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Thyroid Peroxidase Autoantibodies Obtained from Random Single Chain Fv Libraries Contain the Same Heavy/Light Chain Combinations as Occur *in Vivo*

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Three combinatorial libraries were constructed from unpurified, CD19$^+$, and antithyroid peroxidase (anti-TPO) B cells extracted from thyroid tissue of Graves' disease patients. Fifteen of the 41 randomly derived anti-TPO single chain variable region fragments (scFvs), showed VH1–3/Vλ1–51 or VH1–69/Vλ1–40 heavy/light chain pairing similar to that obtained with TPO-specific scFv derived from an in-cell library. One VH1–3/Vλ1–51 scFv, A16, showed exactly the same nucleotide sequence as in-cell scFv ICB7, demonstrating that *in vivo* rearrangement can be obtained from a random combinatorial library. The majority of the scFvs used a heavy chain gene derived from the VH1–3 gene segment, whereas the light chain gene segments used were more heterogeneous, with dominance of the Vκ1–39 and Vλ1–51 gene segments. The anti-TPO scFvs showed high affinities to TPO, with values between 0.77 and 12.3 nM, and defined seven antigenic regions on the TPO molecule. The anti-TPO fragments, particularly VH1–3/Vλ1–51 randomly associated scFv B4, which mimic natural H/L pairing, and VH1–3/Vλ1–40 in-cell-derived scFv ICA5, efficiently displaced the TPO binding of serum autoantibodies from 20 Graves' disease patients. Our study directly demonstrates that antibodies derived from combinatorial libraries are likely to represent *in vivo* pairing, leading to high affinity antibody fragments mimicking the binding of serum autoantibodies to TPO. (*Endocrinology* 142: 4740–4750, 2001)

AUTOIMMUNE THYROID DISEASE is one of the most common organ-specific autoimmune syndromes. In this disease the humoral response is directed against one or more of the thyroid antigens: Tg, thyroid peroxidase (TPO), or the TSH receptor (TSHr). The use of combinatorial libraries for producing recombinant antibodies allowed several laboratories to obtain and characterize numerous recombinant κ and some λ anti-TPO antibodies (Abs) (1–7). One major question when using this technology is the possibility of finding the *in vivo* H/L pairing in recombinant antibodies obtained by a random combinatorial library. Furthermore, it is not clear whether the generation of artificial heavy (VH) and light chain (VL) combinations from random combinatorial library reflects the *in vivo* situation. Although two reports suggest that *in vivo* pairing of the H and L chains is never found in random combinatorial libraries (8, 9), one group hypothesized that Abs from a combinatorial library are closely related to the immune response of the donor (10). Furthermore, the likelihood that chain pairing in a TPO-selected random library contains *in vivo* H/L combinations has been emphasized (11, 12). We recently obtained three λ anti-TPO single chain variable region fragments (scFvs) by an in-cell library (13) that reflect *in vivo* rearrangements. These results point out the need to develop an extended antibody repertoire from combinatorial libraries to definitively discriminate between natural H/L pairing and artificial association.

With these perspectives in mind, we constructed three different random combinatorial libraries using unpurified, CD19$^+$ or TPO$^+$ cells from thyroid-infiltrating B lymphocytes (TIBL) and compared the H/L pairing of the single chain variable region fragments (scFvs) obtained by random combinatorial libraries with those previously produced by in-cell PCR. We provide herein the first direct demonstration that *in vivo* VH1–3/Vλ1–51 or VH1–69/Vλ1–40 H/L pairing can be found in random TPO-specific combinatorial libraries. Secondly, we confirmed that the TPO-specific Ab repertoire shows, independently of the starting B cells, large VH gene restriction to the VH1–3 germline usage, whereas a more heterogeneous VL gene use was observed with dominance of Vκ1–39 and Vλ1–51 genes. Finally, the anti-TPO fragments, particularly VH1–3/Vλ1–51 randomly associated scFv B4 and VH1–3/Vλ1–40 in-cell-derived scFv ICA5, specifically inhibited the binding of autoantibodies (aAbs) to TPO. Our study demonstrates that a large Ab repertoire derived from combinatorial libraries can reflect the *in vivo* situation, and that the effective TPO-specific aAb response in Graves' disease is more diverse than that previously described (1–7).

Abbreviations: aAbs, Autoantibodies; Ab, antibodies; CDR, complementarity-determining region; FR, framework region; H, heavy chain; L, light chain; scFvs, single chain variable fragments; TIBL, thyroid-infiltrating B lymphocytes; TPO, thyroid peroxidase; TSHr, TSH receptor.

Materials and Methods

Isolation and purification of TIBL

The TIBL used for library construction were isolated from biopsies from seven patients (bdw11, bdw16, bdw41, bdw42, bdw43, bdw44, and bdw45) suffering from Graves' disease. These patients were between 23–50 yr of age. The anti-TPO aAbs of these patients' sera were analyzed

by direct ELISA on human purified TPO (purity, >85%; HyTest, Turku, Finland). They had high anti-TPO [titers, 1:2,500 to 1:13,000 (titer defined as the dilution giving an A_{450nm} of 1)]. The sera of 20 Graves' disease patients with high anti-TPO titers (A_{450nm} = 1 for a serum dilution of 1:300 to 1:13,000) were used for ELISA inhibition studies. Most of the patients' sera were further characterized for anti-Tg Abs and for anti-TSHr Abs by ELISA (Bio-Rad Laboratories, Inc., Marnes-La-Coquette, France; Table 1).

TIBL were isolated from the thyroid gland of patients bdw11 and bdw16 and were purified with anti-CD19 magnetic beads as previously described (14). The B cells from patient bdw43 were purified on human TPO with anti-TPO magnetic beads. Briefly, the cells were resuspended in 1 ml PBS and 1% BSA containing 5 μg/ml human TPO and incubated for 30 min at room temperature with gentle shaking. After three washings with PBS/1% BSA, the cell-TPO complexes were resuspended in 1 ml PBS/1% BSA containing anti-TPO-coupled magnetic beads (Estapor, Rhone-Poulenc, France) and incubated for 30 min at room temperature with gentle shaking. The bound cells were washed extensively with PBS using a magnet before cell recovery. Finally, dissociated-thyroid tissues from patients bdw41, bdw42, bdw43, and bdw45 were used as an unpurified B cell suspension (unpurified B cells).

Combinatorial library construction, selection, and expression of scFvs

Different scFv combinatorial libraries were obtained depending on the starting cells, *i.e.* unpurified cells (unpurified library), $CD19^+$ cells ($CD19^+$ library), or TPO^+ B cells (TPO^+ library). After total RNA extraction from cells by using TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD), RT was performed on the total RNA using SuperScript II reverse transcriptase (Life Technologies, Inc.). For construction of the $CD19^+$ library, the PCR amplification protocol was the same as the one we used for construction of the in-cell library (14). For construction of the two other libraries, two PCR steps were performed, one for the amplification of the VH and VL Ig genes and one for the amplification of scFv products after loxP-Cre recombination and the introduction of restriction sites. The amplified and associated scFv genes were cloned and transformed in *Escherichia coli* XL1-Blue-competent cells by electroporation. The loxP site, which allows recombination between VH and VL genes, was removed by *Nhe*I (New England Biolabs, Inc., Beverly, MA) restriction enzyme digestion.

Phage stock solutions were prepared using a standard procedure involving M13K07 helper phages (Bio-Rad Laboratories, Inc., Hercules, CA) routinely yielding phage titers between 10^{11} and 10^{12} titration units/ml. One or two pannings were performed on human TPO as previously described (13). Briefly, immunotubes (Nunc, Roskilde, Denmark) were coated overnight at 4 C with 2 ml 5 μg/ml human TPO in 0.1 M carbonate/bicarbonate buffer, pH 9.6. After washing and a saturation step, 2×10^9 titration unit phage-displayed scFv fragments were incubated in PBS/2% nonfat milk at room temperature with gentle shaking. The bound phages were eluted by adding 0.1 M glycine-HCl (pH 2.2), neutralized, and used to infect the XL1-Blue cells. The bacteria were plated and then used to make a new phage preparation for the next round of panning.

