0013-7227/03/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 88(1):371–378

doi: 10.1210/jc.2002-021073

Thyroglobulin-Thyroperoxidase Autoantibodies Are Polyreactive, Not Bispecific: Analysis Using Human Monoclonal Autoantibodies

FRANCESCO LATROFA, PAVEL PICHURIN, JIN GUO, BASIL RAPOPORT, AND SANDRA M. MCLACHLAN

Autoimmune Disease Unit, Cedars-Sinai Research Institute and the University of California, Los Angeles School of Medicine, Los Angeles, California 90048

Autoantibodies (Ab) to thyroglobulin (Tg) and to thyroid peroxidase (TPO) are reported to share common epitopes, and an assay for bispecific TgPOAb has been developed that may distinguish between different clinical presentations of thyroid autoimmunity. We sought to clone TgPOAb from an Ig gene combinatorial library constructed from B cells infiltrating the thyroid of a patient with TgPOAb. As described for isolating serum TgPOAb, we panned the phage display library by alternating from Tg- to TPO-coated ELISA wells. After panning, the library was enriched for TgPO-binding phage. Of 526 clones tested for expressed Ab, most were negative; 3 clones were specific for Tg, and 5 clones specifically recognized TPO. Antibody from a single clone, encoded by a non-Tg, non-TPO Ig heavy chain gene, bound both Tg and TPO (TgPO activity). However, this antibody also bound equally well to nonthyroid antigens. In conclusion, enrichment for Tg- and TPO-binding phage was largely attributable to phage specific for either Tg or TPO. This finding, albeit from a single patient, questions previous observations of serum TgPOAb prepared by affinity chromatography. Combined with the isolation of a polyreactive monoclonal antibody, our data provide powerful evidence against shared, cross-reactive epitopes on 2 major thyroid autoantigens. (*J Clin Endocrinol Metab* 88: 371–378, 2003)

AUTOANTIBODIES (Ab) to thyroglobulin (Tg) and thyroid peroxidase (TPO) are characteristic serum markers of thyroid autoimmunity in humans. Moreover, Ab to both autoantigens are frequently present in the same patient. One explanation for their concurrence is that they arise independently in response to the release of their respective autoantigens after thyroid cell damage. An intriguing, alternative explanation postulates a role for cross-reactivity in the autoimmune response to Tg and TPO (1). The latter hypothesis could provide important insights into the origins of thyroid autoimmunity.

From the perspective of the immune system, cross-reactivity between different antigens is readily explicable at the level of the T lymphocyte, the cell type necessary to provide help to B cells for antibody production. T lymphocytes recognize short, linear peptides, approximately 10–20 amino acids long. Detailed comparative analysis revealed an 8-amino-acid region in human TPO (amino acids 119–126), which includes 6 identical and 2 conserved residues, when compared with the sequence of human Tg (amino acids 2763–2770) (2). Remarkably, this region of homology conformed to an algorithm for a T cell epitope. Support for a T cell epitope shared by Tg and TPO was obtained by immunizing mice (3). However, although of theoretical interest, there is little evidence that lymphocytes from patients with autoimmune thyroid disease recognize this TgPO T cell epitope (reviewed in Ref. 4).

On the other hand, Tg and TPO may share common Ab epitopes. Accumulating evidence suggests the presence of bispecific TgPOAb in thyroid autoimmunity. For example, TgAb affinity purified from the sera of patients with chronic autoimmune thyroiditis, bound TPO; and this binding was inhibited by Tg (5). Similarly, TgPOAb were isolated from patients' IgG by sequential affinity chromatography on Tg followed by TPO and (unlike TgAb or TPOAb) bound with high affinity to both Tg and TPO (6). Most important clinically, bispecific TgPOAb are reported to distinguish between Hashimoto patients and thyroid autoantibody-positive normals, as well as between patients with thyroid autoimmunity and nonautoimmune thyroid diseases (7–9). Indeed, a 15-team multicenter study, involving 3122 individuals, established a prevalence of TgPOAb in 35–41% of patients with thyroid autoimmunity (Graves' disease and Hashimoto's thyroiditis) (10).

Experimental approaches involving the generation of monoclonal antibodies from immunized mice provide support for the TgPOAb hypothesis (11, 12). However, isolation of bispecific human monoclonal Ab, rather than monoclonals from immunized mice, would definitively establish the existence of TgPOAb in human disease. Consequently, the goal of our present study was to clone TgPOAb from a patient with such antibodies in her serum.

Abbreviations: Ab, Autoantibody (or autoantibodies); CDR3, complementarity determining region 3; Gluc, glucagon; H chain, heavy chain; JH (or JK), joining region for the H (or kappa) chain; K chain, kappa chain; KLH, keyhole limpet hemocyanin; L chain, light chain; LPO, lactoperoxidase; R-I, -II, *etc.*, round I, II, *etc.*; Tg, thyroglobulin; TPO, thyroid peroxidase; VH, variable region of H chain; V gene, variable region gene; VL (VK), variable region of L (or kappa) L chain.

Materials and Methods

Serum TgPOAb assay

We followed the approach of Ruf *et al.* (8). Human TPO was purified from supernatants of transfected Chinese hamster ovary cells (13) and labeled with ^{125}I using iodogen to a specific activity of approximately 50 μCi/μg protein (14). TPO produced in the absence of heme lacks enzymatic activity but is homogeneous on PAGE and is fully recognized

by human Ab (13). Human Tg (>96% pure) was purchased from Calbiochem (San Diego, CA). ELISA wells were coated with Tg (4 mg/ml) or TPO (1 mg/ml, see above) and incubated with patients' sera (diluted $1:10^2$, $1:10^3$, and $1:10^4$), and control serum (diluted $1:10^2$). ^{125}I-TPO (~20,000 cpm) was added to each well; and after incubation and washing, bound tracer was eluted using 1M NaOH and counted. We used this assay to test sera from patient AF (see below) and, as a TgAb negative control, from patient WR (TPOAb-positive, by ELISA, to 1:3000).

