Antigen Receptor Variable Region Repertoires Expressed by T Cells Infiltrating Thyroid, Retroorbital, and Pretibial Tissue in Graves' Disease*

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ABSTRACT

To date, it has remained unclear whether T cells infiltrating thyroid, retroorbital, and pretibial tissue of patients with Graves' ophthalmopathy and pretibial dermopathy represent a primary immune response that is directed against certain antigenic determinants shared among these involved tissues. To characterize these T cells at the molecular level, we compared the T cell antigen receptor (TcR) variable (V) region gene usage in thyroid, retroorbital, pretibial tissue, and peripheral blood mononuclear cells of two patients with Graves' disease, ophthalmopathy, and pretibial dermopathy. Ribonucleic acid was extracted, reverse transcribed, and amplified using the PCR and 22 Vα and 23 Vβ gene-specific oligonucleotide primers. The resulting TcR Vα and Vβ transcripts were verified by Southern hybridization analysis using TcR C region-specific, digoxigenin-labeled oligonucleotide probes. In addition, complementarity determining regions 3 and junctional regions of TcR Vβ genes were sequenced. Marked similarities of intrathyroidal, retroorbital, and pretibial TcR Vα and Vβ gene repertoires were noted with respect to the degree of TcR V gene restriction and the patterns of individual V genes expressed. Sequence analysis of junctional domains of Vβ families revealed oligoclonality of intrathyroidal, retroorbital, and pretibial T cells. In addition, certain conserved junctional motifs were shared by T cells derived the thyroid gland and the extrathyroidal sites. Our results suggest that in the two patients with Graves' disease and extrathyroidal manifestations studied, similar antigenic determinants may have contributed to the recruitment and oligoclonal expansion of T cells both within the thyroid gland and at the involved extrathyroidal sites. (*J Clin Endocrinol Metab* **81**: 3733–3739, 1996)

THE ETIOLOGY of Graves' ophthalmopathy (GO) and pretibial dermopathy (PTD) and the reasons for the preferential involvement of the retroorbital and pretibial space are poorly understood (1–5). During the active stages of GO, infiltration of the retroorbital tissue by mononuclear cells is frequently observed (1, 2). The cellular infiltrate within the retroorbital tissues in GO consists mainly of T lymphocytes, macrophages, and, to a lesser extent, B cells and natural killer cells (6–8). The T cell component includes both CD4$^+$ and CD8$^+$ T lymphocytes, many of which express CD45RO, suggesting that they are major histocompatibility complex (MHC) class I and II restricted memory T cells (8–10). PTD occurs in a small proportion of patients with Graves' disease (GD), most of whom have ophthalmopathy and high serum titers of TSH receptor-directed antibodies (3–5). Both processes are likely to be driven by T cells that access and infiltrate the retroorbital and pretibial space via certain adhesion molecules and release various cytokines capable of stimulating cell proliferation, GAG synthesis, and the expression of immunomodulatory molecules in microvascular endothelial cells and fibroblasts (1, 2, 10–13). In support of this concept, retroorbital and pretibial fibroblasts

have recently been shown to act as targets of the autoimmune process in GO and PTD (14). However, although these mechanisms may be sufficient to explain certain aspects of how GO and PTD may evolve and be propagated, the primary antigen for these presumed autoimmune manifestations has remained elusive. The close clinical association between the hyperthyroidism of GD, GO, and PTD suggests that the antigen responsible for these diverse conditions may be shared by the thyroid gland and extrathyroidal tissues (11).

T cells specifically recognize processed antigens in conjunction with MHC molecules through their T cell receptors (TcR) which consist mostly of α- and β-chain heterodimers. The TcR α-chain consists of variable (V), joining (J), and constant (C) regions, whereas the β-chain consists of V, diversity (D), joining, and constant regions. They are designated Vα, Jα, and Cα or Vβ, Dβ, Jβ, and Cβ, respectively. The antigen specificity of the TcR is defined by the V domains encoded by V, D, and J gene elements that are rearranged and joined during T cell differentiation. The clonotypic rearrangement of germ-line V, D, J, and C genes provides for TcR recognition of a vast array of MHC-antigen complexes. The specific interaction of TcRs with MHC-peptide complexes is mediated by three Ig-like complementary-determining regions (CDR1, CDR2, and CDR3) (15). Germ-line V genes encode both the CDR1 and CDR2 domains, which presumably contact polymorphic MHC determinants. The CDR3 domain is encoded by both germ-line V and J genes and nontemplated nucleotide modifications (N regions) located at the V-J or V-D-J interface of rearranged TcR α and β genes, respectively. The recognition of MHC-peptide complexes by T cells is frequently characterized by the selective or re-