Soluble expression of scFvs was performed in HB2151 *E. coli* cells after induction with 1 mM isopropyl-β-thiogalactopyranoside for 3 h at 25 C. The cells were then pelleted, resuspended at a 1:40 dilution of the culture volume in lysis buffer [20 mM HEPES (pH 8) and 1 mg/ml polymixin B supplemented with protease inhibitors], and incubated for 30 min on ice (15). The suspension was centrifuged for 10 min at 13,000 rpm, and the supernatant containing the scFv fragments was stored or used directly for the immunoreactions.

TABLE 1. Titer of thyroid autoantibodies in serum of Graves' disease patients

Patient with Graves' disease	Age (yr)	Sex	Titer of thyroid antibodies in serum		
			TPO[a]	Tg[a]	TSH-R[b]
bdw05	46	M	500	<100	120
bdw11[c]	29	F	4,000	500	10
bdw16[c]	27	F	>4,000	500	55
bdw17	25	F	300	100	40
bdw20	25	F	800	<100	8
bdw21	33	F	5,000	500	115
bdw24	33	M	750	500	32
bdw25	46	F	1,400	350	32
bdw26	31	F	4,000	300	105
bdw27	21	F	2,000	1,000	18
bdw31	22	F	900	2,800	180
bdw32	30	F	>6,400	>6,400	60
bdw33	27	M	>405	>6,400	2,800
bdw35	?	F	>6,400	390	124
bdw37	27	F	>6,400	ND[d]	20
bdw41[c]	23	F	9,000	<100	ND
bdw42[c]	43	F	8,500	<100	ND
bdw43[c]	50	M	>13,000	6,000	ND
bdw44[c]	23	M	13,000	300	ND
bdw45[c]	32	F	2,500	<100	ND

[a] Antibody titer was determined as the serum dilution showing an absorbance of 1.0 in an ELISA assay using TPO as capture antigen.
[b] Antibody titer was determined using commercial ELISA assay. The titer is given in units per liter.
[c] Patients from whom the cells were extracted for the library construction.
[d] Not determined.

Sequencing of recombinant clones

Sequences were determined by cycle sequencing using the ABI Prism Rhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA). The primers used were the same as those previously described (14). Sequences were run on an ABI Prism 377 electrophoresis system (PE Applied Biosystems). Sequence alignments with germline genes were performed using the IMGT sequence directory (http://imgt.cnusc.fr) (16).

TPO binding activities of scFv fragments and determination of TPO antigenic regions

The binding of soluble scFv fragments to TPO was assessed by ELISA as previously described (13). The microtiter plates were coated overnight at 4 C with human TPO (1 μg/ml in 0.1 M carbonate/bicarbonate buffer, pH 9.6). After washing and saturation, the scFv fragments were added to the plates and incubated for 2 h at room temperature. Bound scFv fragments were detected by the anti-Myc monoclonal antibody 9E10 (17) incubated for 1.5 h at room temperature. Next, an alkaline phosphatase-conjugated Fc-specific antimouse IgG (Sigma, St. Louis, MO) was added and incubated for 1.5 h at room temperature. Enzyme activity was assayed by addition of 4-nitrophenyl phosphate as substrate.

The affinity and the epitope mapping of the anti-TPO scFv fragments were determined using BIACORE 2000 (Biacore AB, Uppsala, Sweden). For the affinity experiments, the anti-Myc monoclonal antibody 9E10 was covalently immobilized on the flow cell of a CM5 sensor chip surface activated with 100 mM *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride and 400 mM hydroxysuccinimide (EDC/NHS). The anti-TPO scFv periplasmic extracts were diluted 1:4 in HBS-EP buffer, pH 7.4, buffer (Biacore AB) and injected at a flow rate of 20 μl/min over the mAb 9E10. The scFv fragments were retained by the c-Myc peptide present at the C-terminal end of each scFv. Known concentrations of TPO were then injected to determine the affinity of the scFv fragments for TPO. The CM5 sensor chip with immobilized 9E10 was regenerated after each kinetic experiment by a 20-sec pulse with 100 mM HCl. The same sensor chip was used for all reported kinetic studies. The kinetic variables were calculated using the BIAevaluation 3.0 software (Biacore AB) (18). For epitope mapping, TPO was covalently immobilized on the flow cell of a CM5 sensor chip surface activated as described above. The epitopes recognized by the scFv fragments were determined by assaying all scFv pairs for their capacity to bind simultaneously to the TPO. The scFv periplasmic extracts diluted 1:2 in HBS, pH 7.4, were successively passed over the immobilized TPO as either first or second Ab. Three injections were required to saturate the epitope of the first Ab so that the binding of the second Ab could be interpreted as being due to the existence of a different epitope. An irrelevant antidigoxin scFv fragment was used as a negative control.

Competition studies between scFv fragments and serum anti-TPO aAbs

scFv fragment binding inhibition by serum anti-TPO autoantibodies of 20 patients suffering from Grave's disease was performed by ELISA (13) with some modifications. Native human TPO (1 μg/ml in carbonate/bicarbonate buffer) was coated overnight at 4 C. After saturation, patients' sera diluted 1:10 in PBS, 0.1% Tween, and 1% nonfat milk were added, and the microtiter plates were incubated for 1.5 h at room temperature. The microtiter plates were washed, and the scFv fragments, diluted 1:10, were added and incubated for 2 h at room temperature. Bound scFv fragments were detected as described above for the binding of soluble scFv to TPO by ELISA.

For inhibition of serum anti-TPO autoantibody binding by scFv fragments, microtiter plates were coated with TPO as described above. After saturation, scFv fragments diluted 1:2 in PBS, 0,1% Tween, and 1% nonfat milk were added and incubated for 2 h at room temperature. Then, the patients' sera, at a dilution giving an absorbance of 1, were added to the wells, and the microtiter plates were incubated for 1.5 h at room temperature. After three washings with PBS/0.1% Tween, bound anti-TPO aAbs were detected by an alkaline phosphatase-conjugated Fc-specific antihuman IgG (Sigma) diluted 1:2000 in PBS, 0.1% Tween, and 1% nonfat milk. Enzyme activity was assayed by addition of 4-nitrophenyl phosphate as substrate.

Results

Library construction and anti-TPO scFv fragment selection

Three distinct combinatorial libraries were constructed from the unpurified, CD19-purified, and TPO-purified B cells. The sizes of these libraries were 2×10^5, 10^6, and 4×10^6 clones, respectively. To obtain at least 80% of colonies specific for human TPO, the $CD19^+$ and the unpurified B cell phage-displayed libraries were subjected to two rounds of panning on highly purified TPO (>95%). In contrast, one round of panning was sufficient to select 100% colonies showing TPO-specific scFv fragments from the TPO^+ library. After sequencing, we obtained 17 different anti-TPO scFv fragments (A1 to A17) of 22 clones sequenced from the $CD19^+$ library, 11 different anti-TPO scFv fragments (B1 to B11) of 23 clones sequenced from the unpurified B cell library, and finally 13 different anti-TPO scFv fragments (T1 to T13) of 15 clones sequenced from the TPO^+ library (Table 2).

H/L pairing of TPO-specific scFv fragments

As shown in Table 2, dominant genes in H/L pairing (VH1–3, Vλ1–51, and Vλ1–40) were used by the anti-TPO scFv whatever their library of origin. Analysis of H/L pairing at the VH/VL family level showed that the VH1–3-derived scFvs were found associated with almost all (8 VL chains of 10) of the light chains (Table 2). Among them, 14 anti-TPO scFvs showed the same VH1–3/Vλ1–51 pairing as the in-cell scFvs ICA1 and ICB7 previously described (13). Similarly, identical VH1–69/Vλ1–40 pairing was found in scFv A10 derived from the $CD19^+$ combinatorial library and in ICA5 scFv obtained from the in-cell library (13).

Analysis of H/L pairing at the VDJ-H/VJ-L level indicates that dominant associations were found in scFvs derived from the unpurified and $CD19^+$ libraries. More precisely, 8 scFvs of 17 from the $CD19^+$ library used the VH1–3 gene associated with the D4–17/JH4 gene segment and paired with the Vλ1–40/Jλ1 or Vλ1–51/Jλ1 rearrangement. This latter pairing corresponds to that described for in-cell scFvs ICA1 and ICB7 (13). The anti-TPO scFv A16, derived from the $CD19^+$ combinatorial library, showed VDJ-H/VJ-L pairing similar to that observed for in-cell ICB7 (13) with an identical nucleotide sequence. Five of 10 scFvs derived from the unpurified library used the VH1–3/D5–24/JH4 gene association paired with 4 different VJ-L genes from either the κ or λ locus. On the other hand, scFvs produced from the TPO^+ library used various VDJ-H rearrangements paired with diverse VJ-κ or -λ genes. These results are indicative of a greater diversity of gene segment usage and H/L pairing for scFvs derived from the TPO^+ library.