Phage display Ig gene library

We constructed an Ig gene combinatorial library from the thyroid gland of a patient (AF) with TgPOAb. Tissue was available, with Institutional Review Board approval and informed consent. The patient had been diagnosed with Graves' disease 2 yr previously and had been euthyroid on 5 mg methimazole per day. However, her thyroid continued to enlarge despite her TSH not being elevated, and subtotal thyroidectomy was performed. Preoperatively, a high titer of TgAb (1:50,000 by hemagglutination assay) suggested concomitant Hashimoto's thyroiditis. Subsequent testing of the preoperative serum, by ELISA, in our laboratory confirmed high autoantibody levels. TgAb and TPOAb were detectable in serum diluted $1:10^5$ and $1:10^4$, respectively. We focused on the kappa (k) light (L) chain repertoire, because the majority of thyroid Ab in this patient had L chains of this type.

Library (IgG1/k) construction was as previously described by us, in detail, for other thyroid tissues (for example, Ref. 15). In brief, we used the phage display vector pComb3H (16) provided by Dr. Carlos Barbas (Scripps Research Institute). Thyroid tissue mRNA was prepared using the mRNA Kit (Amersham Biosciences, Piscataway, NJ), and cDNA was reverse-transcribed (First Strand Synthesis Kit; Stratagene, La Jolla, CA). To ensure amplification by PCR of heavy (H) chains derived from all variable (V)H gene families, we used three new primers based on sequences in the V Base Directory (17) in addition to our previously described panels of VH and VL oligonucleotide primers (18, 19). Kappa-chain DNA was cloned into the Sac1 and Xba1 restriction sites of the vector, followed by introduction of the H chain DNA into the Xho1 and Spe1 sites and electroporation (21 μF, 2,500 V, 400 Ω; Gene Zapper; IBI, New Haven, CT) into electrocompetent XL1Blue cells (Stratagene). Infective phagemid were rescued with M13KO7 helper phage (Life Technologies, Inc., Rockville, MD) and precipitated with polyethylene glycol. The unamplified AF-pComb3H combinatorial library contained approximately 5×10^6 recombinant H and L chain genes.

Combinatorial library panning for monoclonal Ab

Aliquots of the AF phage display library were panned as follows (Fig. 1): 1) on Tg alone (3 rounds); 2) on TPO alone (3 rounds); and 3) on both Tg and TPO in alternating rounds [Tg, round 1(R-I); TPO, R-II; Tg, R-III; and TPO, R-IV].

Each panning round was performed on two ELISA wells (~10^{12} amplified phagemid per well; 1 h, at room temperature). ELISA wells were coated with human Tg (Calbiochem) or human TPO (see above) (both at 10 μg/well). Nonadherent phagemid were removed by washing four times. Bound phagemid were eluted (50 mM glycine, pH 2.2), neutralized with 2 M Tris (pH 10), and used to infect XL1 Blue cells. Aliquots were withdrawn for titering. Culture of the remaining cells was continued overnight at 30 C with M13K07 helper phage. After phagemid precipitation and resuspension, the panning process was repeated.

Ab expression by selected clones

Antibodies in the pComb3H vector are expressed as the Fab (fragment antigen binding) of full-length IgG. Different methods (summarized in Fig. 1) were used to identify TgPO-, Tg-, and TPO-binding clones among colonies: 1) TgPO-binding clones: Colonies in XL1Blue cells, used for titering from the fourth round of alternating panning on Tg and TPO, were grown in 96-well plates (total, 526; 200 ml/well). Aliquots (25 ml) were transferred to fresh medium (175 ml) in a second set of multiwell plates and expanded (4–6 h, 37 C), and Fab expression was induced by overnight incubation (30 C) with isopropyl-thio-galacto-pyranoside (1 mM, Calbiochem). Fab-containing supernatants (50 ml) were assayed on both Tg-coated and TPO-coated ELISA wells (see below). Glycerol stocks of positive clones were prepared from the first set of 96-well plates. 2) Tg-binding Fab were identified by a similar approach using 264 individual colonies from the third round of panning on Tg alone. Fab, expressed by these clones, were tested on Tg-coated wells. 3) Fab, expressed by clones (total 120) isolated after panning on TPO alone, were tested for ^{125}-I-TPO binding using a colony filter lift assay as described previously (15).

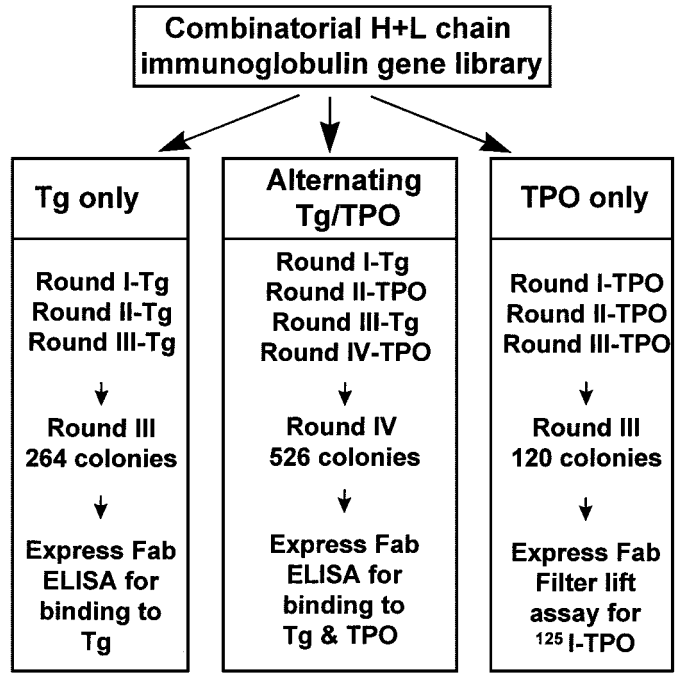

FIG. 1. Strategy for enriching the AF Ig gene combinatorial phage display for TgPO-binding phage. The flow chart outlines the steps for panning and testing expressed Ab (Fab). Clones were isolated from the AF combinatorial library after alternating cycles on Tg and TPO, as well as after panning on Tg alone and TPO alone.

