

Rarity of autoantibodies to a major autoantigen, thyroid peroxidase, that interact with denatured antigen or with epitopes outside the immunodominant region

J. GUO, Y. WANG, J. C. JAUME*, B. RAPOPORT & S. M. MCLACHLAN *Autoimmune Disease Unit, Cedars-Sinai Research Institute and the University of California, Los Angeles School of Medicine, Los Angeles, and *Endocrinology, Veterans' Affairs Medical Center and University of California San Francisco, San Francisco, CA, USA*

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SUMMARY

The nature of the autoantibody repertoire to the dominant autoantigen in human autoimmune thyroid disease is controversial. There is evidence that autoantibodies to thyroid peroxidase (TPO) interact with overlapping conformational epitopes in an immunodominant region and binding to denatured (DN) protein is decreased. Contrary data demonstrate TPO autoantibody reactivity with DN-TPO or polypeptide fragments. However, none of the TPO-specific, human monoclonal autoantibodies isolated to date preferentially recognize denatured autoantigen. We therefore searched an immunoglobulin gene phage display library for human autoantibodies that bind TPO denatured by reduction and alkylation (DN-TPO). Thyroid-infiltrating B cells from a typical TPO autoantibody-positive patient were the source of mRNA for library construction. Surprisingly, the library enriched after panning on DN-TPO, as well as a panel of individual clones, preferentially bound native (N)-TPO. Of 13 clones selected using DN-TPO or N-TPO, 12 clones recognized the TPO immunodominant region. Moreover, regardless of selection with N-TPO or DN-TPO, their heavy and light chains were encoded by similar VDJ and V κ combinations. One clone (DN4), isolated using DN-TPO, did not interact with the TPO immunodominant region and its H chain derives from a different V_H gene. Although DN4 binds specifically to TPO, its affinity is low, unlike the high affinities of other human TPO autoantibodies. In conclusion, human monoclonal autoantibodies that preferentially recognize denatured TPO could not be isolated from an immunoglobulin gene library despite selection with denatured protein. Our findings demonstrate the bias of the human B cell repertoire towards recognition of an immunodominant region on the conformationally intact form of a major thyroid autoantigen.

Keywords autoimmunity autoantibodies human monoclonals thyroid

INTRODUCTION

The nature of the autoantibody repertoire to the dominant autoantigen in human autoimmune thyroid disease is controversial (reviewed in [1]). On the one hand, several studies have shown that autoantibodies to thyroid peroxidase (TPO) interact with epitopes on conformationally intact protein and binding is decreased following protein denaturation [2,3]. On the other hand, other studies have demonstrated serum autoantibody reactivity with TPO after reduction/denaturation (for example [4,5]), suggesting that some TPO epitopes may not be highly conformational. Further, several linear epitopes [6,7], as well as polypeptide fragments [8–11], are recognized by patients' autoantibodies. Indeed, the epitopes of

some autoantibodies are reported to be inaccessible in the native molecule [11].

Unlike the debate regarding autoantibody interaction with denatured versus native autoantigen, there is broad agreement that the component of the B cell repertoire directed against native TPO is restricted. First, the epitopes of patients' autoantibodies overlap with only two of a panel of mouse MoAbs that recognize a diverse range of epitopes on TPO [12,13]. Second, human TPO MoAbs isolated from patients' B cells define a restricted area on TPO that is recognized by TPO autoantibodies in all (>190) patients' sera so far analysed and by approx. 80% of TPO autoantibodies within each individual serum (reviewed in [14]). The concept of an immunodominant region on native TPO has recently been confirmed [15] in a three-way collaborative comparison of the epitopes of the murine TPO MoAb panel [12] with the original human monoclonal autoantibodies described by our laboratory [16] and another panel of

Correspondence: Sandra M. McLachlan, Cedars-Sinai Medical Center, 8700 Beverly Blvd, Suite B-131, Los Angeles 90048, Ca, USA.

E-mail: mclachlans@csmc.org

human MoAbs isolated independently by different investigators [17].

To date, none of the human TPO-specific monoclonal autoantibodies isolated by three different laboratories preferentially recognize denatured autoantigen [17–20]. However, all human monoclonal TPO autoantibodies have been isolated using conformationally intact antigen. Given that autoantibodies to both native and denatured TPO are clearly present in autoimmune thyroid disease, we used the phage display approach (reviewed in [21]) to search an immunoglobulin gene library for human autoantibodies that recognize denatured TPO. Remarkably, even when panned on denatured protein, the enriched libraries as well as a panel of individual clones preferentially bound native TPO. These findings indicate the importance of protein conformation in the selection of the autoimmune B cell repertoire.

MATERIALS AND METHODS

Construction of combinatorial immunoglobulin gene libraries

Thyroid tissue was obtained from a patient (GA) with autoimmune thyroid disease. Typical of the majority of such patients, GA had a moderately high serum TPO antibody titre (1:3000 by ELISA, see below). Thyroid tissue (0.5 g) was used to prepare mRNA (Pharmacia, Piscataway, NJ) for reverse-transcription of cDNA (First Strand Synthesis Kit; Stratagene, La Jolla, CA). IgG1 heavy (H) and κ -chain cDNA were amplified by the polymerase chain reaction (PCR) using panels of 5' V_H (or V _{κ}) oligonucleotide primers capable of cross-priming all V_H (or V _{κ}) gene families [16,22].

A combinatorial library was constructed in the phage display vector pComb3H [23] (provided by Dr C. Barbas, Scripps Research Institute) by cloning the κ -chain DNA into the SacI and XbaI restriction sites and subsequently introducing the H chain DNA into the XhoI and SpeI sites. Electroporation into electrocompetent XL1Blue cells (GIBCO BRL, Gaithersburg, MD) was performed using an IBI Gene Zapper (21 μ F, 2500 V, 400 W). Infective phagemid were generated by rescue with M13K07 helper phage (GIBCO BRL) and precipitated with polyethylene glycol. The unamplified GA-pComb3H combinatorial library contained approx. 5×10^6 recombinants. Using the same H and κ -chain DNA, a combinatorial library containing approx. 30×10^6 recombinants was generated in the bacteriophage λ vector Immunozap (Stratagene) as described previously [18].

Native and denatured TPO

Human TPO secreted by Chinese hamster ovary (CHO) cells was affinity-purified from culture medium as reported previously [24]. An aliquot (10 μ g) of native (N) TPO in PBS pH 7.4 was denatured by reduction and alkylation using 50 mM dithiothreitol (10 min, 20°C) and 40 mM iodoacetamide, 0.2 M Tris, pH 8.3 (10 min, 20°C). This is a standard protocol previously used by Nakajima et al. [2] (but with only 10 mM dithiothreitol) to demonstrate loss of TPO binding activity by patients' autoantibodies. After buffer exchange (to PBS) using Micron 50 columns (Amicon Inc., Beverly, MA), the change in naturation status was assessed by a direct binding assay and reverse immunoblot using TPO autoantibody-positive sera, a human monoclonal TPO-specific autoantibody Fab, WR1.7 [16] and a mouse MoAb (Mab 47) [12] (the latter kindly provided by Dr B. Czarnocka, Medical Centre of Postgraduate Education, Warsaw, Poland). TPO for these assays was labelled using iodogen. Antibody–TPO complexes in the direct binding assay were precipitated for human sera with Protein A (Pansorbin; Calbiochem,

La Jolla, CA) [18] and for the murine MoAb with anti-mouse conjugated beads (Sac-Cel; IDS, Boldon, UK) [15]. The reverse immunoblot protocol is outlined below.