V gene analysis of anti-TPO scFv fragments

The V genes encoding the 41 TPO-specific scFv fragments, selected on the basis of their nucleotide sequence and derived from the three combinatorial libraries, were analyzed and compared with the closest putative germline genes (Tables 2, 3, and 4). The scFvs used 6 different families of VH genes, involving 3 VH1, 2 VH3, and 1 VH5 germline genes. The majority of the H chain V regions were encoded by genes derived from the germline gene VH1–3 (33 clones). These VH1–3 scFvs mainly used D4–17 and D5–24 putative D regions, and all but 4 had the same JH4 segment. However a greater diversity of D regions used by the VH1–3 scFvs obtained from the TPO^+ library was observed by comparison with those derived from the 2 other libraries. The 5 other scFv groups, each represented by 1–3 clones, were derived from VH1–8, VH1–69, VH3–30, VH3–64, and VH5–51 germline genes. No apparent D restriction was observed in these cases. The 2 scFvs using the VH1–69 germline gene were associated with a JH4 segment, and the 3 scFvs using the VH3–64 germline gene were associated with a JH6 segment (Table 2).

The L chain V regions were mainly encoded by genes closest to Vλ1–51 (16 clones), Vλ1–40 (9 clones), and Vκ1–39 (6 clones) germline genes, with dominance of Vλ1–51 and Vκ1–39 germline genes inside each λ and κ locus. The scFvs used Vλ1–51 and Vλ1–40 germline genes in association with either Jλ1 or Jλ3 segments, whereas Vκ1–39 scFvs used Jκ2, Jκ4, and Jκ5 segments. The 10 other scFv fragments were derived from Vλ1–44, Vλ2–14, Vλ2–8, Vκ1–12, Vκ1–5, and Vκ3–11 germline genes and used different J segments (Table 2). Comparison of the unpurified B cell and TPO^+ libraries constructed from cells at different stages of differentiation shows a different VL germline gene usage. Concerning the 6 VL germline genes (Vλ1–51, Vλ2–8, Vλ1–40, Vκ1–5, Vκ3–11, and Vκ1–39) in the TPO^+ library, a majority of Vκ germline genes (8 of 13) was used by the 13 scFvs (T1-T13), whereas among the 6 VL germline genes (Vλ1–40, Vλ2–14, Vλ7–43, Vλ1–51, Vκ1–39, and Vκ1–12) obtained from the unpurified B cell library, only 2 of the 11 scFvs used a κ gene.

All of the anti-TPO scFv sequences showed evidence of somatic hypermutation, with a replacement/silent ratio typically higher in the VH chain than in the VL chain. The replacement/silent ratio was greater in the complementarity-determining region (CDR) regions than in the framework region (FR) regions (Table 2). Although some scFv VL chain sequences showed high somatic hypermutation (A9, B3, B7, T5, and T9), a large majority showed little, if any, mutation. The number of mutations was not dependent on the germline gene used by the

TABLE 2. Anti-TPO scFv fragments isolated from three different combinatorial libraries

Library	Anti-TPO scFv (no. of clones)	Heavy chain[a]					Light chain[a]			
		VH	D	JH	CDR R/S[b]	FR R/S[b]	VL	JL	CDR R/S[b]	FR R/S[b]
Combinatorial library from thyroid-infiltrated CD19$^+$ B cells	A1	VH1–3*01	D5–12*01	JH4*03	6/2	11/6	Vλ1–51*01	Jλ3*01	0/0	0/0
	A2	VH1–3*01	D4–17*01	JH4*03	7/1	12/10	Vλ1–51*01	ND	2/0	0/3
	A3	VH1–3*01	D6–6*01	JH4*02	5/2	8/9	Vλ1–51*01	Jλ3*02	0/1	3/1
	A4	VH1–3*01	D5–24*01	JH4*02	6/2	12/5	Vλ2–14*01	ND	4/0	5/2
	A5 (2)	VH1–3*01	D4–17*01	JH4*03	7/0	8/7	Vλ1–51*01	Jλ3*01	1/0	1/0
	A6	VH1–3*02	D2–21*01	JH4*03	8/1	9/8	Vλ1–40*02	Jλ1*01	5/0	0/1
	A7 (2)	VH1–3*01	D4–17*01	JH4*03	7/1	9/11	Vλ1–51*01	Jλ3*01	0/0	1/4
	A8	VH3–30*04	D3–16*01	JH4*02	10/1	6/4	Vλ1–44*01	Jλ3*01	5/1	5/5
	A9 (2)	VH3–64*01	D1–26*01	JH6*02	4/0	9/1	Vλ1–40*01	Jλ3*01	6/1	12/4
	A10	VH1–69*01	D5–18*01	JH4*02	6/2	10/7	Vλ1–40*02	Jλ1*01	0/0	0/0
	A11 (2)	VH1–3*01	D4–17*01	JH4*03	7/1	12/10	Vλ1–51*01	Jλ1*01	2/1	2/1
	A12	VH3–64*01	D1–26*01	JH6*02	1/0	5/1	Vλ1–51*01	Jλ3*01	0/0	1/2
	A13	VH1–3*01	D4–17*01	JH4*02	7/1	8/10	Vλ1–40*01	Jλ1*01	2/0	2/1
	A14	VH1–3*01	D4–17*01	JH4*02	7/1	9/8	Vλ1–40*01	Jλ1*01	2/0	2/1
	A15 (2)	VH1–3*01	D4–17*01	JH4*03	7/1	12/10	Vλ1–51*01	Jλ1*01	2/1	2/1
	A16	VH1–3*01	D4–17*01	JH4*03	7/1	7/7	Vλ1–51*01	Jλ1*01	1/1	4/3
	A17	VH1–69*01	D5–18*01	JH4*02	6/0	10/3	Vλ-44*01	Jλ1*01	0/0	3/2
Combinatorial library from thyroid-infiltrated unpurified cells	B1	VH1–3*01	D5–24*01	JH4*02	6/3	13/7	Vλ1–40*02	Jλ3*02	0/0	1/2
	B2 (6)	VH1–3*01	D5–24*01	JH4*02	6/3	13/7	Vκ1–39*01	Jκ4*01	0/2	4/1
	B3	VH1–3*01	D5–24*01	JH4*02	6/3	10/5	Vλ7–43*01	Jλ3*02	8/1	2/4
	B4	VH1–3*01	D5–24*01	JH4*02	6/3	14/6	Vλ1–51*01	Jλ3*02	0/0	1/2
	B5 (2)	VH1–3*01	D4–11*01	JH1*01	6/2	10/4	Vλ1–51*01	Jλ3*01	4/0	3/4
	B6	VH1–3*01	D4–11*01	JH1*01	6/2	8/4	Vλ1–51*01	Jλ1*01	2/0	4/2
	B7	VH1–3*01	D4–17*01	JH4*02	7/1	17/5	Vκ1–12*01	Jκ5*01	9/1	8/6
	B8 (6)	VH1–3*01	D5–24*01	JH4*02	6/2	8/7	Vλ1–51*01	Jλ3*02	0/0	0/1
	B9	VH1–3*01	D4–17*01	JH4*03	7/2	17/6	Vλ1–51*01	Jλ3*02	1/1	2/5
	B10	VH1–3*01	D4–11*01	JH4*02	6/2	10/6	Vλ2–14*01	Jλ3*02	1/0	0/0
	B11 (2)	VH5–51*01	D3–16*01	JH4*02	2/0	5/4	Vλ1–51*01	Jλ3*01	3/1	2/2
Combinatorial library from thyroid-infiltrated TPO-purified B cells	T1	VH1–3*01	D5–24*01	JH4*02	6/3	12/7	Vλ1–51*01	ND	4/0	4/5
	T2	VH1–3*02	D2–21*01	JH4*03	8/1	16/12	Vκ3–11*02	ND	4/1	4/3
	T3 (3)	VH1–3*01	D4–17*01	JH4*03	7/1	19/6	Vκ1–39*01	Jκ4*01	0/0	5/2
	T4	VH1–3*01	D4–17*01	JH4*02	6/3	9/11	Vλ2–8*01	Jλ1*01	5/1	5/3
	T5	VH1–8*01	D3–16*01	JH3*02	5/3	8/4	Vκ1–5*03	Jκ2*01	6/2	12/13
	T6	VH1–3*01	D1–20*01	JH4*02	8/1	8/11	Vκ1–39*01	Jκ2*01	0/1	4/2
	T7	VH1–3*01	D3–16*01	JH6*02	7/1	17/9	Vλ1–40*01	Jλ3*02	3/1	2/2
	T8	VH1–3*01	D4–17*01	JH4*03	7/1	19/7	Vκ1–39*01	Jκ4*01	5/2	0/2
	T9	VH1–3*01	D4–17*01	JH4*02	5/2	14/7	Vκ1–39*01	ND	6/2	14/7
	T10	VH3–64*01	D6–19*01	JH6*02	3/4	9/7	Vκ3–11*01	Jκ4*01	2/0	1/2
	T11	VH1–3*01	D1–20*01	JH4*03	6/2	10/12	Vκ1–39*01	Jκ5*01	1/1	7/6
	T12	VH1–3*01	D5–24*01	JH4*03	6/2	9/5	Vλ1–40*02	Jλ3*02	2/0	4/1
	T13	VH1–3*01	D3–16*01	JH6*02	7/1	20/9	Vλ1–40*01	Jλ3*02	6/1	1/3

