by prior injection of TgAb 26, giving an inhibition of 96%. An example of TgAbs that do not compete is also shown. Two injections of TgAb 6 saturated its Tg binding sites. Subsequent injection of TgAb 32 produced an increase of 24 RU (Fig. 3D), which was similar to the signal (25 RU) given by one injection of TgAb 32 without inhibitor (not shown).

Based on 34 competition studies, the TgAb were classified into five types (A to E) (Table 2). Types A, C, D, and E each included one TgAb. Type B included three TgAb (30, 26, and 15) that are derived from the same VH germline gene but differ in terms of their D regions and/or L chains (Table 1). These B-type TgAbs inhibited each other's binding by almost 100%, regardless of their role as the inhibitor or the responder (Table 2, *lower portion*). As inhibitor, the A-type TgAbs competed strongly (75%) for binding by the B-type TgAbs and even more strongly (>95% inhibition) for binding by C-, D-, and E-type TgAb.

The reverse inhibition patterns were not necessarily the same. For example, C-, D-, and E-type TgAbs inhibited the binding of the A-type TgAbs to a lesser extent (48 to 23%). These observations suggest that the A epitope is dominant,

TABLE 2. Epitopic analysis of TgAb by surface plasmon resonance competition assays reveals five different groups

Inhibitor	% Inhibition				
	A	B	C	D	E
All groups					
A (TgAb #37)		74 (±2)[a]	95	98	96
B (TgAb #30/ 26/15)*	50 (±11)[a]		76	100 (±0)[a]	100
C (TgAb #6)	48	15		42	26
D (TgAb #32)	54	87 (±1)[a]	4		93
E (TgAb #18)	23	73	28	79	
Group B	TgAb 30	TgAb 26	TgAb 15		
TgAb #30		95	96		
TgAb #26	93		96		
TgAb #15	100	98			

Data are presented as percentage inhibition by an inhibitor TgAb.
[a] Data for TgAb 30, 26, and 15 are presented as the mean ± SE.

with C-type TgAbs, and particularly D- and E-type TgAbs, on the fringes. Similarly, the B epitope dominates the C epitope. The largest discrepancy occurred for type C-TgAb *vs.* type D-TgAb, 42% (with C-TgAb as inhibitor) *vs.* only 4% (with D-TgAb as inhibitor). The reason for these discrepancies is not fully understood. Possibilities include differences in TgAb affinity and the kinetics of reaction (different on and off rates) as well as possible subtle differences in the binding of individual antibodies to overlapping but nonidentical epitopes.

Schematic representation of TgAb epitopes

On the basis of the findings in Table 2, we propose a model for the epitopes on Tg recognized by the new TgAb panel (Fig. 4). All epitopes overlap, consistent with an immunodominant region. The A epitope appears to lie at one pole and the B epitope at the other pole. Both A and B epitopes overlap the C, D, and E epitopes. The D and E epitopes are closely associated with each other but have minimal overlap with the C epitope.

Overall, TgAb encoded by the VH genes V1–3b and BHBH1 define two major epitopic regions, A and B, respectively. The C region (VH26 gene) is closely associated with the A region and to a lesser extent with the B region. The minor epitopes (D and E) involve the H chain genes VII-5B+ and 9–1, respectively. The outcome of the surface plasmon resonance analysis is consistent with our findings for serum autoantibodies (Fig. 2), namely strong inhibition by TgAb with V1–3b, BHB1, and VH26 H chains (A, B, and C regions) and weaker or variable inhibition by TgAb with VII-5B+ and 9–1+ H chains (D and E region).