Screening the GA-IgG/ κ combinatorial libraries with denatured and native TPO

The GA-pComb3H library was screened as described [20] by incubating approx. 10^{12} phage (1 h, 37°C) in ELISA wells coated with denatured (DN)-TPO or N-TPO (1 μ g/well). The wells were washed (four times), bound phagemid eluted with 0.1 M HCl pH 2.2, neutralized with 2 M Tris pH 7.5 and used to infect XL1 Blue cells. Aliquots were withdrawn for titrating and culture of the remaining cells was continued overnight at 30°C with M13K07 helper phage. After phagemid precipitation and resuspension, the panning was repeated twice, increasing the stringency of washing before elution to $\times 5$ in round II and $\times 6$ in round III. Twenty individual colonies from the third round of panning on DN-TPO (or N-TPO) were screened in filter lift assays as described [25] for binding to 125 I-DN-TPO (or 125 I-N-TPO). Aliquots (3 μ l) of patients' and control sera, as well as murine MoAb 47 [12], were applied to nitrocellulose filters and incubated with the same preparations of radiolabelled N- and DN-TPO (reverse immunoblot). DNA from TPO-binding colonies was digested with the frequent cutting enzyme BstNI (Stratagene) to identify clones with different restriction patterns to avoid analysing multiple identical colonies arising from the amplification involved in library panning.

Screening of the GA Immunozap library was as previously described [18] using conventional filter lifts and 125 I-N-TPO. Following plaque purification, plasmids were excised from the Immunozap vector using helper phage R408 (Stratagene). Nucleotide sequencing of double-stranded plasmid cDNA for TPO-binding colonies from pComb3H and Immunozap libraries was performed by the dideoxynucleotide termination method or by PCR-based automated sequencing. Sequences were analysed using the V Base Directory [26] to determine the putative germ-line genes encoding the TPO binding H and L chain V regions.

Fab expression from TPO-binding colonies in pComb3H

Fab were expressed as soluble proteins without removal of the cpIII gene from the pComb3H plasmid, as we have shown that this approach provides sufficient Fab for analysis [20]. Plasmid-bearing XL1 Blue cells were incubated (37°C, 6–7 h) and protein synthesis was induced by overnight incubation with isopropyl-thio-galactopyranoside (1 mM; Sigma Chemical Co., St Louis, MO). After four freeze–thaw cycles, the cells were centrifuged and the cell pellets were resuspended in PBS containing protease inhibitors.

Fab recognition of N versus DN TPO

A 'phage' ELISA was performed by modifying an ELISA previously reported for TPO-specific Fab [18]. In brief, 100- μ l aliquots of libraries after one, two or three rounds of panning (diluted 1:10²–1:10⁴) were applied to ELISA wells coated with N-TPO or DN-TPO (1 μ g/ml). Bound phage were detected with biotinylated sheep antibody to M13 (5 Prime to 3 Prime, Inc., Boulder, CO) and horseradish peroxidase (HRP)-conjugated streptavidin (Pharmingen, San Diego, CA). ELISA for Fab was performed using similarly coated wells and detection, as in previous studies [18], with murine monoclonal anti-human κ (QE11; Recognition Sciences, Birmingham, UK) and affinity-purified anti-mouse IgG conjugated to HRP (Sigma). In both assays, the substrate was o-phenylenediamine + H₂O₂ and optical densities (OD) were read

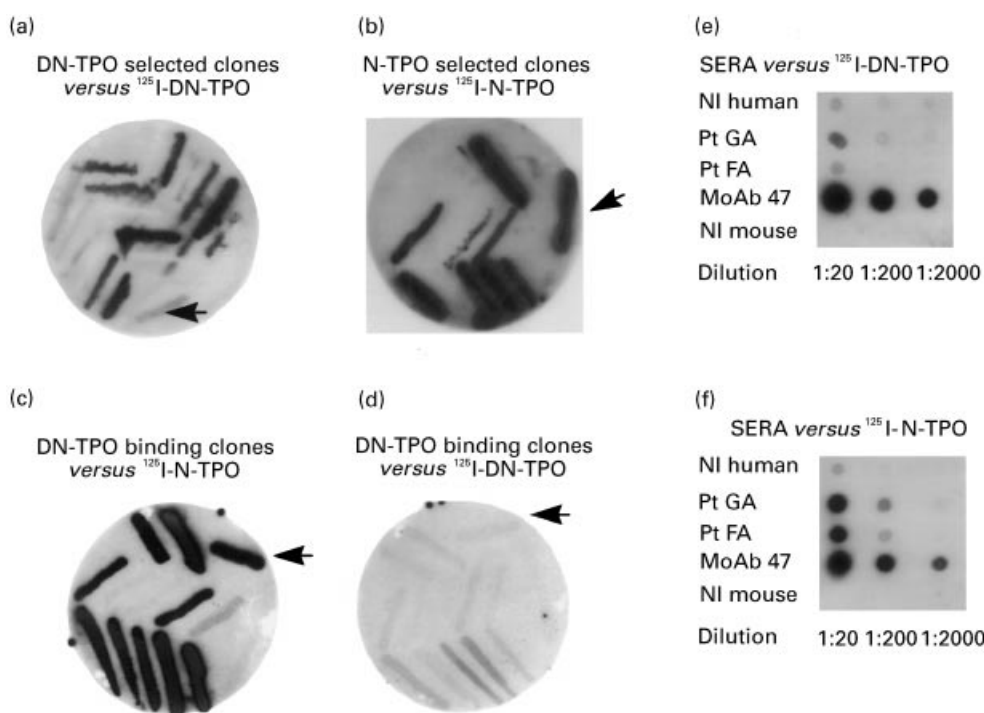


Fig. 1. Interaction of thyroid-derived clones with native (N) versus denatured (DN) thyroid peroxidase (TPO) after three rounds of panning a pComb3H phage display library constructed from thyroid tissue of a patient with autoimmune thyroid disease. Individual clones from the third round of panning against DN-TPO or N-TPO were tested in filter lifts for binding of radiolabelled antigen. As anticipated, DN-TPO-selected clones bind ^{125}I -DN-TPO (a) and N-TPO-selected clones bind ^{125}I -N-TPO (b). Unexpectedly, DN-TPO binding clones interact better with ^{125}I -N-TPO (c) than with ^{125}I -DN-TPO (d). ^{125}I -DN-TPO used in these studies is recognized very well by mouse MoAb 47, but only weakly or not at all by serum autoantibodies (patients GA and FA) (e). In contrast, ^{125}I -N-TPO is recognized by both serum antibodies and mouse MoAb 47 (f). Controls include normal (NI) human serum and normal mouse serum (e,f). The arrows indicate binding by the human monoclonal TPO autoantibody Fab WR1.7 (as a bacterial streak) that preferentially recognizes N-TPO [16].

at 492 nm. Fab specificity was established by comparing OD values for ELISA wells coated with TPO versus the following proteins (1 $\mu\text{g}/\text{ml}$): bovine serum albumin (BSA; Sigma); myeloperoxidase (MPO; Calbiochem), lactoperoxidase (LPO), transferrin, keyhole limpet haemocyanin (KLH) (all from Sigma) and human thyroglobulin (Tg) prepared from Graves' thyroid tissue by gel filtration and ion exchange chromatography.

Recognition of TPO expressed on eukaryotic cells

Flow cytometry of TPO expressed on CHO cells was studied as described [27]. In brief, CHO-TPO cells grown in monolayer were detached by mild trypsinization and incubated with TPO-binding Fab followed by anti-human κ conjugated to PE (Caltag, Burlingame, CA). Fluorescence was analysed using the Becton Dickinson FACScan-CELLQuest system (Mountain View, CA).

