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Human Anti-Thyroid Peroxidase Single-Chain Fragment Variable of Ig Isolated from a Combinatorial Library Assembled In-Cell: Insights into the In Vivo Situation[1]

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In an attempt to explore the natural variable heavy and light chain (V_H/V_L) pairing of autoantibodies involved in Graves' disease, we constructed a phage-displayed Ab library obtained by in-cell PCR of thyroid-infiltrating cells. We report here the molecular cloning and characterization of human single-chain fragment variable regions (scFv) specific for thyroid peroxidase (TPO) generated from this library. On the basis of the nucleotide sequences, three different scFvs were obtained (ICA1, ICB7, and ICA5). All were encoded by genes derived from the VH1 and Vλ1 gene families. Using BIACORE for epitope mapping and kinetic analysis, we showed that these scFvs exhibited high affinity (K_d = 1 nM) for TPO and recognized three different epitopes. The biological relevance of these scFvs as compared with serum anti-TPO autoantibodies was assessed by competition studies. Sera from all the 29 Graves' disease patients tested were able to strongly inhibit (60–100%) the binding of the 3 scFvs to TPO. These data demonstrate that the in-cell PCR library generated human anti-TPO scFvs that retained the V_H/V_L pairing found in vivo and that the different epitope specificities defined by these scFvs overlapped with those found in the sera of patients with autoimmune thyroid disease. *The Journal of Immunology,* **2000, 114: 4162–4169.**

Thyroid peroxidase (TPO)[3] is the major thyroid autoantigen recognized by serum autoantibodies from patients with Graves' disease or Hashimoto's thyroiditis. The epitope specificity of the TPO disease-associated autoantibodies has been extensively studied using mouse mAbs (1, 2). However, because of the limited value of mouse mAbs for the study of human autoimmunity, particularly in terms of the respective genetic and epitopic repertoires (reviewed in Ref. 3), recombinant human Ab fragments have been produced using phage library technology (4–6). Several groups have used this technology to generate specific human anti-TPO Fabs (7–11). These Fabs have been widely analyzed in terms of epitope specificity and variable genes usage. Chazenbalk et al. (9) demonstrated that the anti-TPO Fabs showed a restriction in variable Ig heavy (V_H) and light (V_L) chain gene usage and also in epitopic recognition. Recently, Guo et al. (12) investigated the relationship between epitopes and gene usage of TPO Fab autoantibodies and concluded that epitope specificity and O2/O12 κ light chain gene usage are correlated.

Despite the large number of applications of combinatorial library methods to the study of human disease-associated autoantibodies and although the high affinity and the epitope specificity of TPO recombinant Abs are similar to autoantibodies in the serum, one major question raised by many investigators remains: Does the V_H and V_L chain pairing of these antibodies from random combinatorial libraries reflect the in vivo pairing (13–16)? The production of human mAbs by cell fusion or by transformation of B cells with EBV could provide answers to this question. However, to date only one human mAb against TPO has been reported (17, 18).

It is our view that it is important to analyze the natural V_H/V_L pairing of the disease-associated autoantibodies in relation to their epitope specificity to understand the pathological process leading to autoimmune thyroid disease, and particularly in the phenomena implicated in the maintenance of B cell tolerance and induction of autoimmunity, known as receptor editing, that occurs at the level of V_H/V_L pairing (19–22).

To address this issue, we previously applied an in-cell PCR protocol, originally described by Embleton et al. (23), to human thyroid-infiltrating $CD19^+$ B cells to obtain in vivo V_H/V_L gene pairing information (24). In the present study, we report the isolation and characterization of recombinant anti-TPO scFvs generated from this in-cell combinatorial library. Three specific human anti-TPO scFvs were obtained, each directed against a different epitope on human TPO. Using competition studies with serum autoantibodies, we have shown that the epitope specificities expressed by these single-chain fragment variable regions (scFvs) were present in the sera of all Graves' disease patients tested. We present here the first human anti-TPO scFv from an in-cell library that retains the V_H/V_L pairing present in vivo.

Methods

Patients

Thyroid-infiltrating B lymphocytes used for the library construction were isolated from biopsies from three women suffering from Graves' disease.

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[3] Abbreviations used in this paper: TPO, thyroid peroxidase; scFv, single-chain fragment variable, CDR, complementary-determining regions; FR, framework regions; RU, resonance unit.

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These patients were named bdw17, bdw26, and bdw31 and were 25, 31, and 22 years old, respectively. The sera of 29 Graves' disease patients with high anti-TPO titers were used for the competition studies.

Construction of the in-cell combinatorial library

The isolation and purification of the $CD19^+$ B cells as well as the in-cell PCR procedure have been described in detail (24). The three samples were amplified and cloned separately and then pooled for panning on TPO. Briefly, the in-cell-amplified and -associated scFv genes were cloned into the pHEN1 vector (25) between the *Sfi*I and *Not*I restriction sites and transformed in *Escherichia coli* XL1-Blue competent cells by electroporation. The *lox*P site, which allows the recombination between V_H and V_L genes, was removed by *Nhe*I restriction enzyme digestion.

Selection of phage-displayed scFv

2xTY medium (50 ml containing 100 μg/ml ampicillin, 25 μg/ml tetracycline, and 2% glucose) was inoculated with 0.5 ml scraped colonies from the library and incubated overnight with shaking at 37°C. The preculture was diluted 100-fold in the same medium and grown until the $OD_{600\ nm}$ reached 0.5; 5 ml of this culture was added to 45 ml of the same medium containing 5×10^{10} PFU M13KO7 helper phage (Bio-Rad, Hercules, CA) and incubated for 40 min at 37°C without shaking, followed by 1 h with shaking. The culture was pelleted; resuspended in 30 ml 2xTY medium containing 100 μg/ml ampicillin, 25 μg/ml tetracycline, and 50 μg/ml kanamycin; and then incubated overnight at 37°C with shaking. Bacteria were pelleted and the phages in the supernatant were precipitated by adding a volume of 20% polyethylene glycol, 2.5 M NaCl equal to one-fifth of the volume of the supernatant. After incubation on ice, the phages were pelleted by centrifugation at 11,000 rpm for 30 min at 4°C, resuspended in 1 ml of distilled water, and filtered through a 0.45-μm pore size filter to remove the remaining cell fragments.