Discussion

The information in this study extends the database of information about human monoclonal TgAbs isolated to date. To avoid characterizing replicate TgAbs isolated from a thyroid-derived combinatorial Ig gene library, we focused on seven autoantibodies reflecting the diversity in the repertoire based on their unique combinations of H and L chain genes. The TgAb preferentially recognize native (rather than denatured) Tg with estimated dissociation constants between 10^{-10} and 10^{-9} M, and they inhibit the binding to Tg

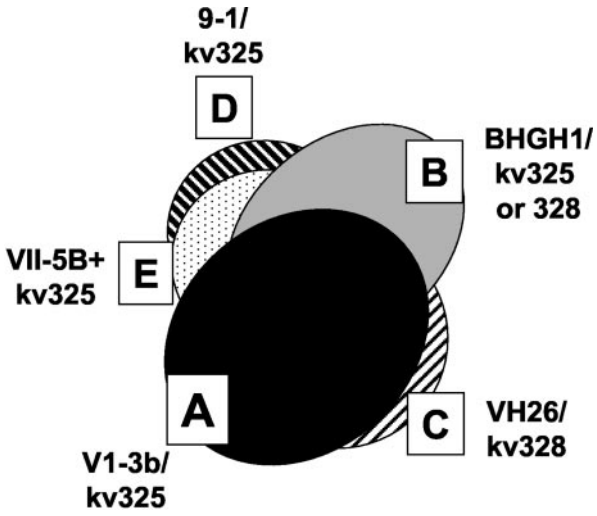

FIG. 4. Schematic representation of the epitopes recognized by the panel of TgAb in relation to the H chain genes. The TgAb (groups A–E) bind to a set of closely overlapping epitopes, compatible with the two major epitopic regions on Tg.

of autoantibodies in serum from the donor of the thyroid gland and in two other unrelated patients. Moreover, as shown previously, they bind specifically to Tg and not to other antigens or autoantigens (16). Overall, the properties of the new TgAb panel are similar to those of other human monoclonal TgAbs derived from patients with autoimmune thyroid disease (6, 7, 10, 11).

The novel information provided by our TgAb panel relates to the expansion of the H chain gene repertoire of TgAb and to the relationship between genes and Tg epitopes. Four H chain genes encoding our TgAb belong to the large VH1 and VH3 families (like most previous monoclonal TgAbs), but one belongs to the small VH2 family. More importantly, four of five VH genes encoding the new TgAbs had not previously been described for TgAbs (16). The V1–3b gene, although new for TgAb, is used by numerous TPO autoantibodies (reviewed in Ref. 35). However, neither 9–1+ nor BHGH1 genes are used by TPO autoantibodies. Remarkably, the VH26 gene is used by several TgAbs cloned in the same (11) or different laboratories (10 and present data). In addition, these VH26-encoded TgAbs were isolated in different ways using lymphocytes from different tissues: Epstein Barr virus transformation of peripheral blood lymphocytes followed by cell fusion (6); direct fusion of thyroid infiltrating lymphocytes (7); and Ig gene combinatorial libraries generated from thyroid tissue (8 and present data) or thyroid-draining lymph nodes (10) (summarized in Table 3). Finally, some human TgAbs have long CDR3 regions, 14 amino acids (10), or 22 amino acids (11) in length, compared with approximately nine amino acids for most antibodies. Our TgAb panel included short and long CDR3 regions (from seven to 18 amino acids; Table 3), suggesting that a long CDR3 is not a prerequisite for autoantibody binding to Tg.

It has long been known that, compared with multiple epitopes recognized by immunized rabbits (*e.g.* Ref. 36, 37) or mice (38, 39), human serum autoantibodies recognize a restricted range of conformational epitopes on the large (~660 kDa) Tg molecule. Extensive investigations performed