The amplification cycles inherent in panning phage displays libraries frequently leads to the selection of multiple, identical clones (for example, Ref. 20). To reduce unnecessary nucleotide sequencing, we digested plasmid DNA prepared from antigen-positive colonies, with the enzyme BstN I (Stratagene), to determine their restriction patterns. Automated sequencing of double-stranded plasmid DNA was performed by Cedars-Sinai Research Institute core facility. Ig H and L chain genes were classified according to the V Base sequence directory (17). Larger-scale Fab expression was performed on selected clones (10-ml cultures).

ELISA for binding to Tg and TPO

Three types of ELISA were performed for phage, Fab, and serum autoantibody binding. For phage ELISA, aliquots of the library (before and after panning; diluted 1:10 or $1:10^2$) were incubated on ELISA wells coated with Tg (4 mg/ml) or TPO (1 mg/ml) as described (15). Bound phage was detected with biotinylated sheep antibody to M13 (5 Prime to 3 Prime, Inc., Boulder, CO) and horseradish peroxidase-conjugated streptavidin (PharMingen, San Diego, CA). ELISA for Fab was performed using similarly coated wells. Detection was with murine monoclonal antihuman k (QE11; Recognition Sciences, Birmingham, UK) and affinity-purified antimouse IgG-conjugated to horseradish peroxidase (Sigma, St. Louis, MO). Serum autoantibody binding was detected with horseradish peroxidase-conjugated goat antihuman IgG (ICN Biomedicals Inc., Costa Mesa, CA) or using antihuman k (or antihuman l; Recognition Sciences) and horseradish peroxidase-conjugated antimouse IgG (as above). In all assays, the substrate was o-phenylene diamine + H_20_2, and OD were read at 490 nm. In some studies, ELISA wells were coated (4 mg/ml) with the following antigens: lactoperoxidase (LPO), myeloperoxidase, BSA, keyhole limpet hemocyanin (KLH), and glucagon (Gluc) (all from Sigma). On ELISA, serum from patient AF ($1:10^2$) weakly recognized these antigens (ODs ~ 0.1), no different from serum from normal individuals.

Results

Serum TgPOAb in the donor of the thyroid-derived phage display library

In attempting to clone TgPOAb, it was first necessary to identify a suitable patient for Ig gene phage display library construction. TgPOAb are reported to be detectable in most patients with high titers of TgAb and TPOAb but absent in patients without TgAb (8). Patient AF, whose thyroid tissue was available, had high titers of both TPOAb and TgAb, by ELISA (see *Materials and Methods* and Fig. 2, A and B), and was, therefore, an excellent candidate. Examples in this assay of a patient with only TPOAb and not TgAb, as well as serum from a normal individual, are also shown.

To test for serum TgPOAb, we used an established assay (8) based on the principle that a bivalent antibody captured by one arm to immobilized, unlabeled antigen can bind via the other arm to the same labeled antigen or to a different labeled antigen. We used the identical antigen coating conditions and serum dilutions (Fig. 2, A and B) to test for detection of captured antibody with ^{125}I-TPO. As expected, serum from patients AF and WR, captured on TPO-coated ELISA wells, bound ^{125}I-TPO (Fig. 2C). Most important, serum Ab from patient AF (but not WR), captured on Tg, bound ^{125}I-TPO (Fig. 2D), conforming to the previous definition of TgPOAb.

Enrichment of phage display library for TgPO binding

We adapted the conventional phage display panning protocol to parallel, as far as possible, the process of sequential affinity chromatography used by other investigators to isolate TgPOAb from patients' IgG (6). Thus, we panned the AF combinatorial Ig gene library alternately on Tg, TPO, Tg, and then TPO, for a total of four rounds (Fig. 1). To evaluate the quality of the thyroid-derived phage display library, we also panned on Tg alone or on TPO alone (three rounds for each antigen).

To assess library enrichment for antigen binding consequent to panning, we performed phage ELISAs. Before panning, phage in the library did not provide a detectable signal on Tg- or TPO-coated ELISA wells (Fig. 3). After panning on Tg alone, we observed phage binding to Tg-coated wells after R-II and even more after R-III (Fig. 3A). Likewise, after panning the library on TPO alone, enrichment for binding to TPO-coated wells was seen with the R-II and R-III phage (Fig. 3B). The library that had been panned alternately on Tg and TPO (two rounds on each antigen) was tested for binding to both Tg and TPO. The phage ELISA signal on TPO-coated wells rose and fell in accordance with the alternate panning on TPO and Tg (higher values after R-II and R-IV) (Fig. 3C). In contrast, and suggestive of the presence of TgPOAb, the modest level of R-III phage binding to Tg-coated wells was retained in R-IV despite panning in R-IV on TPO (Fig. 3D).

Identification of TgPOAb clones

Having established autoantigen binding by pools of phage, we next expressed Ab from individual colonies to identify bispecific TgPO clones. Synthesis of Ab (in the form of Fab) was induced from individual plasmid-containing