The selection of anti-TPO clones was achieved by panning on TPO. Immunotubes (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 2 ml of a 5-μg/ml purified human TPO (HyTes, Turku, Finland) solution in carbonate-bicarbonate buffer (pH 9.6), washed three times with PBS, and blocked with 2 ml PBS, 2% nonfat milk for 2 h at 37°C. The phage-displayed scFvs were incubated at 10^9 TU/ml in 2 ml of the same buffer for 2 h at room temperature with low shaking. After extensive washing with PBS, 0.1% Tween, the bound phages were eluted with 0.1 M glycine-HCl (pH 2.2), for 10 min, neutralized by adding 2 M Tris base, and used to infect XL1-Blue cells. These bacteria were plated on 245 $\times$ 245-mm dishes of 2xTY medium containing 100 μg/ml ampicillin, 25 μg/ml tetracycline, and 2% glucose and were grown overnight at 37°C. Colonies were scraped off and used to make a new phage preparation for the next round of panning.

Screening of phage-Abs by ELISA

The microtiter plates were coated overnight at 4°C with TPO at a concentration of 1 μg/ml in carbonate-bicarbonate buffer (pH 9.6), washed three times with PBS, 0.1% Tween, and blocked with PBS, 0.1% Tween, 2% nonfat milk for 2 h at 37°C. Different dilutions of phages were added in the same buffer for 2 h at room temperature. After three washings with PBS, 0.1% Tween, bound phages were detected by adding sheep anti-M13 Abs (5 Prime $\rightarrow$ 3 Prime, Boulder, CO) diluted 1:4000 in PBS, 0.1% Tween, 1% nonfat milk followed by alkaline phosphatase-conjugated anti-sheep IgG Abs (Sigma, St. Louis, MO) and 4-nitrophenyl phosphate as substrate (Sigma).

Sequencing of the recombinant clones

Sequences were determined by cycle sequencing using ABI Prism Rhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). The primers used on both sides of the pHEN1 cloning site were: 5′-TGATTACGCCAAGCTTGC-3′ for the forward primer and 5′-TTGTCGTCTTTCCAGACG-3′ for the reverse primer. Sequences were run on an ABI Prism 377 electrophoresis system (Applied Biosystems). Sequence alignments with germline genes were performed using the IGMT sequence directory (26).

Diversity determination by Bst*NI restriction enzyme pattern*

As described by Marks et al. (27), the scFv genes were amplified and the PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Chatsworth, CA). Then, the scFv genes were cut for 2 h at 60°C by 10 U of *Bst*NI restriction enzyme (New England BioLabs, Beverly, MA). The scFv gene restriction patterns were visualized by ethidium bromide on an 2% agarose gel.

Expression of soluble scFv and periplasmic extraction

Soluble expression was performed in HB2151, a nonsuppressive *E. coli* strain as previously described (28). An overnight culture of recombinant bacteria was diluted 1:100 in 2xTY medium containing 100 μg/ml ampicillin and 0.1% glucose and grown until the OD_{600} was ~0.6. The induction of scFv expression was achieved by adding 1 mM isopropyl-β-D-thiogalactopyranoside and shaking for 3 h at 25°C. The bacteria were pelleted, resuspended in 1:40 dilution of the culture volume of lysis buffer (20 mM HEPES (pH 8), 1 μg/ml polymyxin B supplemented with protease inhibitors), and incubated for 30 min on ice (29). Cells were centrifuged for 10 min at 13,000 rpm, and the supernatant containing the scFvs was stored and used directly for immunoreactions.

Binding of soluble scFv to TPO by ELISA

The protocol was the same as for the phage ELISA test except that the detection of the soluble scFv was performed using the murine mAb 9E10 which is directed against the myc-tag (30) sequence present at the C-terminal end of scFv. The mAb 9E10 was used at a concentration of 4 μg/ml in PBS, 0.1% Tween, 1% nonfat milk and incubated for 1.5 h at room temperature. Next, an alkaline phosphatase-conjugated Fc specific anti-mouse IgG (Sigma) diluted 1:1000 in the same buffer was added to each well and incubated for 1.5 h at room temperature. Enzyme activity was assayed by addition of 4-nitrophenyl phosphate as substrate.

Affinity determination and epitope mapping by real time analysis

The affinity and the epitope mapping of the anti-TPO scFvs were determined using BIACORE 2000 (Biacore, Uppsala, Sweden). For the affinity experiments, an anti-myc mAb, 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. The anti-TPO scFv periplasmic extracts and an irrelevant scFv (1C10), used as a negative control, were diluted 1:4 in HBS-EP buffer (10 mM HEPES, pH 7.6, 150 mM NaCl, 3 mM EDTA, 0.005% polysorbate 20 (v/v)) (Biacore), pH 7.4, and injected at a flow rate of 20 μl/min over the mAb 9E10. The scFvs 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 for TPO. The CM5 sensor chip with immobilized 9E10 was regenerated after each kinetic experiment by a 20-s 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 (31). 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 scFvs 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-EP buffer, pH 7.4, were successively passed over the immobilized TPO, either as first (Ab inhibitor) 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 due to the existence of a different epitope. The 1C10 scFv was used as a negative inhibitor control.

Spot multiple peptide synthesis and scFv assay

We synthesized 307 overlapping hexadecapeptides, frameshifted by 3 residues and representing the complete human TPO sequence, on a cellulose membrane (Abimed, Langenfeld, Germany) by the spot technique (32, 33) using the ASP 412 spotter (Abimed). The membrane-bound peptides were probed by incubation with the scFv, and the binding was detected by anti-*myc* mAb followed by addition of an alkaline phosphatase-conjugated Fc-specific anti-mouse IgG (Sigma). A phosphatase substrate (5-bromo-4-chloro-3-indolyl phosphate and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) was then added. A blue precipitate on the spots was indicative of binding.

scFv binding inhibition by serum anti-TPO autoantibodies

Inhibition ELISA was performed as previously described (34) with some modifications. Native human TPO was coated overnight at 4°C at a concentration of 1 μg/ml in carbonate-bicarbonate buffer, pH 9.6. After saturation with PBS, 0.1% Tween, 2% nonfat milk for 2 h at 37°C, patients' or normal sera diluted 1:10 in PBS, 0.1% Tween, 1% nonfat milk were added, and the microtiter plates were incubated for 1.5 h at room temperature. Plates were washed three times with PBS, 0.1% Tween, and then the scFv, at a dilution giving 50% binding, was added followed by incubation for 2 h at room temperature. Bound scFv was detected as described above for the binding of soluble scFv to TPO by ELISA.