TABLE 3. Ig H and L chain genes encoding TgAb autoantibodies

VH family	VH gene	VL family	VL gene	Approach	Source	Ref.
Tg antibodies with Kappa L chains						
VH1	HG3	VKIII	A27/humkv325	Combinatorial lib	Thyroid	11
	V1-3b+/DP25	**VKIII**	**A27/humkv325**	Combinatorial lib	Thyroid	Present study
VH2	**VII-5b+**	**VKIII**	**A27/humkv325**	Combinatorial lib	Thyroid	Present study
VH3	VH26/DP47*	VKI	O2/O12	Fusion	Thyroid	11
	VH26/DP47*	VKIII	Vg	Fusion	Thyroid	11
	VH26/DP47*	VKI	L12a	Combinatorial lib	Lymph node	10
		VKI	O12			
		VKIII	A27/humkv325			
		VKIII	Vg			
		VKIII	humkv328h5+**			
	VH26/DP47[a]	**VKIII**	**humkv328**	Combinatorial lib	Thyroid	Present study
	BHGH1	**VKIII**	**A27/humkv325**	Combinatorial lib	Thyroid	Present study
		VKIII	**humkv328h5+**[b]			
	9-1+/DP38	**VKIII**	**humkv328h5+**[b]	Combinatorial lib	Thyroid	Present study
Tg antibodies with Lambda L chains						
VH3	VH26	VL-?	IGLV12	Fusion	Thyroid	10
VH4	DP65	VL-I	Iv117	EBV & fusion	Blood L's	11

TgAb obtained in the search for TgPOAb (16) and described herein are indicated in **bold**. lib, Library; EBV, Epstein Barr virus; Blood L's, peripheral blood lymphocytes.

[a] Also known as V3–23.

[b] Also known as DPK21.

with panels of mouse monoclonal TgAbs have provided important information on human TgAb binding to a variable number of clustered epitopes (reviewed in Refs. 40, 41). More recently, competition studies with human monoclonals *vs.* either serum autoantibodies (11) or mouse monoclonals (32) indicated that human autoantibodies recognize two major epitopic regions on Tg. Our present data are consistent with these findings. Epitope mapping of the new TgAbs establishes a group of overlapping epitopes, with two major regions (Fig. 4). TgAbs encoded by the VH26 gene interact with a major Tg epitopic region in our panel as in previous studies (11, 32). In contrast, the epitopes of two new TgAbs overlapped minimally with the epitopes of other TgAb and were less well recognized by autoantibodies from three patients. It is possible that this lesser degree of overlap is related to their unusual H chain genes (9–1+ and VII-5b+) and may be the result of antibody epitopic spreading. Incidentally, it should be appreciated that the A and B domains for TgAb epitopes (Fig. 4) are unrelated to the domains we and others have described (using the same letters) for the autoantibody immunodominant region on TPO (*e.g.* Refs. 42, 43).

A point of interest arising from our data regards the properties of antibodies encoded by H chains paired with different L chains. In an earlier study, a set of closely related H chains was combined promiscuously with 5 κ- and 9 λ-chains (10). Competition assays with murine monoclonal Tg antibodies revealed that, despite subtle differences relating to the L chain types, these human TgAbs bound to overlapping epitopes (32). Among the seven representative TgAbs that we characterized in detail, only one H chain was promiscuously paired with several L chains: H chains derived from the BHG1 gene with the same or different CDR3 regions combined with two different L chains (Table 1). Nevertheless, these three types of TgAbs, involving two different CDR3 regions and two L chains, all recognized the same epitopes (Fig. 4).

The Ig gene library used in this study was constructed from thyroid tissue of a high-titer TgAb-positive patient. Would the outcome for TgAb epitopes (Fig. 4) been different had we used thyroid tissue from a moderate-titer TgAb patient or another high titer TgAb patient? We think this possibility is unlikely for the following reasons. First, substantial evidence (see above) showed that TgAb in different patients bind to the same, overlapping epitopic domains on Tg. Second, in isolating and characterizing a very large panel of TPO autoantibodies from Ig gene libraries (reviewed in Refs. 35, 44–46), we learned that factors besides antibody titer play major roles. Antibody number and gene diversity depends on the intensity of the screening process as well as on the oligonucleotide primers used to amplify H and L chain gene DNA for library construction (47, 48). Third, surprisingly but most importantly, antigen manipulation or masking is required to bias the repertoire away from the autoantibodies that recognize immunodominant epitopes and toward other regions of TPO (44, 45). Although neither antibody titer nor a different patient is likely to have produced major differences, the diversity of our TgAb panel may account for the presence of antibodies to both the major and minor overlapping, epitopic domains on Tg.