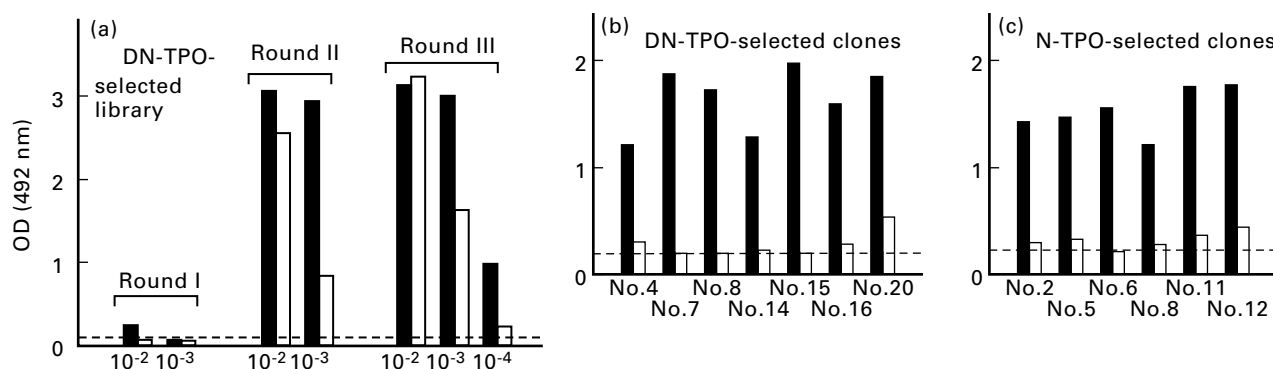


Fig. 2. Preferential recognition of native (N) (■) versus denatured (DN) thyroid peroxidase (TPO) (□) by the phage display library panned on denatured TPO (a) and by Fab expressed by individual clones selected by panning on either DN-TPO (b) or N-TPO (c). Dilutions of phage from the GA-pComb3H library after rounds I, II and III or individual expressed Fab were applied to ELISA wells coated with N- or DN-TPO. Phage were detected with biotinylated anti-M13 phage and horseradish peroxidase (HRP)-conjugated streptavidin and Fab with anti-human κ and HRP anti-mouse IgG. The upper limit of binding to bovine serum albumin (BSA)-coated wells is indicated by the broken lines. OD, Optical density.

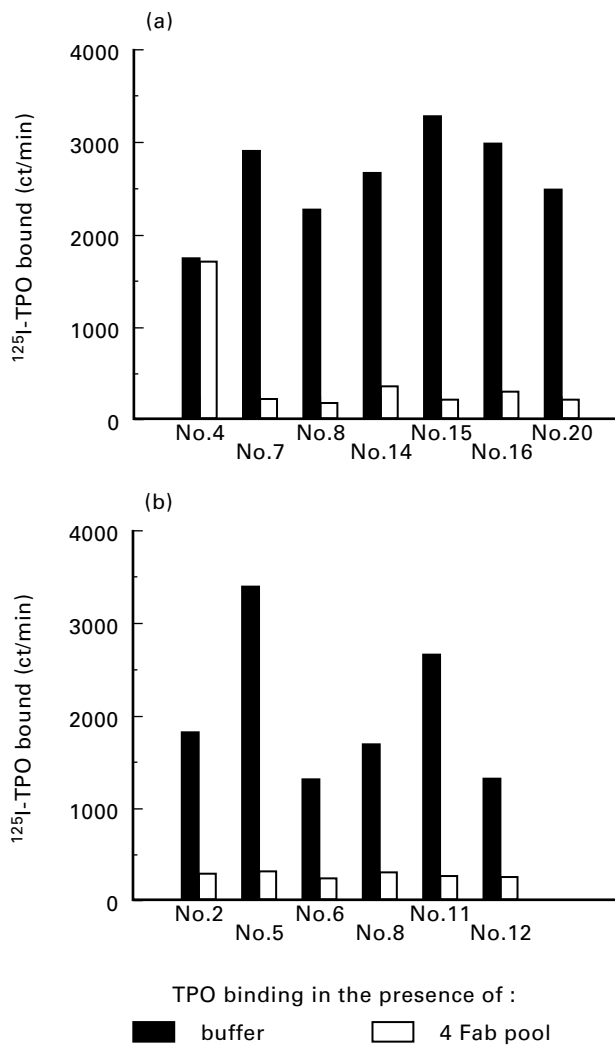


Fig. 3. The epitopes of thyroid peroxidase (TPO)-binding clones selected by panning on denatured (DN)-TPO (a) or on native (N)-TPO (b), with one exception (clone DN4), lie within the immunodominant region. Competition assays for radiolabelled TPO binding were performed between newly cloned TPO-binding Fab and four previously isolated Fab that define the immunodominant region [16]. Data are presented as ct/min ¹²⁵I-TPO bound.

Fab binding to ¹²⁵I-TPO and Fab affinities for TPO

TPO-specific Fab with κ -chains were incubated with ¹²⁵I-TPO (approx. 20 000 ct/min) and murine monoclonal anti-human κ (QE11) as described [18]. After addition of anti-mouse IgG-coated beads (Sac-cel) to capture the immune complexes, the beads were sedimented and counted to determine the percentage ¹²⁵I-TPO bound. The Fab affinities for TPO were determined by Scatchard analysis of ¹²⁵I-TPO binding in the presence of increasing concentrations of unlabelled TPO.

Interaction of TPO-specific Fab with the immunodominant domain on TPO

A modification of the above assay was used to determine the interaction of newly cloned Fab with epitopes in the TPO immunodominant region (reviewed in [14]). As detailed previously [18], test Fab were immobilized with anti-human κ (QE11) and anti-mouse IgG-coupled beads (Sac-cel). Remaining anti- κ binding

sites were saturated with normal human serum. Aliquots of ¹²⁵I-TPO (approx. 20 000 ct/min) preincubated in buffer or with a pool of four Fab that define the immunodominant region (SP1.4, WR1.7, TR1.8 and TR1.9; each at 10^{-8} M) [16] were added to the immobilized test Fab pellets. After incubation and washing, radioactivity bound to the Sac-cel was counted. Non-specific binding (approx. 3% of total counts) was subtracted to provide specific binding to TPO.

RESULTS

Phage screened on denatured TPO preferentially recognize native TPO

The yield of phage eluted after panning the GA-pComb3H library on DN-TPO increased progressively; approx. 10^{-4} %, approx. 5×10^{-4} % and 2×10^{-3} % after rounds I, II and III, respectively. Comparable yields were observed for phage from the same library panned on N-TPO (approx. 10^{-4} %, approx. 10^{-3} % and 4×10^{-3} %). After three panning rounds, filter lift assays indicated that approx. 50% individual colonies selected using DN-TPO recognized ¹²⁵I-DN-TPO (Fig. 1a) and approx. 50% colonies selected using N-TPO recognized ¹²⁵I-N-TPO (Fig. 1b). Surprisingly, in a cross-comparison, the DN-selected colonies recognized N better than DN ¹²⁵I-TPO (Fig. 1c versus Fig. 1d).

The efficacy of denaturation of DN-TPO used in panning and as tracer in these studies was confirmed in two ways. First, ¹²⁵I-TPO binding by patients' autoantibodies (sera diluted 1:200) was markedly lower to DN-TPO than to N-TPO, $8.9 \pm 4.6\%$ (mean \pm s.e.m.; $n = 11$ sera) versus $35.4 \pm 4.5\%$ ($t = 14.41$, $P < 0.001$, paired t-test). This magnitude of reduction in TPO autoantibody binding is comparable to values previously obtained by others for Graves' and Hashimoto sera [2]. In contrast, the same DN-TPO was bound to a greater extent than N-TPO (46.5% versus 17.6%, respectively) by murine MoAb 47 (diluted 1:10 000) that recognizes denatured and native TPO [12]. Second, after direct application to filters, sera from patients GA and FA (1:20 and 1:200) bound ¹²⁵I-DN-TPO (Fig. 1e) to a much lesser extent than N-TPO (Fig. 1f). Similarly, a streak of TPO-specific Fab WR1.4 bacteria also gave strong signal with N-TPO (Fig. 1b,c) and a much weaker or negative signal with DN-TPO (Fig. 1a,d). Consistent with the direct binding assay, murine MoAb 47 recognized both native and denatured TPO even at a dilution of 1:2000 (Fig. 1e,f).

The autoradiographic observations for TPO selected colonies were confirmed quantitatively by ELISA at different stages of library panning on DN-TPO, as well as for individual clones after the third panning round. Enrichment for TPO-binding phage was evident in the library after round II, at which stage there was preferential recognition of N-TPO versus DN-TPO (Fig. 2a). A similar preference for N-TPO was seen after round III. The greater enrichment at this stage required greater library dilution to overcome saturation of available binding sites on ELISA wells.

To reduce the likelihood of characterizing multiple identical clones, we tested 13 DN-TPO-selected and 11 N-TPO-selected clones for differences in BstN1 restriction patterns of their DNA (data not shown). Quantification of binding activity of soluble Fab expressed from individual clones revealed significantly greater recognition of N-TPO versus DN-TPO for 7/7 clones selected with DN-TPO (Fig. 2b, $t = 11.369$, $P < 0.0003$; paired t-test), as well as for 6/6 clones selected with N-TPO (Fig. 2c, $t = 17.05$, $P < 0.00001$). Therefore, regardless of whether they were selected on DN-TPO or on N-TPO, all clones preferentially recognized N-TPO.