Table I. *Analysis of anti-TPO scFv V(D)J heavy and VJ light chain germline genes: usage and homologies*

	Heavy Chain Germlines				Light Chain Germlines		
Clones	V_H	Homology	D	J_H	V_L	Homology	J_L
ICA1	VH1-3*01	(90.6%)	D4-17*01	JH4*02	Vλ1-51*01	(91.3%)	Jλ-1*01
ICB7	VH1-3*01	(90.9%)	D4-17*01	JH4*02	Vλ1-51*01	(96.9%)	Jλ-1*01
ICA5	VH1-69*01	(88.7%)	D1-1*01	JH4*02	Vλ1-40*02	(94.5%)	Jλ-3*01

Results

Selection of anti-TPO scFv from in-cell library

One million human thyroid-infiltrating B $CD19^+$ lymphocytes were subjected to in-cell PCR in association with the Cre-*lox*P recombination system to obtain in vivo pairing of V_H and V_L genes (24). The scFv genes (V_H-linker-V_L) were ligated into the pHEN1 vector and used to transform *E. coli* XL1b cells resulting in 10^5 clones.

Freshly prepared phages from the library were subjected to three consecutive rounds of panning on highly purified human TPO. Phage pools from each round of panning were tested by ELISA on TPO. The best result was obtained from panning 2 in which 88% of the clones obtained expressed an scFv able to recognize TPO. A second screening was performed based on the *Bst*NI digestion pattern of the clones. The scFvs could be divided into two groups on the basis of their restriction patterns (data not shown). All the scFvs in the first group were sequenced, leading to two different V_H/V_L sequences, represented by anti-TPO scFvs ICA1 and ICB7. In the second group, 16 scFvs sequenced showed similar V_H/V_L sequences, represented by ICA5.

Genes encoding TPO-specific in-cell scFvs

The nucleotide sequences were compared to the closest known germline genes (Fig. 1). The three scFvs were found to use V genes from the VH1 and Vλ1 gene families. The most closely related V_H germline genes were VH1-3*01 for ICA1 and ICB7 (with 90.6 and 90.9% homology, respectively) and VH1-69*01 for ICA5 (with 88.7% homology). These two scFvs used the same D4-17*01 segment in association with the JH4*02 gene, whereas ICA5 resulted from the rearrangement of VH1-69*01 with the D1-1*01/JH4*02 gene segments. For the V_L chain genes, the ICA1 and ICB7 scFvs showed 91.3 and 96.9% homology, respectively, with the Vλ1-51*01 germline gene linked to the Jλ1*01 segment. Finally, the ICA5 scFv had 94.5% homology with the Vλ1-40*02 germline gene and used the Jλ3*01 segment (Table I).

For all three scFvs, somatic hypermutations were found in both the V_H and V_L sequences. In the H and L chain genes of ICA1, ICB7, and ICA5, the ratio of replacement vs silent (R/S) mutations was much higher in the complementarity-determining regions (CDRs) than in the framework regions (FRs), except for the CDRs of the ICB7 light chain which showed the same low R/S ratio as the FRs (Table II). These results suggest that scFvs ICA1 and ICA5, in particular, were generated by an Ag-driven autoimmune response. Further, the ICA1 and ICB7 scFvs used the same V, (D), J genes but could be differentiated by the number of the replacement mutations localized in their LCDR3s. In the case of ICA1 scFv, 16 replacement mutations were observed, whereas LCDR3 of ICB7 scFv presented the same nucleotide sequence as the germline. Comparison of the amino sequences of these two LCDR3s (Fig. 2) showed that among the 16 replacement mutations in LCDR3 of ICA1, three encoded the amino acids cysteine, lysine, and alanine, the physicochemical properties of which are completely different from those (glycine, tryptophan, and aspartic acid) of the germline genes, leading to inversion of the global charge of the LCDR3.

Although we previously verified that the Cκ and Vκ primers are able to amplify different κ genes (24), no κ-chain type was seen in any of our anti-TPO scFvs. Additional controls have since been done and demonstrate the low efficiency of the Cκ *Ext For* primer used in the oligonucleotide pool for the RT-PCR and PCR1 (data not shown).

Characterization of the anti-TPO scFvs

The binding of scFv to TPO was determined by ELISA. As shown in Fig. 3, strong binding was obtained for both ICA5 and ICA1, whereas ICB7 presented weaker anti-TPO activity. The binding of these scFvs to adsorbed TPO was strongly inhibited by soluble TPO and weakly by structurally related molecules such as lactoperoxidase and HRP, whereas no cross-reactivity was observed with human thyroglobulin (Fig. 4).

The affinity of the three scFvs for TPO was determined by BIACORE 2000. The resonance unit (RU) values at 190 s were 104, 77, 54, and 4 for scFvs ICA5, ICA1, ICB7, and an irrelevant scFv (1C10), respectively. The binding curves for these scFvs were different, with strong binding of ICA5 as shown by the sensorgrams (Fig. 5). The kinetic constants (dissociation rate (K_d) and association rate (K_a)) determined on BIACORE 2000 by real time interaction analysis, are presented in Table III. The ICA1 and ICA5 scFvs had affinities for TPO in the nanomolar range (K_d = 4.2 nM and K_d = 1.82 nM, respectively). The affinity of ICB7 scFv was lower (K_d = 12 nM) than the other two clones due to a lower association rate and a higher dissociation rate (see Table III).

To determine whether the three scFvs recognized similar or different epitopes on TPO, all scFv pairs were assayed by injecting them sequentially on TPO covalently linked to the dextran matrix. The RU values of ICA1 binding, alone or in the presence of ICA5, and reciprocally, were similar (Table IV) suggesting that the binding of first scFv, under saturating condition, did not affect the

FIGURE 1. Nucleotide sequences of the V(D)J heavy and VJ light chain regions of the three TPO-specific in-cell scFvs. *A*, ICA1 and ICB7 scFv genes compared with the closest germline genes VH1-3*01/D4-17*01/JH4*02 and Vλ1-51*01/Jλ1*01 according to IMGT sequence, analysis software (26). *B*, ICA5 scFv genes compared with the closest germline genes VH1-69*01/D1-1*01/JH4*02 and Vλ1-40*02/Jλ3*01. Identities are shown by dashed lines. The replacement mutations are indicated by capital letters and the silent mutations by small letters. These sequence data are available from EMBL/GenBank/DNA Data Base in Japan under accession numbers AJ238326 and AJ238329 for the heavy and light chains of clone ICA1, respectively; AJ238327 and AJ238330 for the heavy and light chains of clone ICA5, respectively; AJ238328 and AJ238331 for the heavy and light chains of clone ICB7, respectively.