[a] Putative closest germline genes.
[b] Somatic hypermutation in complementarity determining regions (CDR) and in framework regions (FR). R, Replacement mutation; S, silent mutation.

anti-TPO scFvs, as the scFvs derived from the same germline genes, Vλ1–51, Vλ1–40, and Vκ1–39, were either highly mutated or completely unmutated. The fact that 80% of the characterized scFvs showed the same VH1–3 germline gene, whereas a wider VL gene usage was observed, suggests that the sequences encoding anti-TPO heavy chains are more restricted than those encoding light chains.

Amino acid sequence analysis of anti-TPO scFv fragments

Hypermutations were analyzed in detail for the 33 different VH1–3-derived scFv sequences, including those showing H/L pairing similar to that which occurred *in vivo*, and revealed 3 distinct patterns (Table 3). First, somatic gene mutations, mainly located in the CDRs, were common to most of these anti-TPO scFvs, whatever the patient and the library of origin. In CDR1, the threonine in positions 29 and 31 was mainly replaced by a serine, and the alanine in position 34 was replaced by an asparagine or a glycine; in FR2, the methionine in position 39 was replaced by an isoleucine. In CDR2, the alanine in position 58 was replaced by a glutamine, and the asparagine 60 was replaced by a threonine in most of the anti-TPO scFvs (Table 3). Second, some mutations were restricted to anti-TPO scFvs derived from only 1 random library. For example, the arginine in position 49 in FR2 was replaced by a glycine, and the threonine in position 63 in CDR2 was replaced by an arginine in most of the scFvs from the CD19$^+$ library. Mutation of valine 12 to methionine was also restricted to scFvs obtained from the CD19$^+$ library (Table 3). Third, similar hypermutations were observed in scFvs derived from TPO$^+$ and unpurified B cell combinatorial libraries, *i.e.* residue mutations in positions 6, 13, 14, 42, 50, 53, 62, 89, and 92. On the other hand, replacement of asparagine 57 by histidine was mainly represented in

TABLE 3. Amino acid sequences of TPO-specific scFv heavy chains aligned with the putative closest germline genes[a]

IGVH gene	FR1-IMGT (1-26)	CDR1-IMGT (27-38)	FR2-IMGT (39-55)	CDR2-IMGT (56-65)	FR3-IMGT (66-104)	CDR3-IMGT (105-...)	IGHJ
	1 10 20	30	40 50	60	70 80 90 100	110 120	130
X62109,IGHV1-3*01	QVQLVQSGA EVKKPGASVKVSCKAS	GYTFTSYA	MHWVRQAPGQRLEWMGW	INAGNGNT	KYSQKFQ GRVTITRDTSASTAYMELSSLRSEDTAVYYC	AR	
,A1	E..... ..R.....	..M..N	I.....T.....	.H..TS..	.....L. ....F.....N.....G.IP....I...	..DTYSDFDY	WGQGTLVTVSS
,A2/A11/A15	E..... .M.....T.....	..S.SV.N	I.....G.....	.H..T..R	....M.. D....S.....R.S.L.....T.....	..DPNYGDFDY	WGQGTLVTVSS
,A3	E.....	..A.A...	I.....	.H..T..R	....T.. D...L....TT.....K.....	..VPTGWVAEFDS	WGQGTLVTVSS
,A4	E..... .....RI..E..	..A.S..D	I.....S.....	.HG.T...	....F.....A.....T..T.....M.F.	..DTYRDFDY	WGQGTLVTVSS
,A5	E..... .....T.	..A.ST.I	V.....	.HG.T...	...E... .....A....V.N.....F.	..DPNYGDLDY	WGQGTLVTVSS
,A7	E.....	..S.SI.N	I.....G.....	.H..T..R	....V.E D..S.....S.....T.....	..DPNFGDFDS	WGQGTLVTVSS
,A13	E..... .M.....	..S.SI.N	I.....G.....	.H..T..R	....V.. D.....S.....T.....	..DPNFGDFDS	WGQGTLVTVSS
,A14	L..... .M....T.....	..S.SI.N	I.....G.....	.H..T..R	....V.. D.....S.....T.....	..DPNFGDFDS	WGQGTLVTVSS
,A16	L.....	..S.SI.N	I.....G.....	.H..T..R	....V.. D.....S.....T.....	..DPNFGDFDS	WGQGTMVTVSS
,B1	E....E...	..S.S..G	I.....	..G.T.F.	....N.. ...SL.....S.L..NN.T....G..F.	..DPYNDYAAELDY	WGQGTLVTVSS
,B2	E....E...	..S.S..G	I.....	..G.T.F.	....N.. ...SL.....S.L..NN.T....G..F.	..DPYNNYAAELDY	WGQGTLVTVSS
,B3	E....E...	..S.S..G	I.....	..G.T.F.	....N.. ....L.....T....L..N.....GL...	..DPYNNNAAELDY	WGQGTLVTVSS
,B4	..N.RE...	..S.S..G	I.....	..G.T.F.	....N.. ...SL.....S.L..NN.T....G..F.	..DPYNNYAAELDY	WGQGTLVTVSS
,B5	E....E...	..S.S..G	I.....	..G.T.F.	....N.. ....L.....LD.N.....GL...	..DPYNNYAAELDH	WGQGTLVTVSS
,B6	....	..S.S..G	I.....	..G.T.F.	....N.. ....L.....LD.N.....GL...	..DPYNNYAAELDH	WGQGTLVTVSS
,B7	....RR.....R.....	..S.ST.N	I..L.....P..V.L	.HS.T...	.SLE.LR D.....T.V.....P.....	..EFYGDFAY	WGQGTLVTVSS
,B8	E....E...	..S.S..G	I.....	..G.T.F.	....N.. ....L.....L..N.....G....	..DPYNNYAAELDY	WGQGTLVTVSS
,B9	....RR.....R.....	..S.ST.N	I..L.....P..V.L	.HS.T...	.SLE.LR D.....T.V.....P.....	..EFYGDFAY	WGQGTLVTVSS
,B10	....L..A.	..S.R..G	I.....	..G.T.F.	....I.. ....L....A.....L..N.....GL...	..DPYNNYAAEFDY	WGQGTLVTVSS
,T1	.....E...	..S.S..G	I.....	..G.T.F.	....N.. ...SL.....S.L..NN.T....G..F.	..DPYNNYAAELDY	WGQGTLVTVSS
,T3	E....E... ..RR.....RI.....	..S.ST.N	I..L.....P..V.L	.HS.T...	.SLE.LR D.....T.V.....P.....	..EFYGDFAY	WGQGSLVTVSS
,T4	.M.....	..S..P.S	I.....A..S.....L	.Y..T.A.	...DSW. ....F.....	..GLHPEIDY	WGQGTLVTVSS
,T6	L.....	..I..G.T	I.....P.....	V.T.S.K.	...E... D.....I.....T...A.....	.GTGTTCYMCLDY	WGQGTLVTVSS
,T7	L..... ..RR.....IT....	..S.ST.G	IQ.....P..L..	.H..T.G.	...R... ..I.....N.V.LD.N..T.....	..DLDPFGGGMDV	WGQGTTVTVSS
,T8	E....E... ..RR.....RI.....	..S.ST.N	I..L.....P..V.L	.HS.T...	.SLE.LR D.....T.V.....P.....	..EFYGDFAY	WGQGSLVTVSS
,T9	E..... ..RR.....R.....	..S..T.T	I..L.....P.....	.HS.....	..LE.LR D.....T.....P.....	..EFYGDFAH	WGQGTLVTVSS
,T11	E...LE...	....G.T	I.....P.....	V.T...K.	...E... D.....I.....T...A.....	.GTGTTCYMCLDY	WGQGTTVTVSS
,T12	....L..A.	..S.S..G	I.....	..G.T.F.	....N.. ....L.....L..N.....GL...	..DPYNNYAAELDY	WGQGTLVTVSS
,T13	E....E.A. ..RR.....IT....	..S.ST.G	IQ.M.....P..L..	.H..T.G.	...R... ..I.....N.V.LD.N..T.....	..DLDPFGGGMDV	WGQGTTVTVSS
X62107,IGHV1-3*02	QVQLVQSGA EVKKPGASVKVSCKAS	GYTFTSYA	MHWVRQAPGQRLEWMGW	SNAGNGNT	KYSQEFQ GRVTITRDTSASTAYMELSSLRSEDMAVYYC	AR	
,A6	E..... .M.....	..S.SI.N	I.....G.....	IH..T..R	....V.. D.....S.....T...T.....	..DPNFGDFDS	WGQGTLVTVSS
,T2	E..... ..RR.....IT....	..S.ST.G	I.....I....P..L..	IH..T.A.	...R.L. ..I.L.....T.....R.....T.....	..DLDPFGGGMDV	WGPGTLVTVSS
M99637,IGHV1-8*01	QVQLVQSGA EVKKPGASVKVSCKAS	GYTFTSYD	INWVRQATGQGLEWMGW	MNPNSGNT	GYAQKFQ GRVTMTRNTSISTAYMELSSLRSEDTAVYYC	AR	
,T5	....M.....	EF...H..	.....	.....R.	..G.N.. ....I...S.T..I.....I.....	V.NSKAFRALEI	WGQGTMVTVSS
L22582,IGHV1-69*01	QVQLVQSGA EVKKPGSSVKVSCKAS	GGTFSSYA	ISWVRQAPGQGLEWMGG	IIPIFGTA	NYAQKFQ GRVTITADESTSTAYMELSSLRSEDTAVYYC	AR	
,A10	E..... .....L.....	.D....RP	V.....	...L.R.T	....L....MI...L...G.T.D.....	.TTRMKITVFASTFDY	WGQGTLVTVSS
,A17	E..... .....L.....	.D....RP	V.....	.....R.T	....MT...L..RG.T.D.....	.TTRMKITVFASTFDY	WGQGTLVTVSS
L06615,IGHV3-30*04	QVQLVESGG GVVQPGRSLRLSCAAS	GFTFSSYA	MHWVRQAPGKGLEWVAV	ISYDGSNK	YYADSVK GRFTISRDNSKNTLYLQMNSLRAEDTAVYYC	AR	
,A8	E.....	..A.DAFT	.....T.....	..SA.TKT	.F..... .....V.....T....I...	..DGDPIGYYFDY	WGPGTLVTVSS
M99682,IGHV3-64*01	EVQLVESGG GLVQPGGSLRLSCAAS	GFTFSSYA	MHWVRQAPGKGLEYVSA	ISSNGGST	YYANSVK GRFTISRDNSKNTLYLQMGSLRAEDMAVYYC	AR	
,A9	....	.....T	VY.....G	..GH....	S..... .....V...L....T..T...F.	..EWQLPNFYSYGMDV	WGQGTLVTVSS
,A12	.....A.	.....	.Y.....G	...H....	S..... .....T..T.....	..EWQLPNFYSYGMDV	WGQGTLVTVSS
,T10	.D..... .....V..	.....	.....S...	..GH..T.	.F..... D...M....A.....D.T.....	..SQWLDRAWGGYFGLDV	WGHGTLVTVSS
M99686,IGHV5-51*01	EVQLVQSGA EVKKPGESLKISCKGS	GYSFTSYW	IGWVRQMPGKGLEWMGI	IYPGDSDT	RYSPSFQ GQVTISADKSISTAYLQWSSLKASDTAMYYC	AR	
,B11	.....E... ....S.....S.	..Y.....	.....	.....	....A.....I...	V.SFGAFRHTSYYFDY	WGQGTLVTVSS