(a)

SPYSDL D REGION

		FR1	CDR1	FR2
DN	16	VQLLEQSGAQVKKPGASVKVSCASGYSFT	IYPIHWVRQAPGQRLEWMMG	LIHAGNSNTKYSQKFQG
DN	20	VQLLEQSGAQVKKPGASVKVSCXSGYSFT	IYPIHWVRQAPGQRLEWMMG	LIHAGNSNTKYSQKFQG
N	11	VQLLEQSGAEVKKPGASVKVSCASGYSFT	IYPIHWVRQAPGQRLEWMMG	WIHAGNGNTKYSQKFQG
N	2	-----VSCTVCGYSFN	IYXIHWRQAPGLWHEWMMG	LIHVGNSDTKYSQFLC
N	5	VQLLEQSGAEVKKPGASVKVSCASGYSFT	IYPIHWVRQAPGQRLEWMMG	WIHAGNGNTKYSQEFQG
N	8	VQLLRQSGAEVKKHGASVKVSCASGYSFT	IYPIHWVRQAPGERLEWMMG	LIHAGNSNTKYSQKFQG
N	12	VQLLEESGAEVKKPGASVKVSCASGYSFS	SYGVHWVRQAPGQSLEWMMG	WIHAGTGNTKYSQKFQD
G (N)	2	VKLLLEQSGLEVKKPGASVKVSCASGYSFT	IYPLHWVRQAPGQNLEWMMG	WIHAGTGNSKYSQKFQG
G (N)	3	VKLLLEQSRAEVKKPG-SVKVSCASGYSFT	IYPMHWVSQAPGQRLEWMMG	LIHSGNGNTKYSQKFQG
G (N)	6	VKLLLEQSGRLKPKPGASVKVSCASGYSFT	IYPIHWVQAPGQRLEWMMG	LIHAGNSNTKYSQKFQG
G (N)	9	-----	-----	-----GNTKYSQKFQG
V1-3b		VQL-VQSGAEVKKPGASVKVSCASGYTFT	SYAMHWVRQAPGQRLEWMMG	WINAGNGNTKYSQKFQG
		FR3	CDR3	FR4 (JH4)
DN	16	RVTFTTRDTSASTAYMELSSLRYEDTAMYFCAR	SPYSDL DI	WGQGTTLVQVSS
DN	20	RVTFTTRDTSASTAYMELSSLRYEDTAMYFCAR	SPYSDL DI	WGQGTTLV----
N	11	RVTFTTRDTSASTAYMELSSLRYEDTAMYFCAR	SPYGDY	WGQGTTLVTS-
N	2	RVTFTTRYTSASTAYMVLNIRYEDTAMYFCAR	SPYSDF DI	WRQGTTLVTVSS
N	5	RVTFTTRDTSASTAYMELSSLRYEDTALYFCAR	SPYGDY	WGQGTTLVTVSS
N	8	RVTFTTRDTSASTAYMELSSLRHEDTAMYFCAR	SPYNDI DI	WGQGTTLVRVSS
N	12	RVTIIRDTSASTAYMELSSLRSEDVAVYFCAR	DPYSWGDEL DY	WGQGTTLVTVSS
G (N)	2	RVTFTTRDTSASTAYMELSSLRYEDTALYFCAR	SPYGDY	WGQGTTLVTVSS
G (N)	3	RVTFTTRDTSASTAYMELSSLRYEDTAMYFCAR	SPYSDL DY	WGQGTTLVTVSS
G (N)	6	RVTGTRDTSASTAYMELSSLRYEDTAMYFCAR	SPYSDL DI	WGQGTTLVSVSS
G (N)	9	RVTGTRDTSASTAYMELSSLRYEDTAMYFCAR	SPYSDL DI	WGQGTTLVTVSS
V1-3b		RVTITRDTASTAYMELSSLRSEDVAVYFCAR	YFDY	WGQGTTLVTVSS

Fig. 4. (See pp 24 and 25) Similar H chain V regions encode thyroid peroxidase (TPO) binding Fab selected using native (N) or denatured (DN)-TPO. Derived amino acid sequences are shown for: (i) clones selected from the GA-pComb3H phage display library by panning on DN-TPO (DN clones); (ii) clones selected from the same library by panning on N-TPO (N clones); (iii) clones isolated using ¹²⁵I-N-TPO from the GA-bacteriophage λ library (G(N) clones). Clone grouping is based on their D regions: (a) 'SPYSDL' group; (b) 'ENGLNLAFF' group; (c) 'VAAPAYNFAV' group and the single clone with D region VERAPEAYYYRGGT. Included for comparison are the amino acid sequences of their closest germ-line genes, namely V1-3b, V1-2 and hv1263 [26]. Note, because of their high degree of mutation, the D region germ-line genes could not be assigned and the DJ junctions shown, except for clone DN4, are arbitrary.

Recognition of the TPO immunodominant region

Despite recognizing conformationally intact TPO, it was possible that clones selected using DN-TPO might differ in other aspects from clones identified using N-TPO. Consequently, we investigated whether the epitopes of the newly isolated TPO-binding clones lay within the TPO immunodominant region recognized by all patients' sera, as previously defined by a pool of four human monoclonal autoantibody Fab [16]; reviewed in [14].

Competition studies with the four Fab pool revealed that, with one exception, the newly isolated TPO-binding Fab interacted with epitopes in the TPO immunodominant region. Thus, these four Fab inhibited ¹²⁵I-TPO binding by Fab generated from 6/7 clones selected on DN-TPO (Fig. 3a) and from 6/6 clones selected on N-TPO (Fig. 3b). Quantitatively, the mean inhibition for the six DN-TPO-selected and six N-TPO-selected clones was similar:

91 ± 3% and 84 ± 5% inhibition of ¹²⁵I-TPO binding, respectively. In contrast, in two separate experiments (one shown in Fig. 3), TPO binding by DN-selected clone no. 4 (DN4) was unaffected by the presence of the four Fab pool. Consequently, unlike the epitopes of the other 12 clones from patient GA, the epitope of DN4 lies outside the TPO immunodominant region.

H and L chain variable region genes for clones selected with DN-TPO and N-TPO

We determined the H and L chain V region nucleotide sequences of the seven DN-TPO-selected clones ('DN-clones') and the six N-TPO-selected clones ('N-clones') isolated from the GA-pComb3H library. In addition, we characterized 12 clones from a bacteriophage λ (ImmunoZap) library constructed using H and κ-chain cDNA from the same patient. These clones, termed 'G(N)', were

(b)