Table II. *Analysis of the replacement (R) and silent (S) mutation ratio in the V_H and V_L chain genes of TPO-specific in-cell scFvs*

Clones	CDRs			FRs		
	R	S	R/S	R	S	R/S
H chains						
ICA1	8	1	8.0	10	8	1.2
ICB7	8	1	8.0	11	7	1.6
ICA5	14	1	14.0	14	7	2.0
L chains						
ICA1	18	0	Infinity	4	3	1.3
ICB7	1	1	1.0	4	3	1.3
ICA5	10	1	10.0	3	2	1.5

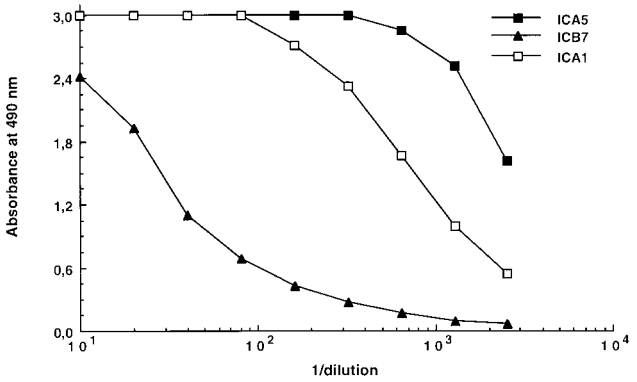

FIGURE 3. Binding curves of anti-TPO scFv ICA1, ICA5, and ICB7 to adsorbed TPO.

binding of the other. Similarly, when the ICA1 or ICA5 scFv was injected first, the binding of the ICB7 scFv to TPO was not inhibited as shown in Table IV. As example, sensorgrams of epitope mapping of ICA1 and ICB7 are represented in Fig. 6. Sensorgram 1 (binding of ICB7 without ICA1) and sensorgram 2 (binding of ICB7 after binding of ICA1 injected three times) were baseline matched. The difference observed between these sensorgrams was due to the dissociation level of ICA1 binding which was not subtracted from ICB7 binding, thus leading to a lower apparent RU value. These results indicate that the three scFvs were able to bind to different epitopes on TPO.

Using spot synthesis (32) to identify amino acid sequences of epitopes recognized by the 3 scFvs, 307 overlapping 16-aa-long peptides spanning the entire sequence of TPO were synthesized on cellulose membrane supports. The absence of binding of any of the three scFvs to the synthetic peptides (data not shown) suggested that they probably recognize discontinuous epitopes on TPO.

Competition studies between scFv and serum TPO autoantibodies

Twenty-nine sera from patients suffering from Graves' disease and 20 normal sera were used to test their ability to inhibit the binding of the three scFvs to TPO. Strong inhibition (60–100%) was obtained with every patient's serum (Fig. 7), and no inhibition was observed with normal sera.

Discussion

Phage-displayed combinatorial library techniques have been used to characterize gene families and epitope specificities of human autoantibodies (3). Several TPO Fabs have been produced using either bacteriophage λ vector (7, 9) or phage display vector pComb3 (10, 11, 16, 35, 36). Despite the fact that individual H and L chain genes of the TPO Fab already described are clearly those used in vivo, it has yet to be proved whether separate amplification of heavy and light chains and their random recombination produce the same H and L chains pairing as in vivo. Caton and Kaprowski (37) demonstrated in an immunized mouse model that most of V_H and V_L chain combinations obtained from a combinatorial library are similar to those observed among hybridoma Abs. However, such evidence is not available for human autoantibodies.

To clarify this issue, we have used a novel PCR method (in-cell PCR) developed by Embleton et al. (23) which allows the intracellular PCR amplification of V_H and V_L genes within B cells and the generation of Ab fragments that retain the H and L chain pairing that occurs in vivo. Our present study describes the production and characterization of the first human recombinant scFvs specific for TPO using this methodology. The starting lymphocytes were thyroid-infiltrating B cells from patients with Graves' disease. These lymphocytes are enriched in activated B cells secreting thyroid autoantibodies, as demonstrated by McLachlan et al. (38). We purified the $CD19^+$ B cells from this lymphocyte population. The leukocyte surface marker $CD19^+$ is expressed at all stages of B cell development (39) but especially in memory cells, which are a useful source of Ab-secreting cells because of their long life (40). Fujikawa et al. (41) demonstrated that the percentage of $CD19^+$ B cells is increased within the thyroid gland in Graves' disease. In addition, an association between CD19 overexpression and autoimmunity in humans has been suggested (42, 43).

From these B cells, we isolated and sequenced 21 TPO scFv Ab fragments. On the basis of their nucleotide sequences, three different scFvs (ICA1, ICB7, and ICA5) were obtained and characterized. In agreement with previously reported results (reviewed in Ref. 44), our anti-TPO scFvs are encoded by hypermutated genes. The scFv VH genes are derived from the V_H1 family germline genes. The ICA1 and ICB7 scFvs are encoded by the VH1-3*01 germline gene, and the scFv ICA5 is derived from the VH1-69*01 germline gene. The majority of heavy genes used by anti-TPO autoantibodies (reviewed in Ref. 44) are derived from either the V_H1 or the V_H3 gene families. Our results show that the anti-TPO scFvs selected from the in-cell library used the same V_H germline genes and J_H segments as most of the anti-TPO Fabs selected from random libraries. In particular, the association of VH1-3 with JH4,

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                          FR1            _____CDR1___        FR2          ___CDR2___
IGLV1-51*01     QSVLTQPPS.VSAAPGQKVTISCSGS SSNIGNNY.... VSWYQQLPGTAPKLLIY DNN.......65
      ICA1      QSVLTQPPS.VSAAPGQRVTISCSGS SSSIGNKY.... VSWYQQLPGTAPKLLIF DNN.......65
      ICB7      QSVVTQPPS.VSAAPGQKVSISCSGS SSNTGNNY.... VSWYQLLPGTAPKLLIY DNN.......65

                          FR3                                    __CDR3_
IGLV1-51*01      KRPSGIP.DRFSGSK..SGTSATLGITGLQTGDEADYYC GTWDSSL   111
      ICA1       KRPSGIP.DRFSASK..SGTSATLGITGLQTEDEADYYC CSKAAGN   111
      ICB7       ERPSGIP.DRFSGSK..SGTSATLGITGLQTGDEADYYC GTWDSSL   111
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FIGURE 2. Derived amino acid sequences, compared with the closest germline gene, of the λ light chains of TPO-specific ICA1 and ICB7 scFvs. Amino acid differences are indicated by bold letters.