stricted use of particular TcR germ-line (V and J genes) and CDR3 sequence combinations. Such biases may reflect the need for a conserved structural framework and peptide contact sites in TcR recognition events (16).

Simultaneous screening of samples for TcR gene rearrangements or their transcription of messenger ribonucleic acid (RNA) has been facilitated greatly by the availability of PCR-based TcR V region gene phenotyping. Using this technique, in both humans and animal models, restricted heterogeneity of the T cell response was detected in several autoimmune diseases (17–20), including thyroid autoimmune diseases and GO (21–24). In addition, we recently demonstrated restricted usage of TcR V region gene families in T lymphocytes derived from pretibial biopsies of patients with active PTD (25). Although these studies suggested that specific antigens are targeted by T lymphocytes present at these distinct sites, analysis of intrathyroidal, retroorbital, and pretibial TcR V gene repertoires in individual patients has been prohibited by the lack of appropriate study material. Comparative analysis of local TcR V gene usage at the involved sites of individual patients with GD may help to determine whether the hypothesis of a shared antigen between the thyroid gland and the extrathyroidal manifestations is supported at the molecular T cell level.

In this study, using PCR-based phenotyping of TcR V region genes and sequencing of junctional regions of dominant Vβ genes, we analyzed the expression of the known Vα and Vβ gene families in T cells infiltrating the thyroid gland and various extrathyroidal sites of two patients with GD, severe ophthalmopathy, and PTD. Our results indicate oligoclonality of TcRs and striking similarities of TcR V gene repertoires at the intra- and extrathyroidal manifestations in these two patients with GD.

Materials and Methods

Study subjects

Patient 1, a 54-yr-old white female, presented to her local physician in June 1993 with palpitations, sweating, irritability and a 9-lb weight loss. She had previously been healthy except for chronic bronchitis due to heavy smoking (25 cigarettes/day). Graves' hyperthyroidism was diagnosed based on typical clinical signs and symptoms, the presence of mild bilateral ophthalmopathy, and heterogeneous, markedly elevated technetium uptake of 9.6% on thyroid scintigraphy. Thyroid function tests were as follows: basal TSH, less than 0.03 mEq/L; free T$_4$, 580 pg/L; free T$_3$, 14.7 fg/L; thyroglobulin antibodies, 480 mU/L; thyroid peroxidase antibodies, 1600 mU/L; and TSH receptor stimulating index, 17.8, as determined by stimulation of cAMP production in rat thyroid cells. Thyroid ultrasound revealed a large multinodular goiter (78 mL) with marked hypoechogenicity. Antithyroid medication with carbimazole (20 mg, orally, twice daily) was started; however, the patient was still hyperthyroid after 3 weeks of therapy. A euthyroid state was achieved only after the carbimazole dose had been increased to 60 mg/day. Near-total thyroidectomy was performed without complications, and a small piece of thyroid tissue was immediately frozen in liquid nitrogen. Four months later, while the patient was euthyroid with replacement therapy of 125 µg/day levothyroxine, progression of ophthalmopathy was noted by her ophthalmologist; however, treatment with glucocorticosteroids or orbital irradiation was not initiated. Smoking was strongly discouraged, with minimal success. Two months later, the patient presented with an infiltrative dermopathy that extended over both anterior pretibial areas. Lesional biopsies, taken from both legs for immunohistochemical studies, were consistent with pretibial dermopathy. One deep biopsy specimen was quickly frozen in liquid nitrogen and reserved for further study. In January 1994, the patient was referred

for progressive severe ophthalmopathy. Marked bilateral proptosis, periorbital edema, diplopia, left optic neuropathy, and incipient exposure keratitis of the left eye due to incomplete lid closure were noted. Urgent surgical decompression of the left orbit was performed, resulting in rapid recovery of optic nerve function and restoration of lid closure. During the surgical procedure, a small biopsy of retroorbital connective tissue was frozen in liquid nitrogen, and a blood sample was taken for preparation of peripheral blood mononuclear cells. Postoperatively, the patient underwent orbital irradiation and oral glucocorticoid therapy, with acceptable long term success. Human leukocyte antigen (HLA) haplotyping of patient 1 revealed HLA class I (A2, B7, Bw57, Cw6), HLA class II (DR2 DR7).