ENGPLNTAFF D REGION					
		<u>FR1</u>	<u>CDR1</u>	<u>FR2</u>	<u>CDR2</u>
DN	14	EVQLLES-GAELKKPGASVKVSKASGYTFT	AYYIH	WVRQAPGQGLEWMG	WINPNSGGTNYAQKFQG
N	6	EVQLXEQSGAEVKKPGASVKVSKXSGYTFS	AYYVH	WVRQAPGQGLEWMG	WINPNSGGTNYPPQKFQG
G (N)	1	-----GASVKVSKASGYTFT	AYYIH	WVRQAPGQGLEWMG	WINPNSGGTNYAQKFQG
G (N)	4	-----GASVKVSKASWYTFT	AYYIH	WVRQAPGQGLEWMG	WINPNSGGTNYAQKFQG
G (N)	17	-----EVKKLGASVNVSKASGYTFT	AYYIH	WVRQVPDQGLEWMG	WINPNSGGTNYAQNFQG
G (N)	19	-----QSGAEVKKLGLSVKVSCKASGYTFT	GYIYH	WVRQAPVQGLEWMG	WINPNSGGTNYAQKFQG
G (N)	22	-----	-----	-----WMG	WINPYCGGTNITQKFQG
V1-2/ hv1L1		QVQL-VQSGAEVKKLGLSVKVSCKASGYTFT	GYMH	WVRQAPGQGLEWMG	WINPNSGGTNYAQKFQG
		<u>FR3</u>	<u>CDR3</u>		<u>FR4 (JH6)</u>
DN	14	RVTMTRDTSSTAYMDLSRLTSDDTAVYYCAR	ENGPLNTAFF	YGLDV	WGQGTTVTVSS
N	6	RVTLTRDTSINTVYMDLSRLTSDDTAVYYCAR	EGVPINTANS	YGMDV	WGQGTTVTFSS
G (N)	1	RVTMTRDTSSTAYMDLSRLTSDDTAVYYCAR	ENGGNTAFF	SGLAV	WGQGTTVTVSS
G (N)	4	RVTMTRDTSSTAYMDLSRLTSDDTAVYYCAR	ENGPLNTAFF	YGLDV	WGQGTTVTVSS
G (N)	17	RVTMTRDTSSTAYMDLSRLRSDDTAVYYCAR	ESGPINTAFF	YVWNV	WGQGTTVTVSS
G (N)	19	RVTMTRDTSSTAYMDLSRLTSDDTAVYYCAR	ENGPLNTAFF	YGLDV	WGQGTTVTVSS
G (N)	22	RVTMTRDTSSTAYMDLSRLTSDDTAVYYCAR	ENGPLNTAFF	YGLDV	WGQGTTVTVSS
V1-2/ hv1L1		RVTMTRDTSISTAYMELSRRLRSDDTAVYYCAR		YYYYGMDV	WGQGTTVTVSS

Fig. 4. continued (See previous page for caption)

identified by filter lift assays using ^{125}I -labelled N-TPO. All TPO binding clones were classified in terms of the derived amino acid sequences of their H chain D regions. Regardless of whether they were selected with DN-TPO or N-TPO (pComb3H vector) or by using ^{125}I -N-TPO (Immunozap vector), the TPO binding clones comprise four groups: (i) the largest group has 'SPYSDL' D regions, $V_{\text{H}}1$ family genes derived from V1-3b/DP-25 germ-line genes [26] and $J_{\text{H}}4$ regions (Fig. 4a). This group includes two DN-TPO clones, five N-TPO clones and four G(N) clones obtained by screening with ^{125}I -N-TPO; (ii) the second group has 'ENGPLNTAFF' D regions, V_{H} regions related to the germ-line gene V1-2 [26], also known as hv1 I1, and modified $J_{\text{H}}6$ regions (Fig. 4b). This group includes one DN clone, one N clone and five G(N) clones; (iii) the third group has 'VAAPAYNFAV' D regions, $V_{\text{H}}1$ genes related to V1-3b/DP-25 [26] and truncated $J_{\text{H}}6$ regions (Fig. 4c). Three DN clones and three G(N) clones are within this group; (iv) finally, DN4, the unique clone that interacts with an epitope outside the immunodominant region also uses genes distinct from all the other TPO-binding clones. Thus, DN4 has a unique D region, 'VERAPEAYYGGGYT', as well as a V_{H} region related to the germ-line gene hv1263 [26] and a complete $J_{\text{H}}6$ region (Fig. 4c). The combination of this D region and a non-truncated $J_{\text{H}}6$ creates a very long CDR3.

In terms of their κ -chains, partial sequencing of the CDR2 regions (Fig. 5) showed that the majority of the 'SPYSDL' D region group, as well as all of the 'VAAPAYN' D region group, have O12/O2 [26]-like L chains. In contrast, all clones with 'ENGPLNTAFF' D regions have V_{κ} III chains derived from germ-line gene Vg [26]. One other Fab, N12, with a greatly modified 'SPYSDL' D region, has a V_{κ} III chain derived from

A27 [26]. Although the VDJ region of clone DN4 is unique among TPO-binding clones isolated from this patient, it has an O12-like κ -chain (like many other TPO-binding Fab in this panel).

Affinity for TPO and specificity of clone DN4 compared with other DN-TPO-selected clones

Because of its unusual VDJ region, and because its epitope lies outside the TPO immunodominant region, we examined other immunological properties of the Fab expressed by clone DN4 Fab: (i) on flow cytometry, Fab DN4, like another representative Fab (DN14), bound native TPO expressed on intact eukaryotic cells (mean fluorescence intensity (MFI) value > 110 units for both Fab compared with 30 units in the presence of second antibody only); (ii) unlike other Fab representative of the three major D region groups (DN8, DN14, and DN16), ^{125}I -TPO binding by DN4 was only marginally inhibited by concentrations of unlabelled TPO as high as $2 \times 10^{-8}\text{ M}$ unlabelled TPO (Fig. 6a). Fab DN8, DN14 and DN16 had high affinities for TPO, as calculated by Scatchard analysis of the binding data, namely $K_{\text{d}} 1.5 \times 10^{-10}\text{ M}$, $2.6 \times 10^{-10}\text{ M}$ and $1.2 \times 10^{-10}\text{ M}$, respectively. In contrast, the affinity of Fab from DN4 was too low to be measured; (iii) despite its low affinity, Fab DN4 bound specifically to TPO (Fig. 6b). Thus, like Fab DN14, N2 and N6, Fab DN4 gave a strong signal on ELISA wells coated with TPO, but not with the related enzymes LPO and MPO, Tg, and with two non-autoantigens, namely transferrin and KLH.

DISCUSSION

The phage display system provides a powerful means of searching

(c)

VAAPAYN D REGION

		FR1	CDR1	FR2	CDR2
DN	8	EVQLLEQSGAEVKKPGASVRVSVCKASGYMIS	TYTMH	WVRQAPGHRPEWMG	WINADNGRTRYSQRFQG
DN	15	EVQLLEQSGAEVKKPGASVRVSVCKASGYMIS	TYTMH	WVRQAPGHRPEWMG	WINADNGRTRYSQRFQG
DN	7	EVQLLEQSGAEVKKPGASVRVSVCKASGYMIS	TYTMH	WVRQAPGHRPEWMG	WINADNGRTRYSQRFQG
G (N)	7	-----GASVRVSVCKASGFMYIS	TYTIH	WVLQAPGHRPEWMG	WINADNAKTRYSQRFQG
G (N)	5	--QL-EQSGAEVKKPGASVKVSVCKASGYTFT	TYTMH	WVRQAPGQGLEWMG	WINADNGRTRYSQRFQG
V1-3b		QVQL-VQSGAEVKKPGASVKVSVCKASGYTFT	SYAMH	WVRQAPGQRLEWMG	WINAGNGNTKYSQKFKQG
		FR3	CDR3	FR4 (JH6)	
DN	8	RVTITRDTIANTAYMELSSLSSDDTAVYYCAR	VAAPAYN	FAVDV	WGQGTTVIVSS
DN	15	RLTITRDTIANTAYMELSSLTSDDTAVYYCAK	VAAPAYN	FAVDV	WGQGTTVIVSS
DN	7	RVTITRDTIANTAYMELSSLSSDDTAVYYCAK	VAAPAYN	FAVDV	WGQGTTVIVSS
G (N)	7	RVTITRDKIANTAYMELSSLRSEDVAVYYCAK	VAAPAYN	FAVDV	WGQGTTVTVSS
G (N)	5	RVTITRDTIANTAYMELSSLSSDDTAVYYCAK	VAAPAYN	FAMDV	WGQGTTVTVSS
V1-3b		RVTITRDTSASTAYMELSSLRSEDVAVYYCAR		YYYYGMDV	WGQGTTVTVSS

VERAPEAYYRGGGYT D REGION

		FR1	CDR1	FR2	CDR2
DN	4	EVQLLEQSGAEVKKPGSSVKVSVCKASGGTFS	KDGIS	WVRQAPGQGLEWMG	RIITMFDVANYAQKFKQG
hν1263		QVQLV-QSGAEVKKPGSSVKVSVCKASGGTFS	SYAIS	WVRQAPGQGLEWMG	RIIPILGIANYAQKFKQG
		FR3	CDR3	FR4 (JH6)	
DN	4	RVTITADKSTSTAYMELSNLRSDDTAVYYCAR	VERAPEAYYRGGGYT	YYSPMDV	WGQGTTVTRTS
HV1263		RVTITADKSTSTAYMELSSLRSEDVAVYYCAR		YYYYGMDV	WGQGTTVTVSS

Fig. 4. continued. (See p 23 for caption)

an immunoglobulin gene combinatorial library for rare antibody specificities. We therefore used this approach to search for human autoantibodies preferentially reactive with denatured autoantigen in a combinatorial library generated from thyroid-infiltrating plasma cells of a typical patient with autoimmune thyroid disease. Surprisingly, both the library enriched by panning on denatured (reduced and alkylated) TPO, as well as individual clones from this enriched library, interacted preferentially with native TPO. The efficacy of reduction and alkylation in changing the TPO natural status was confirmed by a direct binding assay and by reverse immunoblot using human sera, a human TPO-specific autoantibody Fab [16] and a mouse MoAb that recognizes N- and DN-TPO [12]. We cannot exclude the possibility that a small proportion of TPO remained in its native form. However, the presence of minimal amounts of N-TPO is unlikely to have influenced the outcome of the study, since previous studies have indicated that antigen-specific phage present at frequencies of less than 1 in a billion can be isolated from a phage display library by repeated rounds of selection [21].