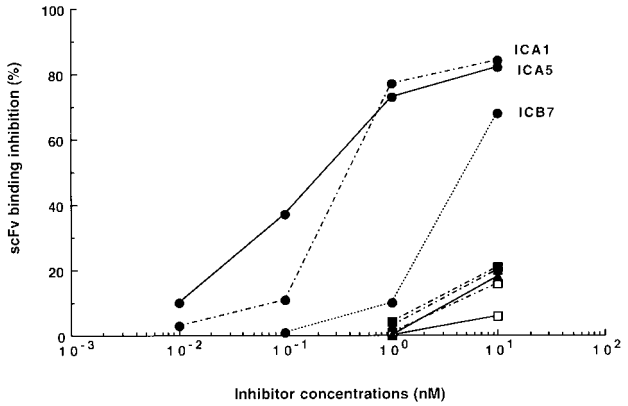

FIGURE 4. Binding inhibition of anti-TPO ICA1, ICA5, and ICB7 scFvs to adsorbed TPO by increasing concentrations of soluble TPO (●), Tg (□), lactoperoxidase (■), and HRP (▲).

expressed by two of our anti-TPO scFvs, is found in five of the reported anti-TPO Fabs. However, these five anti-TPO heavy chains are paired with κ light chains and most frequently with the gene VκO12, whereas the scFvs described here are paired with λ chains. Even though we were unable to amplify the κ light chain genes (due to the inefficiency of our Cκ *Ext For* primer), we demonstrated the presence of anti-TPO scFvs with λ light chains in thyroid-infiltrating B cells from three patients. This is not an isolated phenomenon as shown by the generation of 14 anti-TPO λ light chains and 10 anti-TPO κ light chains from new recombinant libraries constructed using B cells from additional patients (our manuscript in preparation). On the basis of these results, we can affirm that both λ and κ light chains are expressed by thyroid-infiltrating B cells.

The VL chain of ICA5 is encoded by the Vλ1-40 germline gene (HumLv1042) that is expressed in other autoantibodies such as rheumatoid factor (45) and thyroglobulin autoantibodies (reviewed in Ref. 44). In addition, the germline gene Vλ1-51 (Humlv117) that encodes the light chain of the other two TPO scFvs (ICA1 and ICB7) is also used in autoimmune anti-DNA Abs (46) and in acetylcholine receptor autoantibodies (47). Only four anti-TPO Fab/λ light chain combinations have been described (16, 48). One of them (TR1.41) used the VH1-69 germline gene that is used by one of our TPO scFvs (ICA5). A striking feature of the nucleotide sequence of these two anti-TPO Ab fragments is that they have eight identical somatic mutations in common on the heavy chain gene, suggesting that these V_H Igs, which are from different patients with a thyroid disorder, have undergone the same somatic

Table III. *Kinetic parameters of anti-TPO scFvs determined by BIACORE 2000*

Clones	k_a (M^{-1} s^{-1})	K_d (s^{-1})	K_D (M)
ICA1	2.79×10^5	1.16×10^{-3}	4.17×10^{-9}
ICB7	1.82×10^5	2.18×10^{-3}	1.20×10^{-8}
ICA5	3.66×10^5	1.22×10^{-3}	1.82×10^{-9}

Table IV. *Epitope mapping of anti-TPO scFvs by BIACORE 2000*

	Anti-TPO scFv Binding (RU)[b]		
Inhibitor[a]	ICA5	ICA1	ICB7
None	171	137	68
ICA5		129	63
ICA1	158		65
ICB7	ND	ND	

[a] First anti-TPO scFv, injected three times to saturate its binding site on TPO.
[b] RU values were corrected by subtracting the base line.

maturation. Both Abs are paired with λ light chains but from a different light chain gene family. In view of this, it would be instructive to compare their epitope specificities.

Despite the predominance of κ light chains in V_H and V_L pairing found in human IgG-B cells in general (49), several groups have demonstrated the use of Igλ light chain genes in autoimmune diseases. Two dsDNA mAbs expressing the λ light chain type have been reported (50). More recently, Ravirajan et al. (51) generated two IgG/λ chain DNA-binding mAbs and, interestingly, one of them (IgG1/λ) is nephritogenic. In addition, Prummel et al. (52) described several clones with a diverse spectrum of λ light chain variable genes in Graves' ophthalmopathy and have suggested a role for λ autoantibodies in the pathogenesis of this autoimmune disease. Our results and those in the literature raise numerous interesting questions concerning the expression of the λ light chain such as whether they are the result of secondary rearrangement, or receptor editing, resulting in a light chain replacement as observed in human mature B cells (53). Similar observations concerning the rearrangement of lambda chain genes in murine germinal center B cells have been made by Hikida and Ohmori (54). In addition, it has been suggested that receptor editing plays a role in establishing B cell tolerance and in induction of pathogenic autoimmunity (55). In view of these data, the physiopathological role of these λ light chain autoantibodies remains an important question to be clarified.

The affinities of the anti-TPO scFvs were measured by BIACORE. ICA5 and ICA1 have relatively high affinity for

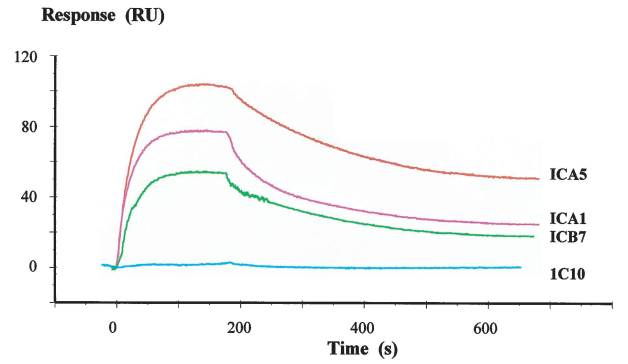

FIGURE 5. Surface plasmon resonance analysis of the binding of scFvs ICA1, ICB7, and ICA5 and control scFv IC10 to immobilized TPO. Sensorgrams for the kinetic analysis of the interaction of these scFvs with TPO.

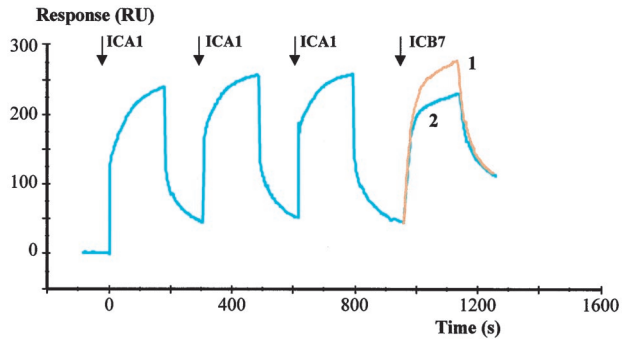

FIGURE 6. Sensorgrams of epitope mapping of scFvs ICA1 and ICB7. Sensorgram 1 corresponds to the binding of ICB7 to TPO in the absence of ICA1, and sensorgram 2 corresponds to the binding of ICB7 after three injections of ICA1.