Patient 2, a 44-yr-old white female, presented for evaluation of progressive periorbital swelling and visual impairment. She had been diagnosed with Graves' hyperthyroidism 3 yr ago and undergone successful treatment with radioiodine, followed by supplementation with 75 µg/day levothyroxine. Thyroid function tests confirmed a euthyroid state (TSH, 1.2 mU/L; free T$_4$, 140 pg/L; free T$_3$, 2.9 fg/L) and a markedly elevated TSH receptor stimulating index of 21. Eye examination revealed the presence of severe ophthalmopathy, disfiguring bilateral periorbital edema, marked proptosis of the right globe, diplopia due to profound extraocular muscle dysfunction, and right optic neuropathy. Computed tomography of the orbits suggested compression of the right optic nerve at the orbital apex by markedly enlarged extraocular muscles. Orbital decompression of the right orbit was performed and resulted in rapid improvement of optic nerve functions and periorbital swelling. A small biopsy specimen was obtained from the retroorbital connective tissue and stored in liquid nitrogen for further analysis. In addition, several biopsies were taken from an area of diffuse thickening and redness of the skin that extended from the pretibial area to the forefoot of her left leg. Histology was consistent with pretibial dermopathy. HLA haplotyping of patient 2 revealed HLA class I (A1, A2, B13, Cw6, Cw7), HLA class II (DR2, DR7).

In addition, biopsies of thyroid, retroorbital, and pretibial tissue were obtained from two females (48 and 61 yr of age, respectively) who died from fatal myocardial infarction. None of these individuals had a personal or family history of GD, GO, or systemic or organ-specific autoimmune disease. Thyroid function studies were normal, and thyroid autoantibodies were not detected.

Sample preparations

Biopsies, taken from thyroid gland, retroorbital tissue, and pretibial skin, were rapidly frozen in liquid nitrogen for subsequent RNA extraction. Peripheral blood lymphocytes (PBL) were prepared from heparinized blood by Ficoll-Hypaque density-gradient centrifugation (Pharmacia, Piscataway, NJ) and stored in liquid nitrogen. Immunohistochemical staining of T lymphocytes in frozen sections was performed using monoclonal antibodies directed against CD2, CD3, CD4, and CD8 (Becton Dickinson, Mountain View, CA); CD45RO (Dakopatts Corp., Santa Barbara, CA); α/β TcR (Identi T βF1, β-chain specific) and TcRγ/δ (TcRδ1; T Cell Science Co., Cambridge, MA); TcRVβ2 (Dianova, Hamburg, Germany); and TcRVβ6 (T Cell Diagnostics, Woburn, MA) and biotinylated secondary antibodies in conjunction with the Vectastain Peroxidase ABC elite kit (Vector Laboratories, Burlingame, CA), as described previously (9, 24).

PCR analysis of TcR V genes

TcR V gene analysis was performed as previously described (24, 25). In brief, total RNA was extracted from cryostat sections and PBL, reverse transcribed to complementary DNA (cDNA), and amplified using 22 Vα family-specific primers and 23 Vβ family-specific primers, each paired with a 3'-primer matched to a single constant region of the Vα gene (Cα) and Vβ gene (Cβ). T cell antigen receptor V gene-specific primer sequences, oligonucleotide probe sequences, and the expected sizes of the PCR products were previously reported (24). To avoid technical artifacts, samples were prepared and analyzed together. Southern hybridization was performed to increase the sensitivity of fragment detection and to verify the specificity of PCR products, as previously reported (24). The proportions of TcR V genes expressed were determined by computerized quantitative densitometry of autoradiographs. Each TcR Vα or Vβ gene was expressed as a percentage of all of the Vα or Vβ genes

detected on that scan, respectively. Control experiments were performed as previously described (24). Comparable quantities of TcR-specific cDNA, as indicated by the yield of TcR C region-specific transcripts, were subjected to PCR analysis. Hybridization conditions were of sufficient stringency to allow for specific detection of fragments of defined and predicted size (24).