Competition for Tg binding between human sera and murine monoclonal antibodies revealed epitopic differences between autoimmune thyroid disease patients and euthyroid TgAb-positive individuals (reviewed in Refs. 40, 41). Likewise, two human monoclonal TgAbs competed differently for Tg binding with polyclonal TgAbs in patients' sera *vs.* normal individuals (11). Moreover, TgAbs in Graves' and Hashimoto patients discriminate between different tryptic fragments of Tg (49). These observations contrast with the lack of epitopic differences we observed in euthyroid *vs.* hypothyroid TPO autoantibody patients (50, 51). However, all previous TgAb studies were cross-sectional and involved relatively small groups of individuals (52). Our novel TgAb panel could provide valuable tools for future large scale longitudinal investigations of TgAb epitopes to clarify the role of TgAb in health and disease.

In conclusion, we have characterized a new panel of mono-

clonal Tg autoantibodies from a thyroid-derived Ig gene combinatorial library from a patient with autoimmune thyroid disease. The new TgAb panel provides novel information regarding the repertoire of H chain genes encoding human Tg autoantibodies and the relationship between the Ig H chains and the epitopes recognized on this major thyroid autoantigen.

Acknowledgments

We thank Dr. Boris Catz (Los Angeles, CA) for his generous support and Dr. Carlos Barbos (Scripps Research Institute, San Diego, CA) for providing us with the phage display vector pComb3H.

Received December 17, 2003. Accepted April 26, 2004.

Publication of this article was delayed at the request of the authors. Address all correspondence and requests for reprints to: Sandra M. McLachlan, Cedars-Sinai Medical Center, 8700 Beverly Boulevard, Suite B-131, Los Angeles, California 90048. E-mail: Sandra.McLachlan@cshs.org.

This work was supported by National Institutes of Health Grant DK 36182 and a Winnick Family Clinical Research Scholar Award.