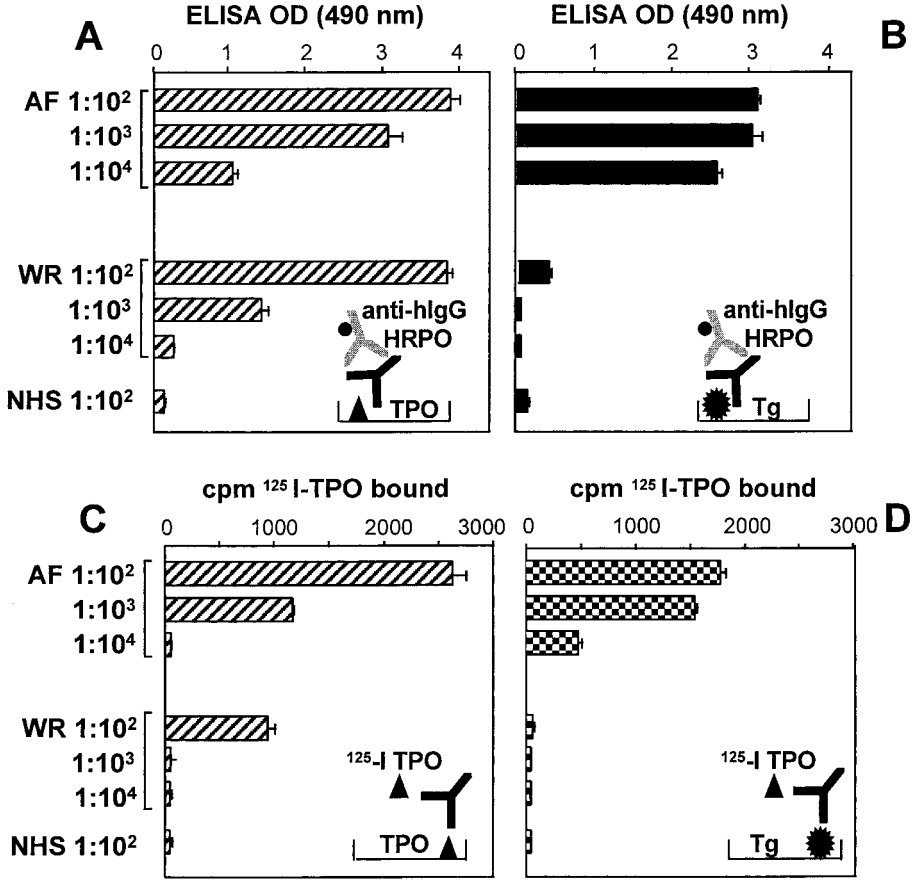

FIG. 2. TgPOAb activity is detectable in serum from patient AF. In a conventional ELISA with TPO- or Tg-coated wells and detection with antihuman IgG conjugate, serum from patient AF is positive for both TPOAb (A) and TgAb (B), whereas serum from another patient (WR) is positive only for TPOAb. Using the same antigen-coating conditions and serum dilutions, ^{125}I-TPO is bound by serum Ab from patients AF and WR captured on TPO-coated wells (C). Serum antibodies from patient AF (but not patient WR), captured on Tg-coated wells, bound ^{125}I-TPO (D), indicating the presence of TgPOAb activity. Sera from patients AF and WR were assayed at three different dilutions (1:10^2, 1:10^3, and 1:10^4). Serum from a control [normal human serum (NHS); diluted 1:10^2] was negative in all four assays. Data are the mean + SD of duplicate determinations. HRPO, Horseradish peroxidase.

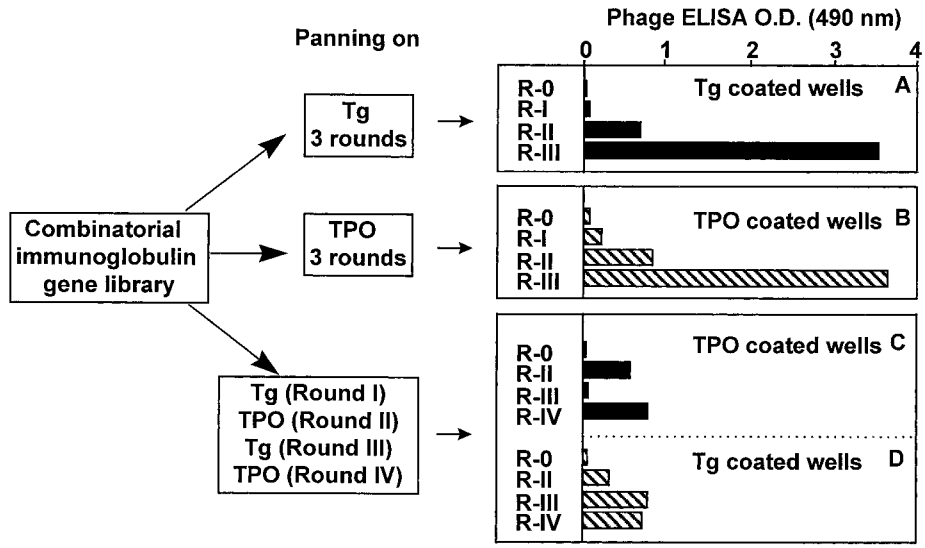

FIG. 3. Enrichment of the AF combinatorial library for binding to Tg, TPO, or both Tg and TPO. Three rounds of panning on Tg produced marked enrichment, particularly after R-III, for phage binding to Tg-coated ELISA wells (A). Similar observations were made for the library panned on TPO alone (B). Phage obtained by alternating panning cycles on Tg and TPO (total, four rounds) were tested for binding to both thyroid autoantigens. This approach produced R-IV phage capable of moderate binding to both TPO-coated wells (C) and Tg-coated wells (D).

bacterial colonies from R-IV after alternate panning on Tg and TPO. In addition we identified Tg-binding clones after panning on Tg- and TPO-binding clones after panning on TPO (both sets from the final R-III).

Of the colonies selected by alternating panning on Tg and TPO, Ab expressed from a large number of clones (526 in total) were tested in parallel for binding to ELISA wells coated with Tg and to wells coated with TPO. Almost all these R-IV clones were negative, and only 2.6% of colonies produced Fab capable of binding to Tg and/or TPO. This low value contrasts with the much higher proportion (45–50%) of positive clones obtained after panning the same library on Tg alone or TPO alone (Table 1). We did not subject R-IV phage to additional pannings on Tg alternating with TPO. An inherent problem in excessive panning is an increase in the proportion of phagemid lacking Ig cDNA (21–23).