Cloning and sequencing of cDNAs encoding TcR Vβ genes

Amplified cDNAs encoding particular TcR V genes from T cells infiltrating the thyroid gland or extrathyroidal were size-fractionated on 2% agarose gels and purified using the Qiaex agarose gel extraction procedure (Qiagen, Chatsworth, CA). Purified DNA fragments were ligated into pCR II T/A cloning vector (Invitrogen, San Diego, CA) and used to transform bacterial cells. Independent recombinants were identified by antibiotic selection and a β-galactosidase colorimetric assay. Individual bacterial colonies were grown overnight in minicultures and screened subsequently by DNA blot hybridization using a TcR Cβ-specific horseradish peroxidase probe. Plasmid DNA was purified from positively hybridizing bacterial lysates using Qiaex silica matrix and eluted in 30 μL dH₂O. Half of the plasmid miniprep was subjected to double stranded DNA sequencing using T7 and SP6 primers, respectively, and the Sequenase V.2 protocol (U.S. Biochemical Corp., Cleveland, OH). Nucleic acid and protein sequence comparisons were conducted using GenBank and EMBL libraries.

Results

Immunoperoxidase detection of TcR chains

Immunohistochemistry of sections derived from thyroid, retroorbital, and pretibial tissue samples of both patients revealed the presence of $CD3^+$ T cells. The vast majority of T lymphocytes (~96-98%) were α/β^+ T cells, as determined by positive immunoreactivity with a β-chain-specific human TcR antibody (data not shown). Except for occasional cells immunoreactive with the human TcR δ-chain-specific monoclonal antibody, $\gamma\delta^+$ T cells were not detected in thyroid, retroorbital, and pretibial specimens of either patient.

T cell antigen receptor V gene analysis

Expression of a broad spectrum of TcR Vα and Vβ genes was detected in cDNA samples prepared from PBL of either patient (Fig. 1; 1:B and 2:B) and two control individuals (data not shown). In these samples, between 19–20 Vα genes and between 20–22 Vβ genes, respectively, were actively transcribed. Compared with PBL, restriction of TcR Vα and TcR Vβ genes (<50% of individual genes rearranged and expressed) was detected in thyroid (7 of 22 Vα gene families; 10 of 23 Vβ gene families), retroorbital (9 of 22 Vα gene families; 10 of 23 Vβ gene families), and pretibial (8 of 22 Vα gene families; 9 of 23 Vβ gene families) tissue obtained from patient 1 (Fig. 1). Similarly, marked restriction of TcR Vα and TcR Vβ genes was detected in retroorbital (8 of 22 Vα gene families; 8 of 23 Vβ gene families) and pretibial (8 of 22 Vα gene families; 8 of 23 Vβ gene families) tissue obtained from patient 2 (Fig. 1). The presence of dominant TcR Vβ2 and Vβ6 gene products in thyroid, orbital, and pretibial tissue of patient 1 and in orbital and pretibial tissue of patient 2 was further confirmed by immunohistochemical staining using monoclonal antibodies directed against human TcR Vβ2 and TcR Vβ6 gene families, respectively (Fig. 2). Further, unrestricted TcR Vα or Vβ gene usage was detected in samples derived from the thyroid gland of two control individuals. TcR V gene analysis of retroorbital and pretibial specimens

derived from these two control individuals failed to yield TcR-specific transcripts (not shown).

Despite obvious heterogeneity of the TcR V gene repertoire between patients 1 and 2, marked similarities of restricted Vα and Vβ gene family usage were noted in T cells infiltrating the thyroid gland, retroorbital connective tissue, and pretibial skin of either patient. For example, the presence of Vα2, Vα3, Vα8, Vα12, Vα15, Vα21, Vβ2, Vβ4, Vβ6, Vβ7, Vβ12, Vβ20, and Vβ24 transcripts, was shared by thyroid, retroorbital, and pretibial tissue of patient 1. Further, Vα3, Vα8, Vα12, Vα14, Vα19, Vβ2, Vβ4, Vβ6, Vβ12, Vβ18, and Vβ23 transcripts were present in both retroorbital and pretibial tissue obtained from patient 2.