[a] Designation of the complementarity determining regions (CDR) and framework regions (FR) are according to the IMGT database (16). Only replaced amino acids are shown.

scFvs from CD19$^+$ and TPO$^+$ libraries. Taken together, the pattern of gene somatic hypermutation in the VH region could be correlated with the starting B cells used for the construction of the libraries. In contrast, no specific assignment of a particular mutation was observed for the VL region (Table 4).

TPO binding activity of scFv fragments

Among the 41 scFv fragments selected on the basis of their gene sequences, 17 Ab fragments presenting a TPO Ab titer, by ELISA, greater than 100 were further characterized (Table 5). The affinity of these anti-TPO scFvs was determined on BIACORE 2000 by real-time interaction analysis, as exemplified by clone T2 (Fig. 1). The scFvs had affinities for TPO ranging from 0.77–12.3 nM (Table 5). As exemplified by clones B2 (VH1–3/Vκ1–39) and B4 (VH1–3/Vλ1–51), the affinity was similar independently of natural *vs.* artificial H/L pairing and of Vλ- *vs.* Vκ-chain usage (Table 5). The scFv-binding domains on TPO were analyzed by competition studies on BIACORE 2000. All scFv pairs were assayed by sequential injections on TPO as previously described (13). Binding of the second scFv to TPO was interpreted as recognition by this scFv of a different antigenic region than that recognized by the first scFv, as exemplified in Fig. 2 for the pair of scFvs B4 and B10. Antigenic regions I and II were previously described as Ab-binding regions on TPO for in-cell scFvs ICA1 and ICA5, respectively (13). Seven other antigenic regions (III–IX) were defined by scFvs obtained from the 3 random combinatorial libraries (Table 5). Antigenic domains VI and VIII, found in the 3 libraries, are closely related and are dominant in the TPO$^+$ and unpurified B cell libraries.

To study more precisely the relationship between antigen specificity and H/L pairing or V chain gene usage, we focused our attention on the scFvs derived from the same VH gene. The same VH1–3-derived gene was found associated

TABLE 4. Amino acid sequences of TPO-specific scFv light chains aligned with the putative closest germline genes[a]