The positive clones in the population of phage enriched for TgPO binding had some unexpected characteristics. Four clones gave moderate binding to both Tg and TPO (OD $>$ 0.5 with both antigens) (Table 1). However, three clones expressed Fab that bound at high levels (OD values $>$ 1.00) to Tg but had negligible binding to TPO. Conversely, Fab from seven clones bound to TPO but not to Tg, five of which were selected for further study.

Overall, 12 Fab-expressing clones isolated by panning on alternating cycles of Tg and TPO were studied in more detail, as well as 64 clones isolated using Tg alone and another 64 obtained using TPO alone. Based on their unique BstN1 DNA fingerprints (see *Materials and Methods*), we determined the H and L chain V gene nucleotide sequences of 18 Tg-only selected clones, 18 TPO-only selected clones, and all 12 Tg and TPO selected clones (Table 1). The final numbers of clones with distinct, unrelated H or L chain genes were: 7 clones selected on Tg alone, 8 clones selected on TPO alone, and 5 clones selected on both Tg and TPO, only 1 of which was a potential TgPOAb clone.

Ig genes encoding Tg- and/or TPO-binding clones

The genes encoding the V regions of the Ig chains are summarized in Table 2. Each H chain V region is described in terms of: 1) its closest VH germline gene; 2) the derived amino acid sequence of a major component of the antigen-binding site, namely the third complementarity-determining region (CDR3); and 3) the gene encoding the joining or JH region. The L chain genes are described in terms of their VK germlines and joining (JK) genes.

Clones from the AF library, isolated by panning on either Tg alone or TPO alone, are encoded by different H and L chain V genes. Among the H chains, only one VH germline gene (V1-3b/DP-25) (24, 25) occurs in both groups, but their CDR3 regions (critical for antigen binding) are completely different. Overall, six types of H chains encode Tg-derived clones, and five types of H chains encode TPO-derived clones. Regarding the five distinct clones obtained by alternate panning on Tg and TPO, the two clones that bound Tg (but not TPO) use H and L chain genes, like those of other clones isolated on Tg only (Table 2). Similarly, two clones that bound strongly to TPO (but not to Tg) have the same VH/VL combination as other clones isolated by panning on TPO alone. Of great interest, the single potential TgPO clone uses a TPO-like L chain VK gene and a Tg-like VH gene (BHGH1) (26), though with a completely different CDR3 region (Table 2).

Antigenic specificity of monoclonal Ab

The crucial question regarding the single potential TgPO clone was the specificity of its recognition of Tg and TPO. We analyzed this clone, as well as all the other clones with characterized genes (Table 2), for binding to Tg, TPO, and a panel of other antigens (BSA, LPO, KLH, or Gluc). Ab (Fab), expressed by clones obtained by panning on Tg alone, bound strongly to Tg and weakly or not at all to other antigens, including TPO (Fig. 4, *top*). Similarly, Ab expressed by TPO-isolated clones bound specifically to TPO and not to Tg or to other antigens (Fig. 4, *middle*).

Turning to the clones isolated by alternate panning on Tg and TPO, Ab generated by two clones (A and C) were specific for TPO, and another two from clones B and D were specific for Tg. Consistent with it being a potential TgPOAb, Fab from clone E did demonstrate apparent bispecificity by binding modestly to both Tg and TPO. However, equal or better

TABLE 1. Summary of clones isolated from the AF combinatorial library by panning on Tg alone, TPO alone, or alternating panning on Tg and TPO

	Combinatorial Ig gene library panned on		
	Tg alone	TPO alone	Tg, TPO, Tg, TPO
Clones expressed	264	120	526
Assay	anti-Tg ELISA	^{125}I-TPO binding (filter lift assay)	anti-Tg ELISA anti-TPO ELISA
% Positive	45%	50%	0.5% (Tg) 1.3% (TPO) 0.8% (Tg & TPO)
Clones digested with BstN1	64	64	12
Clones with unique BstN1 fingerprints	18	18	5
No. of individual clones after DNA sequencing	7	8	5

TABLE 2. H and L chain V region genes that encode antibodies derived from the AF library by panning on Tg alone, TPO alone, or alternating rounds on Tg and TPO

VH	CDR3	JH	VK	JK
Screening on Tg only (three rounds)				
VII-5b+	AGYHRGAFDV	3	A27/humkv325	1
VH26/DP47	HHAPYGDYPPDFDS	4	humkv328h5	4
BHGH1	VNAPYYSGQGLPFDV	3	A27/humkv325	1
			humkv328h5+	5
BHGH1	NHVPYGSARFASDY	4	A27/humkv325	5
9-1+/DP38	APVREVRLGAHSYYIMDV	6	humkv328h5+	2
V1-3b	EGYGGFE	4	A27/humkv325	2
Screening on TPO only (three rounds)				
21-2	PRGEVLGGLYYGVDV	6	O12	1
hv1051k	DNLSYFD(L)	2	O12	4/1
hv1051k	GSSYDALEI	3	A17+	5
V1-3b	NSPWHIFYGMDV	6	O12	1
V1-3b	DPYGGNGELDH	4	L12	1
			O12	4
			L11	4
Screening on Tg, TPO, TG, TPO (four rounds)				
V1-3b	EGYGGFE (Tg)	4	A27/humkv325	2
BHGH1	NHVPYGSARFASDY (Tg)	4	A27/humkv325	5
V1-3b	NSPWHIFYGMDV (TPO)	6	O12	1
V1-3b	DPYGGNSELDH (TPO)	4	humkv328	4
BHGH1	ALSSMPLDAFDV (?TgPO)	3	O12	3

Each H or L chain gene is described in terms of its closest VH (or VK) germline gene, the derived amino acid sequence of the third complementarity determining region (CDR3), and the gene encoding the joining (JH or JK) region. Immunoglobulin VH and Vk germline genes were classified according to the V Base sequence directory (17). Jh and Jk, Joining genes.

binding to four other nonthyroid antigens established that clone E is not a bispecific TgPOAb but is, instead, a polyreactive antibody.