It is possible that extrathyroidal sites of thyroid autoantibody synthesis, such as lymph nodes draining the thyroid gland (reviewed in [28]), may contain B cells specific for denatured TPO. However, TPO-reactive Fab isolated from thyroid and lymph node of the same patient were remarkably similar in terms of their

V_H and V_K gene sequences and recognition of native TPO [17,29]. Our present observations from panning a thyroid-derived library with N- and DN-TPO, together with information from screening thyroid or lymph node libraries with N-TPO (for example [16–20,29]), suggest the rarity of human autoantibodies with specificity for denatured TPO. Consequently, conformationally intact TPO, normally expressed on the surface of thyroid cells, is likely to play a critical role in selection of the autoimmune B cell repertoire in autoimmune thyroid disease, the most common organ-specific autoimmune condition in humans.

The second major finding of our study is that, of 12 new human TPO-specific MoAbs isolated using denatured or native autoantigen, all but one recognized the immunodominant region on native TPO (reviewed in [14]). Combining these observations with data on previously characterized MoAbs obtained by two different laboratories [15], 97% (34/35) of TPO-specific human MoAbs identified from seven different patients interact with epitopes in the immunodominant region. Our findings reinforce the concept that the B cell repertoire in human autoimmune thyroid disease is focused on a restricted set of overlapping epitopes on the native 107-kD TPO molecule.

The basis for restricted epitopic recognition on TPO by spontaneously arising human autoantibodies (reviewed in [14]), versus diverse epitopes in experimentally immunized animals [12],

ENGPLNTAFF D REGION

		CDR1	FR2	CDR2	FR2
DN	14	-----	----KPGQAPRLLIY	DASSRAT	GIPPRFSGSGS-GDFTLTISLLEPEDFAVYY
N	6	-----SNYLA	WYQQKPGQAPRLLIY	HASSRAT	GNPARFRPSGSGTDFTLTISLLEPEDFAVYY
G (N)	1	-----SSYLA	WYQQKPGQAPRLLIY	DASNRAT	GMGARFSGSGSGTDFTLTISLLEPEHFVYY
G (N)	4	-SQSISNYLA	WYQQKPGQAPRLLIY	DASSRAT	GIPARFSGSGSGTDFTLTISRLEPEDFAVYY
G (N)	17	-SQSISNYLA	WYQQ-PGQAPRLLIY	DASSRAT	GIPARFSGSGSGTDFTLTISLLEPEDFAVYY
G (N)	19	-SQSVSSYLA	WYQQKPGQAPRLLIY	DASFRAT	GIPARFSGSGSGTDFTLTISLLEPEDFAVYY
G (N)	22	-----	---QKPAQAPRLLIY	DASSRAT	GIPARFSGSGSGTDFTLTITDLEPEDFAVYY
Vg		-SQGVSSYLA	WYQQKPGQAPRLLIY	DASNRAT	GIPARFSGSGPGTDFTLTISLLEPEDFAVYY

SPYDL D REGION

DN	16	--YLN	WYQQKPAAPKLVIF	AASSLQS	GVPSRFSGSGSGTDFTLTISDLQPEDFATYY
DN	20	-TYVN	WYQQRPGKAPKLLIF	AASSLQS	GVPSRFSGSGSGTHFTLTISLQPEDFATY-
N	11	-----	-----GKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISLQPEAFATYS
N	2	-----	-----NPGTAPKLLIY	AASTLQS	GVTSRFSGSGSGTEFTLTITSLQPEDFATY
N	5	-NYLN	WYQQKPGKAPKLLIY	AASSLES	GVPSRFSGSASGTDFTLTISLQPEDFATY-
N	8	-TYLN	WYQQKPGKAPKSLIY	ATSSLQS	GVPSRFSGSGSETDFTLTISLQPEDFATD-
G (N)	2	-SYLN	WYQQKPGKAPTLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTINSLQREDFGTY
G (N)	3	-SYLN	WYQRKPGKAPNLLIY	AASSLQS	GVPSRFSGSGSPTDFTLTISLQPEDFATYY
G (N)	6	-NYLN	WYQQKPGKAPNLLIY	AASSLQG	GAPSRFSGSGSGTDFTLTISLQPEDFATYS
G (N)	9	-SYLN	WIQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTINSLQPEDFATYY
O12		-SYLN	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISLQPEDFATYY
N	12	-SQSVSSSYKA	WYQQKPGQAPRLVIY	GASSRAT	GIPDRFSGSGSGTANTLTISRLEPEDFAMY-
A27		-SQSVSSSYLA	WYQQKPGQAPRLLIY	GASSRAT	GIPDRFSGSGSGTDFTLTISRLEPEDFAVYY

VAAPAYNFAV D REGION

DN	8	-----	----KPGKAPKLLIY	-AFNLQS	GVPSRFSGSGPGTDFTLTISLQPEDFASY
DN	15	-----	----KPGRAPKLVVF	AASSLQS	GVPSRFSGSGSGTEFTLTISLQPEDFA---
DN	7	-----	----PGKAPKLMIIY	AASSLQS	GVPSRFSGSGSGTDFTLTISLQPEDFATYF
G (N)	5	---SSYVS	WYQQKPGKAPRVLIIY	AASSLQS	GVPSRFSGSGSGTDFTLTISLQREDFGTY
G (N)	7	---SSYLN	WYQQKPGKAPNLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISLQPEDFATYY
O12		---SSYLN	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISLQPEDFATYY

VERAPEAYYRGGGYT D REGION

DN	4	-SSYLN	WYQQKPGKAPKLLIY	AASSFES	GVPSRFSGRSGADFTLTISLQPEDFASY
O12		-SSYLN	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISLQPEDFATYY

Fig. 5. Similar L chain variable regions encode thyroid peroxidase (TPO) binding Fab selected using native (N) or denatured (DN)-TPO. Derived amino acid sequences (partial) are shown for clones selected from: (i) the GA-phage display library panned on DN-TPO (DN clones); (ii) the same library panned on N-TPO (N clones); (iii) the GA-bacteriophage λ library using ^{125}I -N-TPO, 'G(N)' clones. The clones are grouped according to the derived amino acid sequences of their H chain D regions (see Fig. 6). Included for comparison are the amino acid sequence of their closest germ-line genes, namely Vg; O12/O2 and A27 [26].