IG(K/L)V gene	FR1-IMGT (1-26)	CDR1-IMGT (27-38)	FR2-IMGT (39-55)	CDR2-IMGT (56-65)	FR3-IMGT (66-104)	CDR3-IMGT (105-115)	IG(K/L)J
	1 10 20	30	40 50	60	70 80 90 100	110	120
M94116,IGLV1-40*01	QSVLTQPPS VSGAPGQRVTISCTGS	SSNIGAGYD	VHWYQQLPGTAPKLLIY	GNS	NRPSGVP.DRFSGSK SGTSASLAITGLQAEDEADYYC	QSYDSSLSG	
,A9	....A.....T	T.....	.....F..S..Q..FS	.D.	S..A.....A.R .....P.....F.	..F.R.K.PL	FGGGTKVTVLG
,A13/A14	....	.....	.Q.....V... ...	.....	.....	H...N....DV	FGTGTKLEIKR
,T7	....A.V.....	.....D..	.....	D..	.....	.....VA	FGGGTKLEIKR
,T13	.....S.	.....	.....	D.T	.....	.....RHWV	FGGGTKVTVVG
X53936,IGLV1-40*02	QSVVTQPPS VSGAPGQRVTISCTGS	SSNIGAGYD	VHWYQQLPGTAPKLLIY	GNS	NRPSGVP.DRFSGSK SGTSASLAITGLQAEDEADYYC	QSYDSSLSG	
,A6	....S..G	.....	.....	..I	....	H...N....DV	LGTGTKLEIKR
,A10	....	.....	.....	...	....	.....YV	FGTGTKLTVLG
,B1	....	.....	.....V.....	...	....	.....RV	FGGGTKLEIKR
,T12	....T.....	.....	.N.....F...	..T	Y.....	....N....WV	FGGGTKLEIKR
Z73654,IGLV1-44*01	QSVLTQPPS ASGTPGQRVTISCSGS	SSNIGSNT	VNWYQQLPGTAPKLLIY	SNN	QRPSGVP.DRFSGSK SGTSASLAISGLQSEDEADYYC	AAWDDSLNG	
,A8	.P.....S. .....A.....	.....P	...C.....M.	GKS	..... .D.....	.S.....D.PV	FGGGTKLTVLG
,A17	SYE.....	.....	.....	...	....	CV	FGTGTKVTVLG
Z73661,IGLV1-51*01	QSVLTQPPS VSAAPGQKVTISCSGS	SSNIGNNY	VSWYQQLPGTAPKLLIY	DNN	KRPSGIP.DRFSGSK SGTSATLGITGLQTGDEADYYC	GTWDSSLSA	
,A1	....	.....	.....	...	....	VV	FGGGTKLTVLG
,A2	....S.F	.....	.....	...	....	LV	FGGGTKVDIKR
,A3	....M.....	.....	.....R.....	...	Q.....	.....KV	FGGGTKLTVLG
,A5	.....S. ..... T.....	.....	.....	...	....	LV	FGGGTKVEIKR
,A7	....	.....	.....	...	.....S.....	LV	FGGGTKVTVLG
,A11	....	.....	.....	G..	E..... .....S.	.....R...YV	FGTGTKLTVLG
,A12	...V.....	.....	.....	...	....	.....GVV	FGGGTKVEIKR
,A15	....	.....	.....	G..	E..... .....S.	.....R...YV	FGTGTKLEIKR
,A16	...V..... .....S.....	...T....	.....L.....	...	E.....	.....YV	FGTGTKVDIKR
,B4	...V.....	.....	.....	...	....	.....GV	FGGGTKVEIKR
,B5	.... N.....F	.....	.....F.....	..D	.....R	....N....VV	FGGGTKLEIKR
,B6	.A.V..E..	....AD..	.....	...	....S.....	.....GV	FGTGTQLTVLG
,B8	....	.....	.....	...	....	.....GV	FGGGTKLTVLG
,B9	HA.....	.....	.....	.S.	....	.....GV	FGGGTKLTVLG
,B11	....G ..D....F	.....	.....	...	....D.....P.....	.....G.NVI	FGGGTQLTVLS
,T1	...V..... ..... N.....F	.....	.....F.....	..D	.....R .....F..	....N....VV	FGGGTRLEIKR
X97462,IGLV2-8*01	QSALTQPPS ASGSPGQSVTISCTGT	SSDVGGYNY	VSWYQQHPGKAPKLMIY	EVS	KRPSGVP.DRFSGSK SGNTASLTVSGLQAEDEADYYC	SSYAGSNNF	
,T4	.PV..... ..... T...VD...	.....	.....Y.....I..	..N	.....N..... .....C.....	.....FI.V	FGSGTKLEIKR
Z73664,IGLV2-14*01	QSALTQPAS VSGSPGQSITISCTGT	SSDVGGYNY	VSWYQQHPGKAPKLMIY	EVS	NRPSGVS.NRFSGSK SGNTASLTISGLQAEDEADYYC	SSYTSSSTL	
,A4	....A ....T...	.....	.....R.....I..	...	.....Y...G	T..AP...F	FGGGTKLEIKR
,B10	....	.....	.....	D..	.....	.....RV	FGGGTKLTVLG
X14614,IGLV7-43*01	QTVVTQEPS LTVSPGGTVTLTCASS	TGAVTSGYY	PNWFQQKPGQAPRALIY	STS	NKHSWTP.ARFSGSL LGGKAALTLSGVQPEDEAEYYC	LLYYGGAQ	
,B3	.A..... ....P..NT..	.....	.....	N.N	.....R.....	.VH...PRV	FGGGTQLTVLS
X72813,IGKV1-5*03	DIQMTQSPSTLSASVGDRVTITCRAS	QSISSW	LAWYQQKPGKAPKLLIY	KAS	SLESGVP.SRFSGSG SGTEFTLTISSLQPDDFATYYC	QQYNSYS	
,T5	E.VL.HP.....I..... ..VTQ.	.....	.....R.... ...	.....	H.HD..... .....N.....F..	...YT.PYT	FGQGTKLEIKR
X17263,IGKV1-12*01	DIQMTQSPSSVSASVGDRVTITCRAS	QGISSW	LAWYQQKPGKAPKLLIY	AAS	SLQSGVP.SRFSGSG SGTDFTLTISSLQPEDFATYYC	QQANSFP	
,B7	..LL..T..V..... .R.TNL	.....	.....Q..R....	.GY	..... R... ..... T.....	..GY.S.VLS	FGGGTRLEIKR
X59315,IGKV1-39*01	DIQMTQSPSSLSASVGDRVTITCRAS	QSISSY	LNWYQQKPGKAPKLLIY	AAS	SLQSGVP.SRFSGSG SGTDFTLTISSLQPEDFATYYC	QQSYSTP	
,B2	..LL..T.....	.....	.....	...	....N.....	.....LT	FGGGTKVDIKR
,T3	E.VL.....Y.....	.....	.....	...	....N.....	.....LT	FGGGTQLTVLS
,T6	E.V.....G	.....	.....	...	.....V	.....VT	FGQGTKVEIKR
,T8	E.VL.....Y.....	.....	.....	...	....N.....	.....LT	FGGGTKLEIKR
,T9	E.V.....V...S. .N.TN.	.....	.....S.....M.	G..	T...A..... .....G.R.....	...H.R.WT	FGHGTQLTVLS
,T11	E.VL...HP.....	.....	.....	...	.....L..... .....G.....	....N..DT	FGQGTRLAIKR
X01668,IGKV3-11*01	EIVLTQSPATLSLSPGERATLSCRAS	QSVSSY	LAWYQQKPGQAPRLLIY	DAS	NRATGIP.ARFSGSG SGTDFTLTISSLEPEDFAVYYC	QQRSNWP	
,T10	.....G..... ..... TY	.....	.....	...	....	.....LT	FGGGTKLEIKR
.02768,IGKV3-11*02	EIVLTQSPATLSLSPGERATLSCRAS	QSVSSY	LAWYQQKPGQAPRLLIY	DAS	NRATGIP.ARFSGSG SGRDFTLTISSLEPEDFAVYYC	QQRSNWP	
,T2	.TT..... ....S	.....	.....P..... .TA	.....	....T.....	...R...S	FGGGTQLTVLS

[a] Designation of the CDRs and FRs are according to the IMGT database (16). Only replaced amino acids are shown.

with two closely related Vλ1–51-derived genes in scFvs A2 and A11 (Tables 3 and 4); these scFvs recognized two different domains, domains III and V, respectively (Table 5), on TPO. In contrast, scFvs B2 and B4, which used H chain genes close to the VH1–3 germline gene and showed different L chain genes derived from Vκ1–39 and Vλ1–51, respectively, recognized the antigenic region VI on TPO (Table 5). Similarly, scFvs T8 and T13, which used the same H chain genes close to the VH1–3 germline gene (Table 3) and different L chain genes derived from Vκ1–39 and Vλ1–40 (Table 4), respectively, recognized the same domain (namely VIII) on TPO (Table 5). Taken together, these results indicate no relationship between antigenic specificity and variable chain usage or H/L pairing.