Discussion

A unifying hypothesis for the presence of Ab to both Tg and TPO, in many patients with thyroid autoimmunity, arose from evidence for bispecific TgPOAb (1). From the clinical perspective, several studies suggested that TgPOAb might be more useful than measurements of either TPOAb or TgAb, in distinguishing between patients and euthyroid individuals, as well as between patients with Hashimoto's thyroiditis and other thyroid autoimmune conditions (7–9). In this context, it is of interest that there is evidence that the Tg gene is a major susceptibility locus for autoimmune thyroid disease (27). For these reasons, we sought to obtain definitive evidence for the existence of TgPOAb, by cloning such bispecific Ab from a patient with autoimmune thyroid disease.

The Ig gene combinatorial library approach has been successfully used to isolate panels of human monoclonal Ab to TPO and Tg (15, 18, 28–39). Before the development of this technology, some monoclonal TgAb and TPOAb were obtained from patients' B lymphocytes by conventional fusion and/or Epstein-Barr virus infection (40–42). None of the human monoclonal TgAb and TPOAb described to date are bispecific, and their binding is restricted to Tg or to TPO. However, the absence of TgPOAb from these panels of monoclonal autoantibody could be explained in terms of the screening protocol used and/or the particular patient from whom the Ab were obtained.

In the present study, we constructed an Ig gene combinatorial library from a patient with serum TgPOAb. The strategy that we used to screen the phage display combinatorial library was to mimic, as far as possible, the affinity chromatography approach used previously for isolating TgPOAb from patients' sera (6). We panned the library first on Tg, then on TPO, and repeated this alternating pattern for a total of four rounds. As found for serum TgPOAb, we succeeded in obtaining enrichment, albeit modest, for phage that bound both Tg and TPO. It should be emphasized that these studies were performed using a library of IgG1/K genes from the thyroid of a single patient. This K bias was consistent with the virtual restriction of the

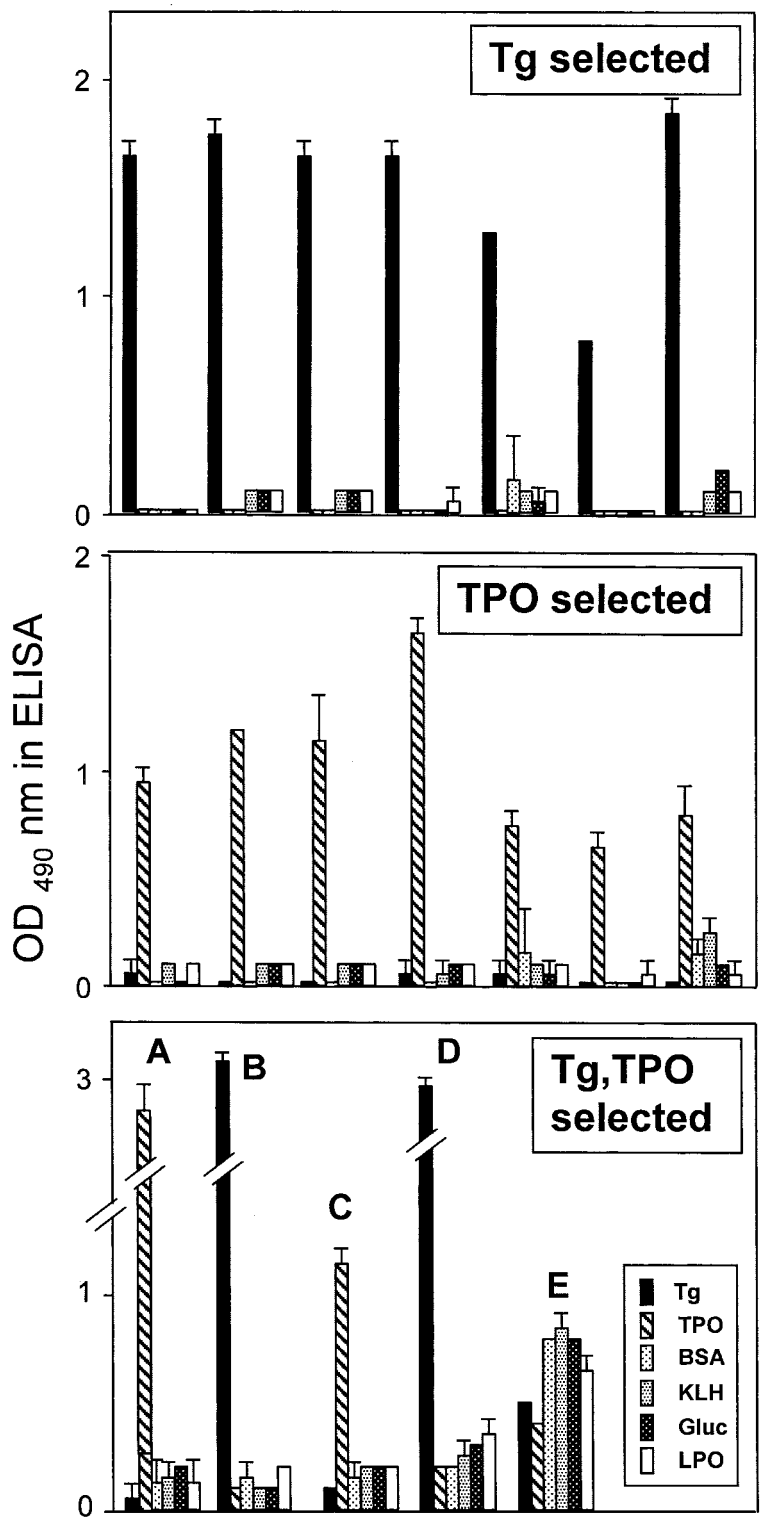

FIG. 4. Ab from clones selected using Tg alone (*top*) or TPO alone (*middle*) bind specifically to Tg or TPO. In contrast, alternating panning on Tg and on TPO (*bottom*) produced clones specific for Tg (B and D) and specific for TPO (A and C), as well as a *polyreactive* clone (E) that binds to Tg, TPO, and other nonthyroid antigens. Autoantibody (Fab) binding was assayed by ELISA, and data are expressed as OD 490 nm (mean + SD for duplicate determinations). Nonthyroid antigens tested: BSA, KLH, Gluc, and LPO.

patient's serum TgAb and TPOAb to L chains of this type. However, it is possible that TgPOAb may be found in subclasses other than IgG1. Despite this potential limitation, we achieved our goal of enriching for TgPO-binding phage.