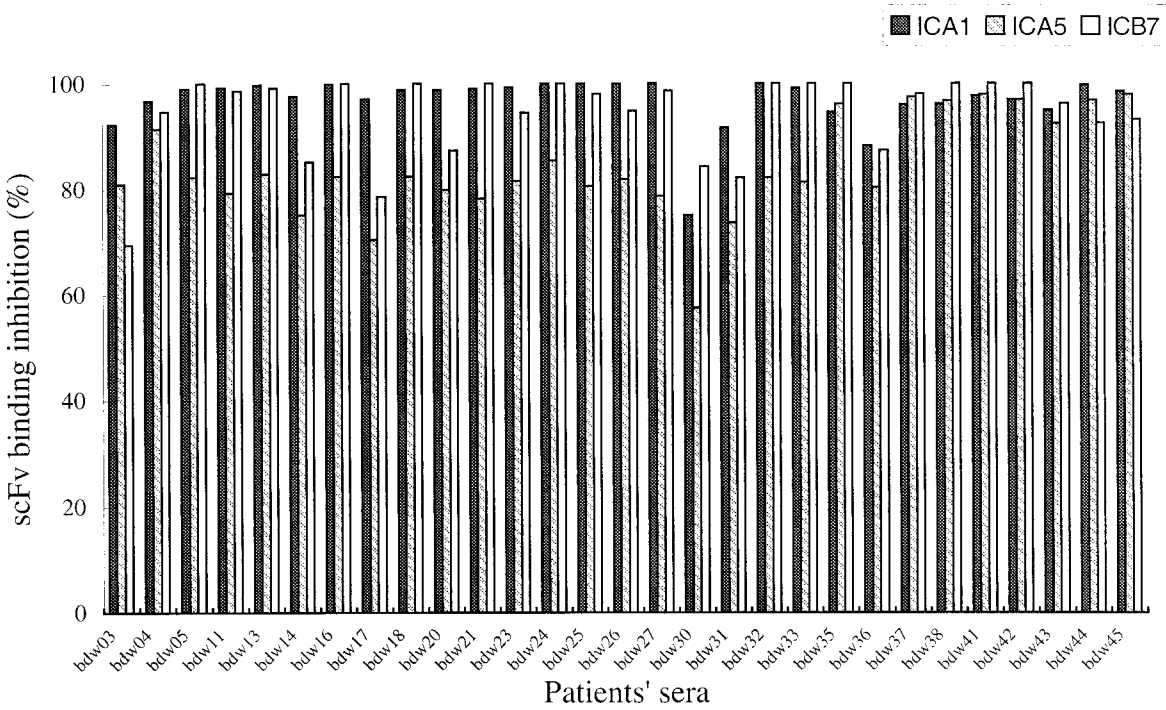

FIGURE 7. Binding inhibition of anti-TPO ICA1, ICA5, and ICB7 scFvs by the sera from the 29 Graves' disease patients. No inhibition was observed with normal sera.

TPO (1.82×10^{-9} M and 4.17×10^{-9} M, respectively), whereas ICB7 has a somewhat lower affinity ($\sim 10^{-8}$ M). These affinities are similar to those of anti-TPO Fabs (λ or κ light chain) already reported (44), although the methods used for their determination are different. Of particular interest is the fact that ICA1 and ICB7 have the same VDJ genes with very similar somatic mutations on the heavy chain. The differences observed between these Abs is in the number of somatic mutations in CDR3 of the light chain of ICA1 (16 nucleic acid mutations vs 1 for ICB7), a factor that may contributed to the increased affinity of ICA1 for TPO as compared with ICB7. Similarly, a great number of mutations (11 R mutations) in the LCDR3 of ICA5, which has a high affinity (1.82×10^{-9} M), was observed. This finding probably indicates that ICA1 and ICA5 have been affinity selected by an Ag-driven immune response and emphasizes the relationship between somatic mutation of light chains and the affinity for TPO. Furthermore, the number of mutations observed in the FR regions is identical in all three light chains, suggesting that the Ag-driven mechanism is operating at the level of the CDRs only.

To determine whether the three scFvs are directed against similar or different epitopes, we used BIACORE technology which allows epitope mapping under optimal conditions, i.e., without any tracer molecule. Three different epitopes on the TPO molecule were recognized by the three anti-TPO scFvs. However, since these scFvs were not able to bind any of the synthetic overlapping hexadecapeptides spanning the sequence of human TPO, we presume that they recognize discontinuous epitopes. This is in agreement with previously published reports which show that the majority of TPO autoantibodies recognize discontinuous epitopes (8). Finally, the three anti-TPO scFv Ab fragments were compared with serum TPO autoantibodies using competition assays. All 29 sera from patients with Graves' disease were able to inhibit the binding of the three anti-TPO scFvs, suggesting that these scFvs and TPO serum autoantibodies share the same or neighboring epitopes on the TPO molecule.

In summary, by in-cell V_H-V_L amplification, we have generated the first anti-TPO scFvs that retain the naturally occurring heavy and light chain pairing. These scFvs recognized three different discontinuous epitopes on TPO with relatively high affinity ($\sim 10^{-9}$ M), similar to those of the majority of the anti-TPO Fab/κ or λ light chains reported (reviewed in Ref. 44). These epitopic specificities were present in all the Graves' disease patients tested. In addition, the V_L germline genes that encoded our scFvs were expressed by the majority of autoantibodies generated in organ-specific or non-organ-specific autoimmune diseases. We have recently obtained data (our manuscript in preparation) showing that the anti-TPO autoantibodies present in intrathyroid-infiltrating B cells possess both λ light and κ chains. This observation is strengthened by the study of Wilkin and Casey (56) on the distribution of Ig-containing cells in human autoimmune thyroiditis, who demonstrated that both types of light chains were present in cells occupying areas of epithelial invasion of the thyroid. The anti-TPO λ scFvs described here add to the information available on human variable λ gene usage and raise the question of the role of this category of autoantibodies in the pathogenesis of thyroid autoimmune diseases.