Competition studies between scFv fragments and serum anti-TPO autoantibodies

For each epitope defined by the BIACORE study, we chose 1 scFv for competition studies with the serum autoantibodies. Twenty sera from patients suffering from Graves' disease and 20 sera from healthy subjects were used to test their ability to inhibit the binding of the scFvs to TPO. Strong inhibitions, ranging between 60–100%, were obtained with

TABLE 5. Characterization of the anti-TPO scFv fragments

Library	Anti-TPO scFv	H/L pairing	TPO-binding antibody titer[a]	Affinity (nM)[b]	Antigenic region[c]
Combinatorial library from thyroid-infiltrated CD19$^+$ B cells	A2	VH1–3/Vλ1–51	400	4.89	III
	A10	VH1–69/Vλ1–40	4,000	5.43	IV
	A11	VH1–3/Vλ1–51	250	8.03	V
	A12	VH3–64/Vλ1–51	100	1.21	VIII
Combinatorial library from thyroid-infiltrated unpurified cells	B2	VH1–3/Vκ1–39	5,000	4.35	VI
	B4	VH1–3/Vλ1–51	90,000	2.83	VI
	B5	VH1–3/Vλ1–51	3,500	1.99	VI
	B6	VH1–3/Vλ1–51	20,000	3.54	VI
	B7	VH1–3/Vκ1–12	1,500	2.17	VI/VIII
	B8	VH1–3/Vλ1–51	600	0.99	VI
	B10	VH1–3/Vλ2–14	900	12.3	VII
Combinatorial library from thyroid-infiltrated TPO-purified B cells	T2	VH1–3/Vκ3–11	5,000	5.09	IX
	T3	VH1–3/Vκ1–39	2,000	1.28	VI/VIII
	T5	VH1–8/Vκ1–5	100	0.77	VI/VIII
	T8	VH1–3/Vκ1–39	4,000	4.50	VIII
	T10	VH3–64/Vκ3–11	100	2.19	VI/VIII
	T13	VH1–3/Vλ1–40	10,000	7.95	VIII

[a] Antibody titer was determined as the scFv dilution showing an absorbance of 1.0 in an ELISA assay using TPO as capture antigen.
[b] Affinity, corresponding to the K_d value, was measured by BIACORE.
[c] Mapping was determined by BIACORE.

each patients' serum, but not with normal sera (data not shown), indicating that the serum aAb from patients with Graves' disease recognized regions on TPO similar to those recognized by the scFvs tested. We also tested the ability of the scFv to inhibit patient serum binding to TPO. As shown in Table 6, the anti-TPO scFv defining the antigenic domains I, III, IV, V, VII, and IX showed moderate inhibition, whereas the scFvs VH1–3/Vλ1–51 B4 (domain VI), VH1–3/Vλ1–40 T13 (domain VIII), and VH1–69/Vλ1–40 ICA5 (domain II) were able to strongly inhibit the binding of most patients' sera to TPO, suggesting that these scFvs recognized immunodominant epitopes.

Discussion

The phage display system has the potential for greatly facilitating the generation and characterization of human Abs. Gene families and epitope recognition of recombinant anti-TPO aAbs (2–7, 19–21) have been characterized using combinatorial libraries. Although two studies stressed the likelihood that H/L pairing in TPO-specific combinatorial libraries reflects the *in vivo* situation (11, 12), the direct demonstration that original H/L pairing can be obtained from a random combinatorial library remained to be elucidated. To clarify this point, we constructed three random combinatorial libraries from different B cell subsets extracted from thyroid biopsies from patients suffering from Graves' disease. The H/L pairing of the anti-TPO aAbs obtained as well as the nucleotide sequence of the scFv fragments were compared with the recombinant aAbs, previously produced from an in-cell library (13), which reflects the *in vivo* situation. The three in-cell anti-TPO scFvs, ICA1, ICA5, and ICB7, used VH1–3 and VH1–69 germline genes associated with Vλ1–51 and Vλ1–40 genes, respectively. ICA1 and ICB7 show the same VDJ-H rearrangement, but differ in VL CDR3. Regarding the H/L pairing, 15 of the 41 scFvs obtained by random pairing used the same association as that found for in-cell scFvs. Particularly, one VH1–3/Vλ1–51 anti-TPO scFv derived from the CD19$^+$ random combinatorial library shows exactly the same nucleotide sequence as in-cell ICB7 (13) and consequently the same H/L pairing. These results clearly indicate that scFv from random combinatorial libraries can be formed by H/L pairs similar as those found *in vivo*, as previously suggested by others (10–12). On the other hand, the VH1–69/Vλ1–40 family pairing, originally found with scFv ICA5, over represented in our in-cell library (13), was obtained with only one scFv derived from the TPO$^+$ random library, but the original VDJ-H/VJ-L pairing was never found in our random combinatorial libraries. It seems that the possibility to obtain recombinant antibodies similar to those produced *in vivo* is strongly dependent on the development of a large random library.

Among the 41 different human anti-TPO scFv fragments selected, 31 possessed a λ light chain, including the 15 anti-TPO scFvs that showed natural H/L pairing. In contrast with the findings of Portolano *et al.* (7), who reported lower affinity for the majority of the λ light chain anti-TPO Fabs than for the κ light chain ones, our anti-TPO scFv fragments showed comparable affinities to TPO, independently of the chain pairing and the light chain type used. In accordance with studies describing the TPO antibody repertoire (6, 7, 19–21), most of the specific anti-TPO scFvs we produced were also derived from the VH1–3 germline gene in association with the Vλ1–40, Vλ1–51, or Vκ1–39 germline genes, indicating that a restriction in gene usage is found in thyroid-infiltrating B lymphocytes from patients with Graves' disease. In addition, we derived scFvs encoded by germline genes VH3–30, VH3–64, VH5–51, Vλ1–44, Vλ2–8, Vλ7–43, Vκ1–5, and Vκ3–11 that have never been described as being implicated in the TPO antibody response. These results extend the repertoire of anti-TPO aAbs and enlarge our knowledge of genes encoding such antibodies. Autoantibodies with λ light chains have been described in various autoimmune diseases (21–25), in particular λ anti-TSHr autoantibodies are involved in thyroid stimulation in Graves' disease

TABLE 6. Serum anti-TPO autoantibody binding inhibition by scFv fragments

Inhibitor	Patient with Graves' disease																			
	bdw05	bdw11	bdw16	bdw17	bdw20	bdw21	bdw24	bdw25	bdw26	bdw27	bdw31	bdw32	bdw33	bdw35	bdw37	bdw41	bdw42	bdw43	bdw44	bdw45	Mean
Irrelevant scFv	6.2[a]	0.0	8.4	2.6	6.6	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0	3.3	0.0	0.2	16.8	11.5	3.7	0.0	3.0
Anti-TPO scFv/epitope																					
A2/III	7.8	4.3	19.8	3.8	1.1	25.5	0.0	39.9	23.8	21.0	0.0	11.0	8.5	3.7	7.1	9.2	22.1	15.9	19.0	0.0	12.2
A10/IV	22.4	5.0	20.7	10.9	14.3	17.3	0.0	35.6	14.1	10.3	0.0	6.4	2.8	7.9	8.9	9.5	22.8	10.7	7.8	0.0	11.4
A11/V	17.1	1.5	22.8	11.3	18.2	29.4	0.0	43.1	26.2	21.1	0.0	9.5	3.8	15.5	13.6	13.0	26.5	10.2	1.8	0.0	14.2
B4/VI	71.4	22.7	60.6	45.2	55.5	65.9	11.6	81.1	74.4	50.2	6.3	24.8	28.6	46.1	38.0	53.4	65.0	37.2	36.2	38.2	45.6
B10/VII	10.4	9.7	15.0	5.9	8.1	19.9	0.0	30.7	20.5	16.9	3.6	9.1	10.3	16.2	13.8	15.3	22.0	18.7	10.7	0.0	12.8
T2/IX	31.2	13.9	27.6	19.6	19.8	29.0	1.1	42.7	28.8	25.5	0.0	3.2	15.4	11.0	14.3	22.6	33.1	20.8	23.6	0.4	19.2
T13/VIII	69.8	34.8	70.6	45.9	58.1	75.3	7.3	87.1	85.0	56.5	7.9	31.6	38.4	50.8	49.3	56.3	63.0	50.7	41.1	40.0	50.1
ICA1/I[b]	34.6	20.1	29.5	10.4	10.5	25.6	20.7	38.7	44.2	30.1	5.4	33.1	22.4	21.7	26.7	16.9	23.2	17.2	31.2	0.0	23.0
ICA5/II[b]	62.7	30.9	60.5	39.3	52.5	81.2	40.7	75.2	78.6	42.8	7.1	52.5	44.8	38.2	51.4	39.2	52.9	39.9	38.2	32.5	48.0

[a] Results are expressed as percent inhibition of the binding of patient's serum to TPO by each anti-TPO scFv.
[b] These in-cell scFvs were previously reported (13).

(26–28). The role of λ autoantibodies in thyroid diseases has recently been emphasized, because 5 anti-Tg antibodies close to the Vλ1–40 germline gene and 1 close to the Vλ1–51 germline gene have been isolated from a combinatorial library constructed from a patient with Hashimoto's thyroiditis (29). Few λ-TPO recombinant antibodies have been reported (7, 13, 21), probably because of the lack of information concerning Vλ germline genes leading to an inadequate choice of amplification primers and the low proportion of TPO autoantibodies present in the sera of patients with Graves' disease (30–33). Our data, obtained from an in-cell VH/VL assembled library (13) or with random libraries, indicate that anti-TPO scFvs derived from the Vλ1 gene family and particularly from the Vλ1–51 and Vλ1–40 germline genes represent one part of the *in vivo* TPO Ab repertoire of patients with autoimmune thyroid diseases.