References

1. **Beever K, Bradbury J, Phillips D, McLachlan SM, Pegg C, Goral A, Overbeck W, Feifel G, Rees Smith B** 1989 Highly sensitive assays of autoantibodies to thyroglobulin and to thyroid peroxidase. Clin Chem 35:1949–1954
2. **Prentice LM, Phillips DIW, Sarsero D, Beever K, McLachlan SM, Rees Smith B** 1990 Geographical distribution of subclinical autoimmune thyroid disease in Britain: a study using highly sensitive direct assays for autoantibodies to thyroglobulin and thyroid peroxidase. Acta Endocrinol (Copenh) 123:493–498
3. **Belyavin G, Trotter WR** 1959 Investigations of thyroid antigens reacting with Hashimoto sera. Evidence for an antigen other than thyroglobulin. Lancet i:648–652
4. **Spencer CA, Takeuchi M, Kazarosyan M, Wang CC, Guttler RB, Singer PA, Fatemi S, LoPresti JS, Nicoloff JT** 1998 Serum thyroglobulin autoantibodies: prevalence, influence on serum thyroglobulin measurement, and prognostic significance in patients with differentiated thyroid carcinoma. J Clin Endocrinol Metab 83:1121–1127
5. **Chiovato L, Latrofa F, Braverman LE, Pacini F, Capezzone M, Masserini L, Grasso L, Pinchera A** 2003 Disappearance of humoral thyroid autoimmunity after complete removal of thyroid antigens. Ann Intern Med 139:346–351
6. **Petersen VB, Fukuma N, McLachlan SM, Bradbury J, Beever K, Devey ME, Bleasdale-Barr KM, Phillips DIW, Baylis P, Ryley H, Rees Smith B** 1989 A human-mouse hybridoma which secretes monoclonal thyroglobulin autoantibody with properties similar to those of the donor patient's serum autoantibody. Autoimmunity 4:89–102
7. **Fukuma N, Petersen VB, McLachlan SM, Pegg CAS, Rees Smith B** 1991 Human monoclonal thyroglobulin autoantibodies of high affinity. I. Production, characterisation and interaction with murine monoclonal thyroglobulin antibodies. Autoimmunity 10:291–295
8. **Hexham JM, Persson MAA, Pegg C, Burton D, Furmaniak J, Rees Smith B** 1991 Cloning and expression of human thyroglobulin autoantibody. Autoimmunity 11:69–70
9. **Hexham JM, Furmaniak J, Pegg C, Burton DR, Smith BR** 1992 Cloning of a human autoimmune response: preparation and sequencing of a human anti-thyroglobulin autoantibody using a combinatorial approach. Autoimmunity 12:135–141
10. **McIntosh RS, Asghar MS, Watson PF, Kemp EH, Weetman AP** 1996 Cloning and analysis of IgG κ and IgG λ anti-thyroglobulin autoantibodies from a patient with Hashimoto's thyroiditis. Evidence for *in vivo* antigen-driven repertoire selection. J Immunol 157:927–935
11. **Prentice L, Kiso Y, Fukuma N, Horimoto M, Petersen V, Grennan F, Pegg C, Furmaniak J, Smith BR** 1995 Monoclonal thyroglobulin autoantibodies: variable region analysis and epitope recognition. J Clin Endocrinol Metab 80:977–986
12. **McIntosh RS, Weetman AP** 1997 Molecular analysis of the antibody response to thyroglobulin and thyroid peroxidase. Thyroid 7:471–487
13. **Kohno Y, Naito N, Hiyama Y, Shimojo N, Suzuki N, Tarutani O, Niimi H, Nakajima H, Hosoya T** 1988 Thyroglobulin and thyroid peroxidase share common epitopes recognized by autoantibodies in patients with chronic autoimmune thyroiditis. J Clin Endocrinol Metab 67:899–907
14. **Ruf J, Ferrand M, Durand-Gorde JM, Carayon P** 1992 Immunopurification and characterization of thyroid autoantibodies with dual specificity for thyroglobulin and thyroperoxidase. Autoimmunity 11:179–188
15. **Estienne V, Duthoit C, Costanzo VD, Lejeune PJ, Rotondi M, Kornfeld S, Finke R, Lazarus JH, Feldt-Rasmussen U, Franke WG, Smyth P, D'Herbomez M, Conte-Devolx B, Persani L, Carella C, Jourdain JR, Izembart M, Toubert ME, Pinchera A, Weetman A, Sapin R, Carayon P, Ruf J** 1999 Multicenter study on TGPO autoantibody prevalence in various thyroid and non-thyroid diseases; relationships with thyroglobulin and thyroperoxidase autoantibody parameters. Eur J Endocrinol 141:563–569
16. **Latrofa F, Pichurin P, Guo J, Rapoport B, McLachlan SM** 2003 Thyroglobulin-thyroperoxidase autoantibodies are polyreactive, not bispecific: analysis using human monoclonal autoantibodies. J Clin Endocrinol Metab 88:371–378
17. **Barbas III CF, Wagner J** 1995 Synthetic human antibodies: selecting and evolving functional proteins. Methods Comp Methods Enzymol 8:94–103
18. **Rapoport B, Seto P, Magnusson RP** 1984 Immunoprecipitation of radiolabeled human thyroid cell proteins by serum from patients with autoimmune thyroid disease. Endocrinology 115:2137–2144