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References

1. Ruf, J., M. E. Toubert, B. Czarnocka, J. M. Durand-Gorde, M. Ferrand, and P. Carayon. 1989. Relationship between immunological structure and biochemical properties of human thyroid peroxidase. *Endocrinology 125:1211.*
2. Ewins, D. L., P. S. Barnett, R. W. Tomlinson, A. M. McGregor, and J. P. Banga. 1992. Mapping epitope specificities of monoclonal antibodies to thyroid peroxidase using recombinant antigen preparations. *Autoimmunity 11:141.*

3. Rapoport, B., S. Portolano, and S. M. McLachlan. 1995. Combinatorial libraries: new insights into human organ-specific autoantibodies. *Immunol. Today 16:43*.
4. Huse, W. D., L. Sastry, S. A. Iverson, A. S. Kang, M. Alting-Mees, D. R. Burton, S. J. Benkovic, and R. A. Lerner. 1989. Generation of a large combinatorial library of the immunoglobulin repertoire in phage λ. *Science 246:1275*.
5. McCafferty, J., A. D. Griffiths, G. Winter, and D. J. Chiswell. 1990. Phage antibodies: filamentous phage displaying antibody variable domains. *Nature 348:552*.
6. Barbas, C. F. 3rd, A. S. Kang, R. A. Lerner, and S. J. Benkovic. 1991. Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. *Proc. Natl. Acad. Sci. USA 88:7978*.
7. Portolano, S., P. Seto, G. D. Chazenbalk, Y. Nagayama, S. M. McLachlan, and B. Rapoport. 1991. A human Fab fragment specific for thyroid peroxidase generated by cloning thyroid lymphocyte-derived immunoglobulin genes in a bacteriophage λ library. *Biochem. Biophys. Res. Commun. 179:372*.
8. Portolano, S., G. D. Chazenbalk, P. Seto, J. S. Hutchison, B. Rapoport, and S. M. McLachlan. 1992. Recognition by recombinant autoimmune thyroid disease-derived Fab fragments of a dominant conformational epitope on human thyroid peroxidase. *J. Clin. Invest. 90:720*.
9. Chazenbalk, G. D., S. Portolano, D. Russo, J. S. Hutchison, B. Rapoport, and S. McLachlan. 1993. Human organ-specific autoimmune disease. Molecular cloning and expression of an autoantibody gene repertoire for a major autoantigen reveals an antigenic immunodominant region and restricted immunoglobulin gene usage in the target organ. *J. Clin Invest. 92:62*.
10. Hexham, J. M., L. J. Partridge, J. Furmaniak, V. B. Petersen, J. C. Colls, C. Pegg, B. Rees Smith, and D. R. Burton. 1994. Cloning and characterisation of TPO autoantibodies using combinatorial phage display libraries. *Autoimmunity 17:167*.
11. McIntosh, R. S., M. S. Asghar, E. H. Kemp, P. F. Watson, A. Gardas, J. P. Banga, and A. P. Weetman. 1997. Analysis of immunoglobulin G κ antithyroid peroxidase antibodies from different tissues in Hashimoto's thyroiditis. *J. Clin. Endocrinol. Metab. 82:3818*.
12. Guo, J., R. S. McIntosh, B. Czarnocka, A. P. Weetman, B. Rapoport, and S. M. McLachlan. 1998. Relationship between autoantibody epitopic recognition and immunoglobulin gene usage. *Clin. Exp. Immunol. 111:408*.
13. Winter, G., A. D. Griffiths, R. E. Hawkins, and H. R. Hoogenboom. 1994. Making antibodies by phage display technology. *Annu. Rev. Immunol. 12:433*.
14. Gherardi, E., and C. Milstein. 1992. Original and artificial antibodies. *Nature 357:201*.
15. Burton, D. R., and C. F. Barbas 3rd. 1992. Antibodies from libraries. *Nature 359:782*.
16. Portolano, S., M. F. Prummel, B. Rapoport, and S. M. McLachlan. 1995. Molecular cloning and characterization of human thyroid peroxidase autoantibodies of λ light chain type. *Mol. Immunol. 32:1157*.
17. Horimoto, M., V. S. Petersen, C. A. Pegg, N. Fukuma, N. Wakabayashi, Y. Kiso, J. Furmaniak, and B. Rees Smith. 1992. Production and characterisation of a human monoclonal thyroid peroxidase autoantibody. *Autoimmunity 14:1*.
18. Hexham, M., C. Pegg, D. Burton, V. B. Petersen, M. Horimoto, J. Furmaniak, and B. Rees Smith. 1992. Variable region sequence of a human monoclonal thyroid peroxidase autoantibody. *Autoimmunity 14:169*.
19. Melamed, D., and D. Nemazee. 1997. Self-antigen does not accelerate immature B cell apoptosis, but stimulates receptor editing as a consequence of developmental arrest. *Proc. Natl. Acad. Sci. USA 94:9267*.
20. Gay, D., T. Saunders, S. Camper, and M. Weigert. 1993. Receptor editing: an approach by autoreactive B cells to escape tolerance. *J. Exp. Med. 177:999*.
21. Klinman, N. R. 1996. The "clonal selection hypothesis" and current concepts of B cell tolerance. *Immunity 5:189*.
22. Radic, M. Z., J. Erikson, S. Litwin, and M. Weigert. 1993. B lymphocytes may escape tolerance by revising their antigen receptors. *J. Exp. Med. 177:1165*.
23. Embleton, M. J., G. Gorochov, P. T. Jones, and G. Winter. 1992. In-cell PCR from mRNA: amplifying and linking the rearranged immunoglobulin heavy and light chain V-genes within single cells. *Nucleic Acids Res. 20:3831*.
24. Chapal, N., M. Bouanani, M. J. Embleton, I. Navarro-Teulon, M. Biard- Piechaczyk, B. Pau, and S. Peraldi-Roux. 1997. In-cell assembly of scFv from human thyroid-infiltrating B cells. *BioTechniques 23:518*.
25. Hoogenboom, H. R., A. D. Griffiths, K. S. Johnson, D. J. Chiswell, P. Hudson, and G. Winter. 1991. Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucleic Acids Res. 19:4133*.
26. Lefranc, M. P., V. Giudicelli, C. Ginestoux, J. Bodmer, W. Muller, R. Bontrop, M. Lemaitre, A. Malik, V. Barbie, and D. Chaume. 1999. IMGT, the international ImMunoGeneTics database. *Nucleic Acids Res. 27:209*.
27. Marks, J. D., H. R. Hoogenboom, T. P. Bonnert, J. McCafferty, A. D. Griffiths, and G. Winter. 1991. By-passing immunization: human antibodies from V-gene libraries displayed on phage. *J. Mol. Biol. 222:581*.
28. Carter, P., H. Bedouelle, and G. Winter. 1985. Improved oligonucleotide site-directed mutagenesis using M13 vectors. *Nucleic Acids Res. 13:4431*.