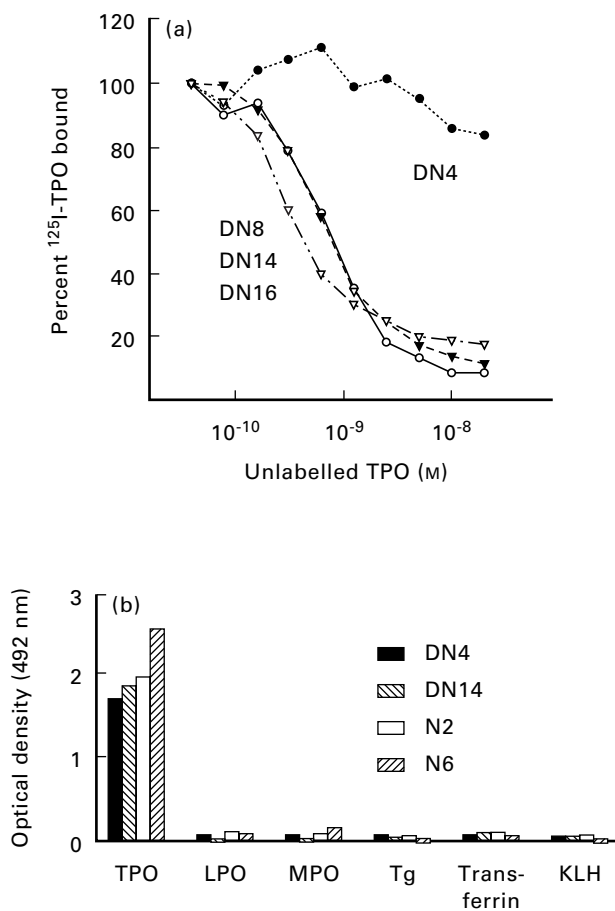


Fig. 6. Fab DN4, that does not recognize the thyroid peroxidase (TPO) immunodominant region, has a low affinity for TPO (unlike other TPO-selected Fab), yet it binds specifically to TPO (like other native (N)-TPO and denatured (DN)-TPO selected Fab). (a) ¹²⁵I-TPO bound (% maximum) in the presence of increasing concentrations of unlabelled TPO by Fab DN4 and three other DN-TPO-selected clones representative of clones with the other D regions. Binding in the absence of unlabelled TPO: DN4, 21.8%; DN8, 27.2%; DN14, 18.3%; DN16, 19.5%. (b) Optical densities (492 nm) for Fab DN4, DN14, N2 and N6 incubated with ELISA wells coated with TPO, lactoperoxidase (LPO), myeloperoxidase (MPO), thyroglobulin (Tg), transferrin (Tsf) and keyhole limpet haemocyanin (KLH). Fab binding was detected with mouse MoAb to human κ -chains. All samples were analysed simultaneously and data are the means of closely agreeing duplicate wells.

is not known. Similar observations have been made for autoantibodies in other autoimmune diseases such as to the acetylcholine receptor in myasthenia gravis [30]. We have suggested [31] that this restricted B cell epitopic recognition may result from the ability of high-affinity antigen-specific B cells to capture and influence the peptides that are ultimately presented to T cells. Several studies have shown that human B cell clones specific for different epitopes on the same antigen (tetanus toxoid) enhance or suppress presentation of different peptides to T cells [32].

The conclusion that clones reactive with native autoantigen dominate the B cell repertoire is supported by our present observation that similar combinations of H and L chain V region genes encode TPO-binding human MoAb isolated from a phage display library using either denatured or native TPO. Similar VDJh and V κ

chain gene combinations were also found in TPO-binding clones isolated from the same thyroid-derived immunoglobulin gene cDNA in a bacteriophage vector using native TPO. Thus, with one exception (see below), all three approaches yielded Fab with three basic VDJh/ κ combinations, regardless of whether the libraries were screened on denatured or native TPO (summarized in Table 1). Incidentally, the presence of the same VDJh and V κ combinations in TPO-binding Fab isolated from two different libraries (pComb3H and Immunozap), each containing random combinations of the same H and L chain cDNA, supports our earlier findings. That is, in 'roulette' studies using a TPO-specific H chain to search the parent L chain library for other L chains compatible with TPO binding, we pulled out the same type of L chains, and vice versa (reviewed in [14]).

Clone DN4 has a VDJh/V κ combination unique for this patient, namely a V κ derived from hv1263, a D region 'VERA-PEAYYYRGGT' and a J κ 6 paired with an O12-like V κ chain (Table 1). Also unique to this autoantibody is that it does not interact with the TPO-immunodominant region. The use of an hv1263-like V κ region is not unusual, as H chains derived from this germ-line gene were used by TPO autoantibodies previously isolated from thyroid tissue of a different patient [16,25]. However, unlike the hv1263/O12 combination in DN4, in previously reported TPO autoantibodies the hv1263-like H chains were paired with L chains related to the V κ III family gene A3. Most striking about TPO clone DN4 is its very long CDR3 region (24 amino acids), longer than those of (i) other Fab cloned from thyroid cDNA of this patient (8–15 amino acids); (ii) the previously reported TPO autoantibodies with hv1263-like H chains (15 amino acids) [16,25]; and (iii) other TPO autoantibodies with J κ 6 regions (12–14 amino acids) [16,29]. Though antigen-specific, DN4 has a very low affinity for TPO, in contrast to the high affinities of other TPO autoantibodies with hv1263-H chains [16,25] and the other TPO autoantibodies characterized in the present study (Kd approx. 2×10^{-10} M). In the context of DN4's low affinity, it is intriguing that hv1263 is a member of the fetally expressed 51P1 group of genes [33]. However, DN4 uses a mutated, not a germ-line, form of hv1263. Instead, the low affinity of DN4 may be related to its unusually long CDR3 region.

Our observations are relevant to B cell repertoire selection in organ-specific human autoimmune diseases in general. For example, besides TPO, preferential interaction with native rather than denatured autoantigen is observed for IgG class autoantibodies to GAD [34] and the acetylcholine receptor (myasthenia gravis) [30]. Moreover, the association between high serum levels of autoantibodies and reactivity to denatured GAD in Stiff Man syndrome versus much lower titres and lack of binding to denatured autoantigen in insulin-dependent diabetes mellitus (IDDM) type I [34] raises the possibility that B cells specific for the denatured form of the protein only arise late in the autoimmune process.

Autoantibodies can directly mediate disease (as in myasthenia gravis and Graves' hyperthyroidism) and provide invaluable pathological markers (IDDM type I). Equally important, autoantibodies are likely to play a vital role in antigen presentation to T cells (as discussed above). Indeed, very recently it has been shown that B cells are crucial antigen-presenting cells in the non-obese diabetic (NOD) mouse model of IDDM type I [35]. T cell activation requires the processing of antigens to relatively simple linear epitopes. In contrast, because conformationally intact autoantigen is likely to be important during the early stage of B cell repertoire selection in human autoimmunity, understanding this

Table 1. Screening of thyroid-derived combinatorial immunoglobulin gene libraries with denatured (DN) or native (N)-thyroid peroxidase (TPO) yields Fab that, with one major exception, are encoded by similar V_H and V_L chain combinations. Fab grouping is based on the derived amino acid sequences of their D regions and the number of clones represented by each V_H/V_L combination is indicated in parentheses

CDR3 region			GA-pComb3H		GA-Immunozap
	Derived amino acid sequence	J _H	Length	DN-TPO V _H /V _L	N-TPO V _H /V _L
ENGPLNTAFFxxxxV	J _H 6	15	V1-2/Vg (1)	V1-2/Vg (1)	V1-2/Vg (3)
SPYSDLDIY	J _H 4	8	V1-3b/O12 (2)	V1-3b/O12 (4)	V1-3b/O12 (4)
DPYSWGGELDY	J _H 4	11	–	V1-3b/A27 (1)	–
VAAPAYNFAVDV	J _H 6	12	V1-3b/O12 (3)	–	V1-3b/O12 (4)
VERAPEAYYYRGGGYTYYYSPMDV	J _H 6	24	hv1263/O12 (1)	–	–

process requires knowledge of the folding, glycosylation and cellular trafficking of full length, native protein.

In conclusion, human monoclonal autoantibodies that preferentially recognize a denatured form of a major thyroid autoantigen (TPO) could not be isolated from an immunoglobulin gene library despite selection with denatured protein. Moreover, unlike the majority of TPO-specific MoAbs isolated in this and previous investigations, only one Fab had its epitope primarily outside the TPO immunodominant region. Our findings demonstrate the bias of the human B cell repertoire towards recognition of an immunodominant region on the conformationally intact form of a major thyroid autoantigen.