19. **Tomlinson IMS, Williams SC, Corbett SJ, Cox JBL, Winter G** 1998 V BASE sequence directory. Cambridge, UK: Center for Protein Engineering, Medical Research Council Center; cpeweb@mrc_lmb.cam.ac.uk
20. **Portolano S, Prummel MF, Rapoport B, McLachlan SM** 1995 Molecular cloning and characterization of human thyroid peroxidase autoantibodies of λ light chain type. Mol Immunol 32:1157–1169
21. **Portolano S, Chazenbalk GD, Seto P, Hutchison JS, Rapoport B, McLachlan SM** 1992 Recognition by recombinant autoimmune thyroid disease-derived Fab fragments of a dominant conformational epitope on human thyroid peroxidase. J Clin Invest 90:720–726
22. **Shin EK, Matsuda F, Nagaoka H, Fukita Y, Imai T, Yokoyama K, Soeda E, Honjo T** 1991 Physical map of the 3′ region of the human immunoglobulin heavy chain locus: clustering of autoantibody-related variable segments in one haplotype. EMBO J 10:3641–3645
23. **Chen PP, Liu MF, Glass CA, Sinha S, Kipps TJ, Carson DA** 1989 Characterization of two immunoglobulin VH genes that are homologous to human rheumatoid factors. Arthritis Rheum 32:72–76
24. **Berman JE, Mellis SJ, Pollock R, Smith CL, Suh H, Heinke B, Kowal C, Surti U, Chess L, Cantor CR, Alt FW** 1988 Content and organization of the human Ig VH locus: definition of three new VH families and linkage to the Ig CH locus. EMBO J 7:727–738
25. **Tomlinson IM, Walter G, Marks JD, Llewelyn MB, Winter G** 1992 The repertoire of human germline VH sequences reveals about fifty groups of VH segments with different hypervariable loops. J Mol Biol 227:776–798
26. **Harmer IJ, Loizou S, Thompson KM, So AK, Walport MJ, Mackworth-Young C** 1995 A human monoclonal antiphospholipid antibody that is representative of serum antibodies and is germline encoded. Arthritis Rheum 38:1068–1076
27. **Liu MF, Robbins DL, Crowley JJ, Sinha S, Kozin F, Kipps TJ, Carson DA, Chen PP** 1989 Characterization of four homologous L chain variable region genes that are related to 6B6.6 idiotype positive human rheumatoid factor L chains. J Immunol 142:688–694
28. **Cox JPL, Tomlinson IM, Winter G** 1994 A directory of human germ-line Vk segments reveals a strong bias in their usage. Eur J Immunol 24:827–836
29. **Radoux V, Chen PP, Sorge JA, Carson DA** 1986 A conserved human germline V-κ gene directly encodes the rheumatoid factor light chains. J Exp Med 164:2119–2124
30. **Straubinger B, Huber E, Lorenz W, Osterholzer E, Pargent W, Pech M, Pohlenz HD, Zimmer FJ, Zachau HG** 1988 The human VK locus: characterization of a duplicated region encoding 28 different immunoglobulin genes. J Mol Biol 199:23–34
31. **Czarnocka B, Janota-Bzowski M, McIntosh RS, Asghar MS, Watson PF, Kemp EH, Carayon P, Weetman AP** 1997 Immunoglobulin G κ anti-thyroid peroxidase antibodies in Hashimoto's thyroiditis: epitope mapping analysis. J Clin Endocrinol Metab 82:2639–2644
32. **Estienne V, McIntosh RS, Ruf J, Asghar MS, Watson PF, Carayon P, Weetman AP** 1998 Comparative mapping of cloned human and murine antithyroglobulin antibodies: recognition by human antibodies of an immunodominant region. Thyroid 8:643–646
33. **Chapal N, Peraldi-Roux S, Bresson D, Pugniere M, Mani JC, Granier C, Baldet L, Guerrier B, Pau B, Bouanani M** 2000 Human anti-thyroid peroxidase single-chain fragment variable of Ig isolated from a combinatorial library assembled in-cell: insights into the *in vivo* situation. J Immunol 164:4162–4169
34. **Chapal N, Chardes T, Bresson D, Pugniere M, Mani JC, Pau B, Bouanani M, Peraldi-Roux S** 2001 Thyroid peroxidase autoantibodies obtained from random single chain FV libraries contain the same heavy/light chain combinations as occur *in vivo*. Endocrinology 142:4740–4750
35. **McLachlan SM, Rapoport B** 2000 Autoimmune response to the thyroid in humans: thyroid peroxidase—the common autoantigenic denominator. Int Rev Immunol 19:587–618
36. **Roitt IM, Campbell PN, Doniach D** 1958 The nature of the thyroid autoantibodies present in patients with Hashimoto's thyroiditis (lymphadenoid goitre). Biochem J 69:248–257
37. **Nye L, Pontes de Carvalho LC, Roitt IM** 1980 Restrictions in the response to autologous thyroglobulin in the human. Clin Exp Immunol 41:252–263
38. **Piechaczyk M, Bouanani M, Salhi SL, Baldet L, Bastide M, Pau B, Bastide JM** 1987 Antigenic domains on the human thyroglobulin molecule recognized by autoantibodies in patients' sera and by natural autoantibodies isolated from the sera of healthy subjects. Clin Immunol Immunopathol 45:114–121
39. **Ruf J, Carayon P, Lissitzky S** 1985 Various expressions of a unique anti-human