We define nine antigenic regions on the TPO molecule by using the anti-TPO scFvs. With regard to the four antigenic domains described by using Fabs (2), it seems that the TPO molecule shows a more diverse set of Ab-binding regions. Furthermore, results obtained for epitope mapping of scFvs on TPO by using BIACORE methodology are difficult to compare with other methods used (2) for defining antigenic domains. Unlike Chazenbalk *et al.* (2), we did not observe any association between gene usage and epitope recognition by the anti-TPO scFvs. More precisely, scFvs with VL genes closest to the same germline gene, notably Vκ1–39, were found to recognize different regions on TPO, and, inversely, scFvs with VL genes closest to different germline genes, including Vκ1–39, were found to recognize the same epitope on TPO. However, other anti-TPO scFv fragments we obtained were encoded by newly described VL genes, which are not represented in the study by Chazenbalk *et al.* (2).

Among the three random combinatorial libraries we prepared, the library constructed from TPO-selected B cells (TPO^+ library) showed the greatest clonal diversity. This is in accordance with Hawkins and Winter (34), who suggested that the construction of Ab fragments from antigen-selected cells gives rise to a more diverse repertoire. As surface Igs are not expressed on plasmocytes, our TPO^+ library is probably composed of antibody fragments from other B cells and, notably, memory cells. On the other hand, the CD19 marker is present at all stages of B cell development, including plasma cells (35), suggesting that the $CD19^+$ and unpurified B cell libraries are derived from the same B cell population. By comparing the $CD19^+$ library with the previously reported in-cell library (13), the clonal diversity of the $CD19^+$ random library is 5 times greater than that of the in-cell library. This difference can be explained by the fact that one single H (or L) chain can be associated with different L (or H) chains and still bind to TPO in a random combinatorial library. This cannot occur in the case of scFvs selected from an in-cell library, where the H/L pairing occurs within the cells.

Amino acid analysis of scFvs derived from the dominant germline gene VH1–3 clearly indicates that certain residue

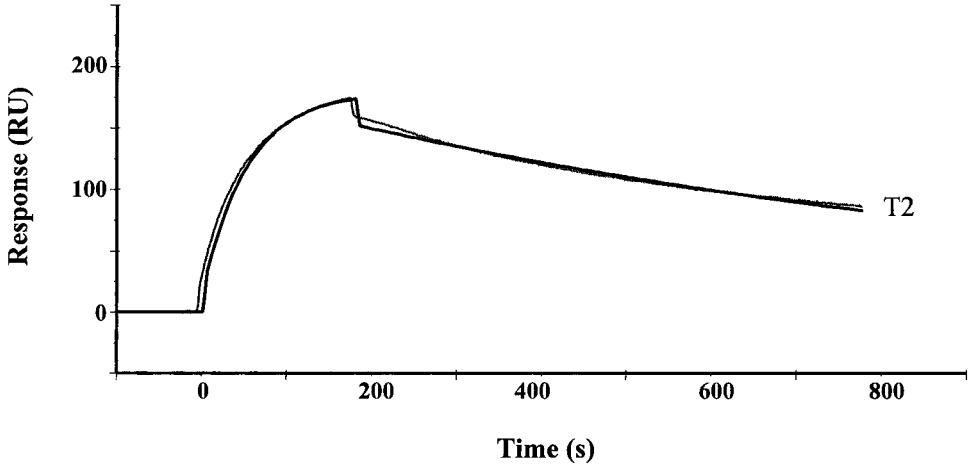

FIG. 1. Affinity measurement of anti-TPO scFvs by BIACORE 2000. Kinetics of TPO binding to scFv T2 immobilized onto a CM5 sensor chip through the anti-Myc mAb 9E10. The experimental curve and the theoretical curve, obtained by the global method using BIAevaluation 3.0 software, are superimposed.

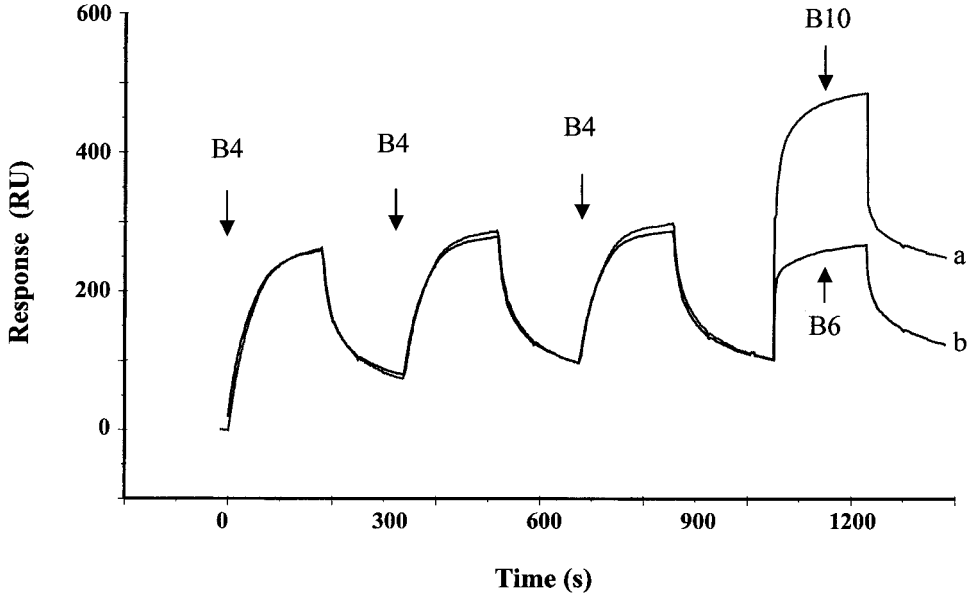

FIG. 2. Epitope mapping of anti-TPO scFvs by BIACORE 2000. After three injections of the scFv B4 to saturate its binding site on TPO, the scFv B10 still bound TPO (a), whereas the scFv B6 did not (b). The scFvs B4 and B6, but not B10, recognized the same domain on TPO. RU, Resonance units.

mutations are systematically found in the majority of anti-TPO recombinant antibodies, whatever the starting cells used for the library construction, but other amino acid replacements are closely related to a given library, suggesting that residue mutations could be a signature of the anti-TPO antibodies. Some of them have been described in TPO-specific Fabs (5–7, 29, 36), but others have never been demonstrated before. Epitope recognition does not appear to be linked to the starting cells, because the three scFvs showing strong inhibition of the autoantibody response to TPO belong to three different random libraries. These observations suggest that particular amino acid patterns in the VH region can be assigned to anti-TPO Abs, with a partial dependence on the library construction.

By competition studies between the anti-TPO scFvs and the serum aAbs from patients with Graves' disease, we have shown that serum aAbs and anti-TPO scFvs recognize the same or closely related domains on TPO. Furthermore, some of our anti-TPO scFvs were able to inhibit more than 80% of the binding of some of the serum aAbs to TPO, independently of serum titer, demonstrating that these recombinant Ab represent the major part of the TPO Ab repertoire. This is in correlation with previous studies described for the anti-TPO Fabs (37) and suggests that our scFvs recognize epitopes similar to those recognized by the Fabs. It would be instructive to compare TPO antigenic regions probed by our scFvs and by recombinant antibodies (1–7) or monoclonal Abs whose epitope has been determined (38). Interestingly, a strong inhibition of the binding of patients' sera to TPO was obtained by the random scFvs B4, T13 and in-cell scFv ICA5, which recognized three different antigenic domains on TPO, suggesting that these scFvs define immunodominant regions on TPO that might be identical to domains A and B (3). In addition, these scFvs use Vλ1–40 or Vλ1–51 genes in association with a VH1–3 gene, reflecting the *in vivo* pairing.

In conclusion, random H/L assortment libraries can give rise to scFvs that reflect the *in vivo* H/L situation and are similar to those obtained with the in-cell scFv library (13), which give rise to high affinity Ab fragments mimicking the binding of serum autoantibodies to TPO.

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