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REFERENCES

- McLachlan SM, Rapoport B. The molecular biology of thyroid peroxidase: cloning, expression and role as autoantigen in autoimmune thyroid disease. *Endocr Rev* 1992; **13**:192–206.
- Nakajima Y, Howells RD, Pegg C, Davies Jones E, Rees Smith B. Structure activity analysis of microsomal antigen/thyroid peroxidase. *Molec Cell Endocrinol* 1987; **53**:15–23.
- Gardas A, Domek H. The effect of sulphhydryl reagents on the human thyroid microsomal antigen. *J Endocrinol Invest* 1988; **11**:385–8.
- Hamada N, Jaeduck N, Portmann L, Ito K, DeGroot LJ. Antibodies against denatured and reduced thyroid microsomal antigen in autoimmune thyroid disease. *J Clin Endocrinol Metab* 1987; **64**:230–8.
- Bermann M, Magee M, Koenig RJ, Kaplan MM, Maastricht J, Johnson J, Baker JR Jr. Differential autoantibody responses to thyroid peroxidase in patients with Graves' disease and Hashimoto's thyroiditis. *J Clin Endocrinol Metab* 1993; **77**:1098–101.
- Finke R, Seto P, Ruf J, Carayon P, Rapoport B. Determination at the molecular level of a B-cell epitope on thyroid peroxidase likely to be associated with autoimmune thyroid disease. *J Clin Endocrinol Metab* 1991; **73**:919–21.
- Libert F, Ludgate M, Dinsart C, Vassart G. Thyroperoxidase, but not the thyrotropin receptor, contains sequential epitopes recognized by autoantibodies in recombinant peptides expressed in the pUEx vector. *J Clin Endocrinol Metab* 1991; **73**:857–60.
- Banga JP, Barnett PS, Ewins DL, Page M, McGregor AM. Mapping of autoantigenic epitopes on recombinant thyroid peroxidase fragments using the polymerase chain reaction. *Autoimmunity* 1990; **6**:257–68.
- Maastricht J, Koenig RJ, Kaplan MM, Arscott P, Thompson N, Baker JR Jr. Identification of localized autoantibody epitopes in thyroid peroxidase. *J Clin Endocrinol Metab* 1992; **75**:121–6.
- Zanelli E, Henry M, Malthiery Y. Use of recombinant epitopes to study the heterogeneous nature of the autoantibodies against thyroid peroxidase in autoimmune thyroid disease. *Clin Exp Immunol* 1992; **87**:80–86.
- Arscott PL, Koenig RJ, Kaplan MM, Glick GD, Baker JR Jr. Unique autoantibody epitopes in an immunodominant region of thyroid peroxidase. *J Biol Chem* 1996; **271**:4966–73.
- Ruf J, Toubert M, Czarnocka B, Durand-Gorde J, Ferrand M, Carayon P. Relationship between immunological structure and biochemical properties of human thyroid peroxidase. *Endocrinol* 1989; **125**:1211–8.
- Czarnocka B, Pastuszko D, Carayon P, Ruf J, Gardas A. Majority of thyroid peroxidase in patients with autoimmune thyroid disease are directed to a single TPO domain. *Autoimmunity* 1996; **23**:145–54.
- McLachlan SM, Rapoport B. Genetic and epitopic analysis of thyroid peroxidase (TPO) autoantibodies: markers of the human thyroid autoimmune response. *Clin Exp Immunol* 1995; **101**:200–6.
- Guo J, McIntosh RS, Czarnocka B, Weetman A, Rapoport B, McLachlan SM. Relationship between autoantibody epitopic recognition and immunoglobulin gene usage. *Clin Exp Immunol* 1998; **111**:408–14.
- Chazenbalk GD, Portolano S, Russo D, Hutchison JS, Rapoport B, McLachlan SM. 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* 1993; **92**:62–74.
- Czarnocka B, Janota-Bzowski M, McIntosh RS, Asghar MS, Watson PF, Kemp EH, Carayon P, Weetman AP. Immunoglobulin G kappa anti-thyroid peroxidase antibodies in Hashimoto's thyroiditis: epitope mapping analysis. *J Clin Endocrinol Metab* 1997; **82**:2639–44.
- Portolano S, Chazenbalk GD, Seto P, Hutchison JS, Rapoport B, McLachlan SM. Recognition by recombinant autoimmune thyroid disease-derived Fab fragments of a dominant conformational epitope on human thyroid peroxidase. *J Clin Invest* 1992; **90**:720–6.
- Hexham JM, Partridge LJ, Furmaniak J, Petersen VB, Colls J, Pegg CR, Rees Smith B, Burton DR. Cloning and characterisation of TPO autoantibodies using combinatorial phage display libraries. *Autoimmunity* 1994; **17**:167–79.
- Portolano S, Prummel MF, Rapoport B, McLachlan SM. Molecular cloning and characterization of human thyroid peroxidase autoantibodies of lambda light chain type. *Molec Immunol* 1995; **32**:1157–69.
- Marks C, Marks JD. Phage libraries—a new route to clinically useful antibodies. *N Engl J Med* 1996; **335**:730–3.
- Jaume JC, Portolano S, Rapoport B, McLachlan SM. Influence of the

- light chain repertoire on immunoglobulin genes encoding thyroid autoantibody Fab from combinatorial libraries. *Autoimmunity* 1996; **24**:11–23.
- 23 Barbas CF III, Wagner J. Synthetic human antibodies: selecting and evolving functional proteins. *Meth: Comp Meth Enzymol* 1995; **8**:94–103.
- 24 Guo J, McLachlan SM, Hutchison JS, Rapoport B. 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. *Endocrinol* 1998; **139**:999–1005.
- 25 Portolano S, McLachlan SM, Rapoport B. High affinity, thyroid-specific human autoantibodies displayed on the surface of filamentous phage use V genes similar to other autoantibodies. *J Immunol* 1993; **151**:2839–51.
- 26 Tomlinson IMS, Williams SC, Corbett SJ, Cox JBL, Winter G. V BASE Sequence Directory. Medical Research Council Center for Protein Engineering 1998.
- 27 Nishikawa T, Rapoport B, McLachlan SM. Exclusion of two major areas on thyroid peroxidase from the immunodominant region containing the conformational epitopes recognized by human autoantibodies. *J Clin Endocrinol Metab* 1994; **79**:1648–54.
- 28 Rees Smith B, McLachlan SM, Furmaniak J. Autoantibodies to the thyrotropin receptor. *Endocr Rev* 1988; **9**:106–21.
- 29 McIntosh RS, Asghar MS, Kemp EH, Watson PF, Gardas A, Banga JP, Weetman AP. Analysis of IgG kappa anti-thyroid peroxidase antibodies from different tissues in Hashimoto's thyroiditis. *J Clin Endocrinol Metab* 1997; **82**:3818–25.
- 30 Tzartos SJ, Seybold ME, Lindstrom JM. Specificities of antibodies to acetylcholine receptors in sera from myasthenia gravis patients measured by monoclonal antibodies. *Proc Natl Acad Sci USA* 1982; **79**:188–92.
- 31 Guo J, Quarantino S, Jaime JC, Costante G, Londei M, McLachlan SM, Rapoport B. Autoantibody-mediated capture and presentation of autoantigen to T cells via the Fc epsilon receptor by a recombinant human autoantibody Fab converted to IgE. *J Immunol Methods* 1996; **195**:81–92.
- 32 Simitsek PD, Campbell DG, Lanzavecchia A, Fairweather N, Watts C. Modulation of antigen processing by bound antibodies can boost or suppress class II major histocompatibility complex presentation of different T cell determinants. *J Exp Med* 1995; **181**:1957–63.
- 33 Sasso EH, van Dijk KW, Bull AP, Milner ECB. A fetally expressed immunoglobulin VH1 gene belongs to a complex set of alleles. *J Clin Invest* 1993; **91**:2358–67.
- 34 Kim J, Namchuk M, Bugawan T *et al*. Higher autoantibody levels and recognition of a linear NH2-terminal epitope in the autoantigen GAD65, distinguish stiff-man syndrome from insulin-dependent diabetes mellitus. *J Exp Med* 1994; **180**:595–606.
- 35 Falcone M, Lee J, Patstone G, Yeung B, Sarvetnick N. B lymphocytes are crucial antigen-presenting cells in the pathogenic autoimmune response to GAD65 antigen in nonobese diabetic mice. *J Immunol* 1998; **161**:1163–8.