thyroglobulin antibody repertoire in normal state and autoimmune disease. Eur J Immunol 15:268–272
40. **Kuppers RC, Bresler HS, Burek CL, Gleason SL, Rose NR** 1992 Immunodominant determinants of thyroglobulin associated with autoimmune thyroiditis. In: Bona CA, Kaushik AK, eds. Molecular immunobiology of self-reactivity. New York: Dekker; 247–284
41. **Tomer Y** 1997 Anti-thyroglobulin autoantibodies in autoimmune thyroid diseases: cross-reactive or pathogenic? Clin Immunol Immunopathol 82:3–11
42. **Chazenbalk GD, Portolano S, Russo D, Hutchison JS, Rapoport B, McLachlan SM** 1993 Human organ-specific autoimmune disease: molecular cloning and expression of an autoantibody gene repertoire for a major autoantigen reveals an antigenic dominant region and restricted immunoglobulin gene usage in the target organ. J Clin Invest 92:62–74
43. **Czarnocka B, Pastuszko D, Carayon P, Ruf J, Gardas A** 1996 Majority of thyroid peroxidase in patients with autoimmune thyroid disease are directed to a single TPO domain. Autoimmunity 23:145–154
44. **Guo J, Wang Y, Jaume JC, Rapoport B, McLachlan SM** 1999 Rarity of autoantibodies to a major autoantigen, thyroid peroxidase, that interact with denatured antigen or with epitopes outside the immunodominant region. Clin Exp Immunol 117:19–29
45. **Pichurin P, Guo J, Yan X, Rapoport B, McLachlan SM** 2001 Human monoclonal autoantibodies to B-cell epitopes outside the thyroid peroxidase autoantibody immunodominant region. Thyroid 11:301–313
46. **Pichurin PN, Guo J, Estienne V, Carayon P, Ruf J, Rapoport B, McLachlan SM** 2002 Evidence that the complement control protein-epidermal growth factor-like domain of thyroid peroxidase lies on the fringe of the immunodominant region recognized by autoantibodies. Thyroid 12:1085–1095
47. **Jaume JC, Guo J, Rapoport B, McLachlan SM** 1997 The epitopic "fingerprint" of thyroid peroxidase-specific Fab isolated from a patient's thyroid gland by the combinatorial library approach resembles that of autoantibodies in the donor's serum. Clin Immunol Immunopathol 84:150–157
48. **Jaume JC, Portolano S, Rapoport B, McLachlan SM** 1996 Influence of the light chain repertoire on immunoglobulin genes encoding thyroid autoantibody Fab from combinatorial libraries. Autoimmunity 24:11–23
49. **Saboori AM, Caturegli P, Rose NR, Mariotti S, Pinchera A, Burek CL** 1994 Tryptic peptides of human thyroglobulin: II. Immunoreactivity with sera from patients with thyroid diseases. Clin Exp Immunol 98:459–463
50. **Jaume JC, Costante G, Nishikawa T, Phillips DIW, Rapoport B, McLachlan SM** 1995 Thyroid peroxidase autoantibody fingerprints in hypothyroid and euthyroid individuals. I. Cross-sectional study in elderly women. J Clin Endocrinol Metab 80:994–999
51. **Jaume JC, Parkes AB, Lazarus JH, Hall R, Costante G, McLachlan SM, Rapoport B** 1995 Thyroid peroxidase autoantibody fingerprints. II. A longitudinal study in postpartum thyroiditis. J Clin Endocrinol Metab 80:1000–1005
52. **Caturegli P, Mariotti S, Kuppers RC, Burek CL, Pinchera A, Rose NR** 1994 Epitopes on thyroglobulin: a study of patients with thyroid disease. Autoimmunity 18:41–49

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.