Unlike the approach using enrichment of polyclonal TgPOAb from patient serum, the combinatorial library approach has the great advantage of being able to dissect out the contribution of individual clones to the binding displayed by a pool of phage. This analysis demonstrated that the individual clones in the enriched library with bispecific phage binding activity were not, in most part, bispecific. Clones that recognized Tg were specific in that they did not recognize TPO, and *vice versa*. In addition, a contribution was also made by polyreactive phage that bound to multiple

antigens (including Tg and TPO). Our observations provide sobering insight into the potential problems associated with solid-phase serum antibody assays (such as ELISA), especially in patients with extremely high titers of TgAb and TPOAb. Furthermore, our data suggest that TgPOAb activity prepared by affinity chromatography from patients' sera may be a mixture of antibodies specific for Tg or specific for TPO (and perhaps also polyreactive antibodies).

Although not our initial goal, our observations provide novel and important information on another aspect of thyroid autoimmunity, namely the genes that encode TgAb and TPOAb in the same patient. In a previous study, several different TPOAb H chains were isolated from a combinatorial library constructed using lymph node B cells from a Hashimoto patient (35). However, all TgAb from this library used a single H chain combined with different L chains (34); and therefore, no definitive conclusions could be drawn regarding gene usage by TgAb and TPOAb in the same patient. In the present study, using thyroid tissue from a Graves' patient, we obtained diverse panels of both TgAb and TPOAb involving multiple H and L chains, as well as H/L combinations. Most important, none of the six different TgAb H chains were the same as the five H chains used by TPOAb. The germline VH gene V1–3b forms the scaffolding for some types of TgAb and TPOAb in this patient, as in monoclonal TPOAb from other patients (summarized in Refs. 4 and 43). However, the CDR3s, crucial regions for antigen binding, are unrelated in the two sets of Ab (see Table 2). Consequently, these findings exclude another potential explanation for the presence of both TgAb and TPOAb in the same patient, namely their derivation from a single B-cell-producing antibody that binds with low affinity to both Tg and TPO and subsequently undergoes affinity maturation (mutation of its CDRs) to form high-affinity TgAb (that no longer recognize TPO) and high-affinity TPOAb (that do not recognize Tg).

Our observations also expand the repertoire of VH genes used by TPOAb and TgAb. Many H chain genes encoding these thyroid Ab are derived from the large VH gene families 1 and 3 (*e.g.* V1-3b and VH26) (summarized in Refs. 4 and 43). More recently, TPOAb encoded by the very small VH5 family have been isolated (37, 39). We now describe the use of additional VH genes: for TPOAb, the VH1 family genes 21–2 (44) and hv105 h (45); for Tg genes, the VH3 family genes 9–1+/DP38 (25, 44) and BHGH1 (26). Moreover, our observation of a VH2 gene (VII-5b+) (24) in a TgAb is the first report of this small family encoding Ab to either Tg or TPO.

Polyreactive antibodies have aroused considerable interest as precursors of specific Ab (for example, Ref. 46). In fact, the potential TgPOAb we isolated that turned out to be polyreactive was derived from one of the germline genes that encode TgAb in this particular patient. However, there was no relationship between the CDR3 regions of the polyreactive Ab and TgAb encoded by the same germline gene. Some studies have suggested that polyreactivity is related to the length of the CDR3 region, as well as to the presence of arginine and asparagine residues (reviewed in Ref. 47). The CDR3 of our polyreactive antibody (Table 2) was not unusually long, and it lacked the residues proposed to be involved in polyreactive binding.

In conclusion, using an Ig gene combinatorial library from a patient with serum TgPOAb activity, we achieved enrichment after alternating panning rounds on Tg and TPO for phage that bound both autoantigens. However, such enrichment was largely attributable to phage specific for either Tg or TPO, and we did not obtain bispecific TgPOAb. It is possible that such bispecific Ab could be isolated from another patient. However, these findings, combined with our isolation of a polyreactive monoclonal human antibody, provide powerful evidence against shared, cross-reactive epitopes on two major thyroid autoantigens.

Acknowledgments

We thank Dr. Orlo Clark, University of California San Francisco, for providing thyroid tissue.

Received July 17, 2002. Accepted September 16, 2002.

Address all correspondence and requests for reprints to: Sandra M. McLachlan, M.D., Cedars-Sinai Medical Center, 8700 Beverly Boulevard, Suite B-131, Los Angeles, California 90048. E-mail: mclachlans@cshs.org.

These studies were supported by NIH Grant DK-36182.