29. Lehrer, R. I., A. Barton, and T. Ganz. 1988. Concurrent assessment of inner and outer membrane permeabilization and bacteriolysis in *E. coli* by multiple-wavelength spectrophotometry. *J. Immunol. Methods 108:153*.
30. Munro, S., and H. R. Pelham. 1986. An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell 46:291*.
31. Karlsson, R., and A. Falt. 1997. Experimental design for kinetic analysis of protein-protein interactions with surface plasmon resonance biosensors. *J. Immunol. Methods 200:121*.
32. Franck, R. 1992. Spot-synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. *Tretrahedron Lett. 33:3296*.
33. Molina, F., D. Laune, C. Gougat, B. Pau, and C. Granier. 1996. Improved performances of spot multiple peptide synthesis. *Pept. Res. 9:151*.
34. Bouanani, M., R. Bataille, M. Piechaczyk, S. L. Salhi, B. Pau, and M. Bastide. 1991. Autoimmunity to human thyroglobulin: respective epitopic specificity patterns of anti-human thyroglobulin autoantibodies in patients with Sjögren's syndrome and patients with Hashimoto's thyroiditis. *Arthritis Rheum. 34:1585*.
35. Portolano, S., S. M. McLachlan, and B. Rapoport. 1993. High affinity, thyroid-specific human autoantibodies displayed on the surface of filamentous phage use V genes similar to other autoantibodies. *J. Immunol. 151:2839*.
36. Jaume, J. C., S. Portolano, B. Rapoport, and S. M. McLachlan. 1996. Influence of the light chain repertoire on immunoglobulin genes encoding thyroid autoantibody Fab from combinatorial libraries. *Autoimmunity 24:11*.
37. Caton, A. J., and H. Koprowski. 1990. Influenza virus hemagglutinin-specific antibodies isolated from a combinatorial expression library are closely related to the immune response of the donor. *Proc. Natl. Acad. Sci. USA 87:6450*.
38. McLachlan, S. M., C. A. Pegg, M. C. Atherton, S. L. Middleton, A. Dickinson, F. Clark, S. J. Proctor, G. Proud, and B. Rees Smith. 1986. Subpopulations of thyroid autoantibody secreting lymphocytes in Graves' and Hashimoto thyroid glands. *Clin. Exp. Immunol. 65:319*.
39. Weng, W. K., N. Shah, D. O'Brien, B. Van Ness, and T. W. LeBien. 1997. Differential induction of DNA-binding activities following CD19 cross-linking in human B lineage cells. *J. Immunol. 159:5502*.
40. Hawkins, R. E., and G. Winter. 1992. Cell selection strategies for making antibodies from variable gene libraries: trapping the memory pool. *Eur. J. Immunol. 22:867*.
41. Fujikawa, M., K. Okamura, K. Sato, T. Mizokami, S. Tanabe, H. Ikenoue, S. Okamura, M. Ohta, and M. Fujishima. 1998. Usefulness of surface phenotype study of intrathyroidal lymphocytes obtained by fine needle aspiration cytology in autoimmune thyroid disease and malignant lymphoma of the thyroid. *Clin. Endocrinol. 49:191*.
42. Inaoki, M., S. Sato, B. C. Weintraub, C. C. Goodnow, and T. F. Tedder. 1997. CD19-regulated signaling thresholds control peripheral tolerance and autoantibody production in B lymphocytes. *J. Exp. Med. 186:1923*.
43. Sato, S., N. Ono, D. A. Steeber, D. S. Pisetsky, and T. F. Tedder. 1996. CD19 regulates B lymphocyte signaling thresholds critical for the development of B-1 lineage cells and autoimmunity. *J. Immunol. 157:4371*.
44. McIntosh, R., P. Watson, and A. Weetman. 1998. Somatic hypermutation in autoimmune thyroid disease. *Immunol. Rev. 162:219*.
45. Williams, S. C., and G. Winter. 1993. Cloning and sequencing of human immunoglobulin V λ gene segments. *Eur. J. Immunol. 23:1456*.
46. Cairns, E., P. C. Kwong, V. Misener, P. Ip, D. A. Bell, and K. A. Siminovitch. 1989. Analysis of variable region genes encoding a human anti-DNA antibody of normal origin: implications for the molecular basis of human autoimmune responses. *J. Immunol. 143:685*.
47. Serrano, M. P., A. Cardona, B. Vernet der Garabedian, J. F. Bach, and J. M. Pleaus. 1994. Nucleotide sequences of variable regions of an human anti-acetylcholine receptor autoantibody derived from a myasthenic patient. *Mol. Immunol. 31:413*.
48. Prummel, M. F., S. Portolano, G. Costante, B. Rapoport, and S. M. McLachlan. 1994. Isolation and characterization of a monoclonal human thyroid peroxidase autoantibody of λ light chain type. *Mol. Cell. Endocrinol. 102:161*.
49. de Wildt, R. M. T., R. M. A. Hoet, W. J. van Venrooij, I. M. Tomlinson, and G. Winter. 1999. Analysis of heavy and light chain pairings indicates that receptor editing shapes the human antibody repertoire. *J. Mol. Biol. 285:895*.
50. Song, Y. W., H. A. Kim, E. B. Lee, H. J. Baek, J. K. Kim, J. H. Chung, E. S. Chung, and K. M. Hong. 1998. Molecular and genetic characterization of two anti-DNA antibodies derived from patients with systemic lupus erythematosus. *Rheumatol. Int. 17:223*.
51. Ravirajan, C. T., M. A. Rahman, L. Papadaki, M. H. Griffiths, J. Kalsi, A. C. Martin, M. R. Ehrenstein, D. S. Latchman, and D. A. Isenberg. 1998. Genetic, structural and functional properties of an IgG DNA-binding monoclonal antibody from a lupus patient with nephritis. *Eur. J. Immunol. 28:339*.
52. Prummel, M. F., G. Chazenbalk, J. C. Jaume, B. Rapoport, and S. M. McLachlan. 1994. Profile of λ light chain variable region genes in Graves' orbital tissue. *Mol. Immunol. 31:793*.
53. Giachino, C., E. Padovan, and A. Lanzavecchia. 1998. Re-expression of *RAG-1* and *RAG-2* genes and evidence for secondary rearrangements in human germinal center B lymphocytes. *Eur. J. Immunol. 28:3506*.
54. Hikida, M., and H. Ohmori. 1998. Rearrangement of λ light chain genes in mature B cells in vitro and in vivo: function of reexpressed recombination-activating gene (RAG) products. *J. Exp. Med. 187:795*.
55. Radic, M. Z., and M. Zouali. 1996. Receptor editing, immune diversification, and self-tolerance. *Immunity 5:505*.
56. Wilkin, T. J., and C. Casey. 1984. The distribution of immunoglobulin-containing cells in human autoimmune thyroiditis. *Acta Endocrinol. 106:490*.