DNA sequencing was used to determine the amino acid sequences of dominant TcR V gene families that contributed 20% or more of the total Vβ gene activity detected at individual sites. TcRs using Vβ6 (patient 1) and Vβ2 (patient 2) genes, respectively, were amplified by PCR and ligated into pCR II T/A cloning vector. Multiple TcRs were then isolated from these libraries and sequenced. The results of this analysis are summarized in Table 1. Overexpressed Vβ2 and Vβ6 gene families from thyroid, orbital, and pretibial tissues of patients 1 and 2, respectively, revealed major populations of clonally expanded TcRs or TcRs whose rearrangements shared significant homologies at the amino acid level of junctional regions. As demonstrated by sequence analysis of 51 Vβ6 TcR cDNA clones derived from the involved tissue sites (thyroid, orbit, and pretibium) of patient 1 and of 29 Vβ2 TcR cDNA clones from the orbit and pretibium of patient 2, single TcR rearrangements accounted for up to 53% (intrathyroidal TcRs in patient 1) of a given TcR family. One TcR clone using Vβ6.2 (Vβ6.2-Jβ2.1) was detected in thyroidal, orbital, and pretibial tissue of patient 1. In addition, a restricted number of Jβ gene family members was detected in distinct tissue sites of either patient. Of the 13 Jβ gene family members, only 4 (Jβ1.6, -2.1, -2.2, and -2.3) were used by intrathyroidal, orbital, and pretibial TcR Vβ6 clones in patient 1, and 5 (Jβ 1.5, -2.1, -2.2, -2.3, and 2.7) were preferentially used by orbital and pretibial TcR Vβ2 clones in patient 2. Further, comparison of the CDR3 domains of junctional regions in expanded Vβ TcR revealed several conserved amino acid motifs that were shared by intrathyroidal, orbital, and pretibial lymphocytes in either patient (Table 1). In Vβ6 TcRs of patient 1, the motif G-L-A was used by the dominant intrathyroidal clonotype, and by one of the clonally expanded Vβ6 orbital and pretibial TcRs, respectively. Similarly, in patient 2, the motifs E-R-G and G-Q-E-L were shared by clonally expanded Vβ2 orbital and pretibial TcRs, respectively. In contrast, none of the dominant rearrangements was found among Vβ2 and Vβ6 TcR cloned from PBL from either patient. These results indicate that certain populations of intrathyroidal, orbital, and pretibial lymphocytes in these 2 patients were oligoclonal, and that several clones were present at a high frequency in these distinct sites.