References

1. **Ruf J, Carayon P** 1995 The molecular recognition theory applied to bispecific antibodies. Nat Med 1:1222
2. **McLachlan SM, Rapoport B** 1989 Evidence for a potential common T-cell epitope between human thyroid peroxidase and human thyroglobulin with implications for the pathogenesis of autoimmune thyroid disease. Autoimmunity 5:101–106
3. **Hoshioka A, Kohno Y, Katsuki T, Shimojo N, Maruyama N, Inagaki Y, Yokochi T, Tarutani O, Hosoya T, Niimi H** 1993 A common T-cell epitope between human thyroglobulin and human thyroid peroxidase is related to murine experimental autoimmune thyroiditis. Immunol Lett 37:235–239
4. **McLachlan SM, Rapoport B** 2000 Autoimmune response to the thyroid in humans: thyroid peroxidase—the common autoantigenic denominator. Int Rev Immunol 19:587–618
5. **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
6. **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
7. **Naito N, Saito K, Hosoya T, Tarutani O, Sakata S, Nishikawa T, Niimi H, Nakajima H, Kohno Y** 1990 Anti-thyroglobulin autoantibodies in sera from patients with chronic thyroiditis and from healthy subjects: differences in cross-reactivity with thyroid peroxidase. Clin Exp Immunol 80:4–10
8. **Ruf J, Ferrand M, Durand-Gorde JM, De Micco C, Carayon P** 1993 Significance of thyroglobulin antibodies cross-reactive with thyroperoxidase (TGPO) antibodies) in individual patients and immunized mice. Clin Exp Immunol 92:65–72
9. **Ruf J, Feldt-Rasmussen U, Hegedus L, Ferrand M, Carayon P** 1994 Bispecific thyroglobulin and thyroperoxidase autoantibodies in patients with various thyroid and autoimmune diseases. J Clin Endocrinol Metab 79:1404–1409
10. **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
11. **Duthoit C, Estienne V, Durand-Gorde JM, Carayon P, Ruf J** 1999 Thyroglobulin monoclonal antibody cross-reacting with thyroperoxidase induces in syngeneic mice anti-idiotypic monoclonal antibodies with dual autoantigen binding properties. The intertope hypothesis. Eur J Immunol 29:1626–1634
12. **Villa M, Durand-Gorde JM, Carayon P, Ruf J** 1996 Idiotypic study of a bispecific thyroglobulin and thyroperoxidase monoclonal antibody. J Autoimmun 9:653–660
13. **Guo J, McLachlan SM, Hutchison JS, Rapoport B** 1998 The greater glycan content of recombinant human thyroid peroxidase of mammalian than on insect cell origin facilitates purification to homogeneity of enzymatically active protein remaining soluble at high concentration. Endocrinology 139:999–1005
14. **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

15. **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
16. **Barbas III CF, Wagner J** 1995 Synthetic human antibodies: selecting and evolving functional proteins. Methods: Comp Methods Enzymol 8:94–103
17. **Tomlinson IMS, Williams SC, Corbett SJ, Cox JBL, Winter G** 1997 V BASE, www.mrc-cpe.cam.ac.uk/vbase-ok.php?menu=901
18. **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
19. **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
20. **Marks JD, Hoogenboom HR, Bonnert TP, McCafferty J, Griffiths AD, Winter G** 1991 By-passing immunization: human antibodies from V-gene libraries displayed on phage. J Mol Biol 222:581–597
21. **Dziegel M, Nielsen LK, Andersen PS, Blancher A, Dickmeiss E, Engberg J** 1995 Phage display used for gene cloning of human recombinant antibody against the erythrocyte surface antigen, rhesus D. J Immunol Methods 182:7–19
22. **Watkins BA, Davis AE, Fiorentini S, di Marzo Veronese F, Reitz MS** 1996 Evidence for distinct contributions of heavy and light chains to restriction of antibody recognition of the HIV-1 principal neutralization determinant. J Immunol 156:1676–1683
23. **Kakinuma A, Portolano S, Chazenbalk G, Rapoport B, McLachlan SM** 1997 Insight into screening immunoglobulin gene combinatorial libraries in a phage display vector: a tale of two antibodies. Autoimmunity 25:73–84
24. **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
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. **Tomer Y, Greenberg DA, Concepcion E, Ban Y, Davies TF** 2002 Thyroglobulin is a thyroid specific gene for the familial autoimmune thyroid diseases. J Clin Endocrinol Metab 87:404–407
28. **Portolano S, Seto P, Chazenbalk GD, Nagayama Y, McLachlan SM, Rapoport B** 1991 A human Fab fragment specific for thyroid peroxidase generated by cloning thyroid lymphocyte-derived immunoglobulin genes in a bacteriophage λ library. Biochem Biophys Res Commun 179:372–379
29. **Hexham M, Persson MAA, Pegg C, Burton DR, Furmaniak J, Rees Smith B** 1991 Cloning and expression of a human thyroglobulin autoantibody. Autoimmunity 11:69–70
30. **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
31. **Portolano S, Chazenbalk GD, Hutchison JS, McLachlan SM, Rapoport B** 1993 Lack of promiscuity in autoantigen-specific H and L chain combinations as revealed by human H and L chain "roulette." J Immunol 150:880–887
32. **Portolano S, McLachlan SM, Rapoport B** 1993 High affinity, thyroid-specific human autoantibodies displayed on the surface of filamentous phage use V genes similar to other autoantibodies. J Immunol 151:2839–2851
33. **Prummel MF, Portolano S, Costante G, Rapoport B, McLachlan SM** 1994 Isolation and characterization of a monoclonal human thyroid peroxidase autoantibody of lambda light chain type. Mol Cell Endocrinol 102:161–166
34. **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
35. **McIntosh RS, Asghar MS, Kemp EH, Watson PF, Gardas A, Banga JP, Weetman AP** 1997 Analysis of IgG κ anti-thyroid peroxidase antibodies from different tissues in Hashimoto's thyroiditis. J Clin Endocrinol Metab 82:3818–3825
36. **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
37. **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
38. **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
39. **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
40. **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
41. **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
42. **Horimoto M, Petersen VB, Pegg C, Fukuma N, Wakabayashi N, Kiso Y, Furmaniak J, Rees Smith B** 1992 Production and characterisation of a human monoclonal thyroid peroxidase autoantibody. Autoimmunity 14:1–7
43. **McIntosh RS, Weetman AP** 1997 Molecular analysis of the antibody response to thyroglobulin and thyroid peroxidase. Thyroid 7:471–487
44. **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
45. **Yang PM, Olee T, Carson DA, Chen PP** 1993 Characterization of two highly homologous autoantibody-related VH1 genes in humans. Scand J Immunol 37:504–508
46. **Kasaian MT, Casali P** 1993 Autoimmunity-prone B-1 (CD5 B) cells, natural antibodies and self recognition. Autoimmunity 15:315–329
47. **Wing MG** 1995 The molecular basis for a polyspecific antibody. Clin Exp Immunol 99:313–315