Discussion

T lymphocytes are thought to play a crucial role in the pathogenesis of GD, GO, and PTD (1, 2, 10, 24, 25). Analysis of lymphocytes infiltrating the retroorbital and pretibial
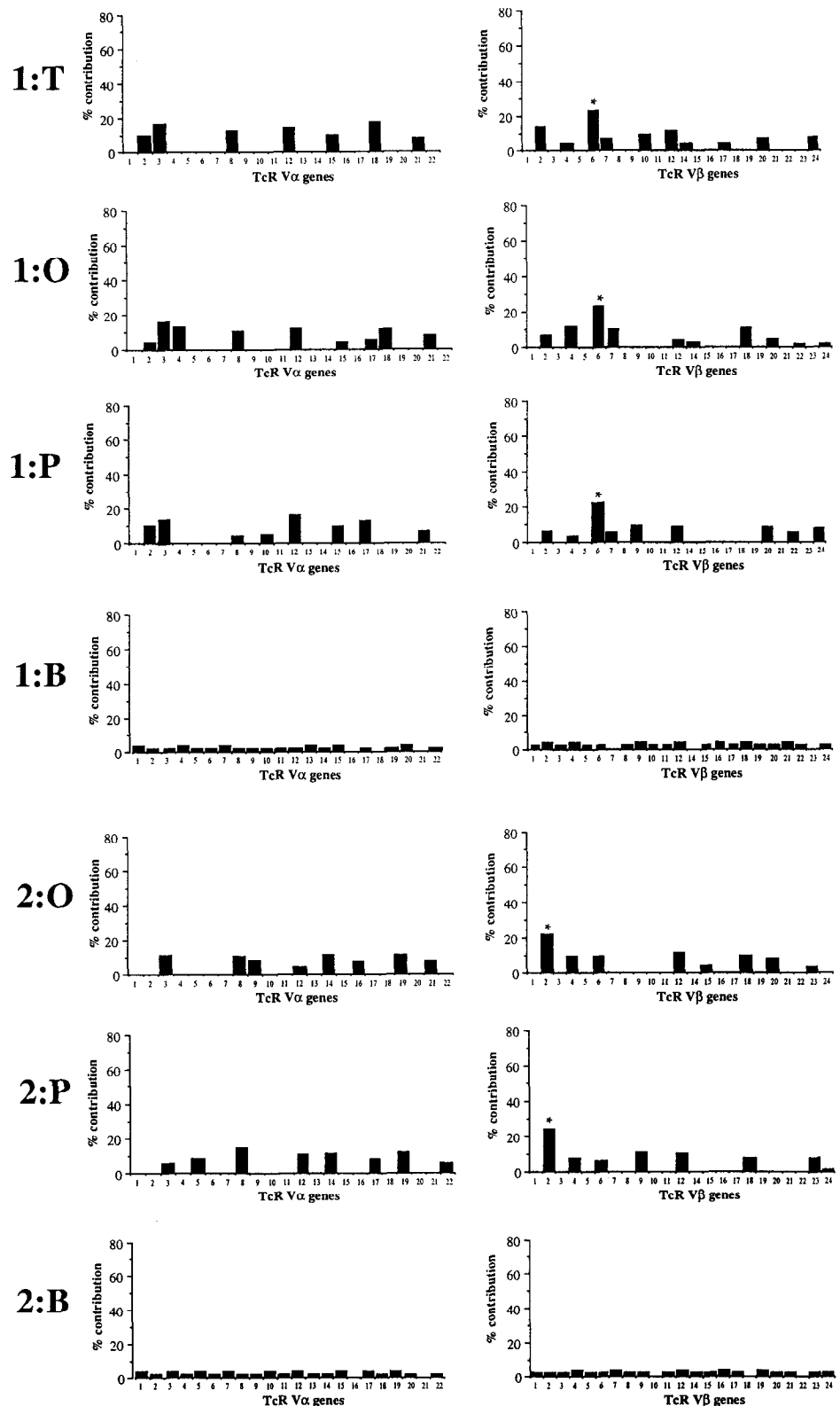
FIG. 1. TcR Vα and Vβ gene family usage in tissue samples derived from thyroid gland, orbital connective tissue, and pretibial skin of patient 1 (*top panel*) and patient 2 (*bottom panel*). RNA was extracted from frozen sections of tissue biopsies, transcribed to cDNA, and amplified by the PCR using 22 Vα and 23 Vβ primer pairs. The resulting transcripts were detected autoradiographically after Southern hybridization with digoxigenin-labeled TcR α- and β-chain-specific constant region oligonucleotide probes. The relative proportions of individual TcR Vα and Vβ genes detected were determined by quantitative densitometry and expressed as percentages (percent contribution) of the 22 Vα and 23 Vβ genes examined. Only TCR Vα and Vβ genes with a more than 1% overall contribution are depicted. In patient 1, intrathyroidal (T), orbital (O), and pretibial (P) TcR Vα and Vβ gene usage is shown. In patient 2, orbital (O), and pretibial (P) TcR Vα and Vβ gene usage is demonstrated. In addition, nonrestriction of peripheral blood (B) TcR Vα and Vβ gene usage was present in both patients.

space of patients with GO and PTD revealed the presence of both CD4^{+} and CD8^{+} T lymphocytes, with a variable predominance of the latter (1, 2, 6, 7). A significant proportion of T lymphocytes, frequently adjacent to blood vessels, are CD3^{+}/CD45RO^{+} cells, reflecting a subset of memory T cells and macrophages (8). However, the autoantigens recognized by autoreactive T cells infiltrating the thyroidal and extrathyroidal manifestations of patients with GD have not been
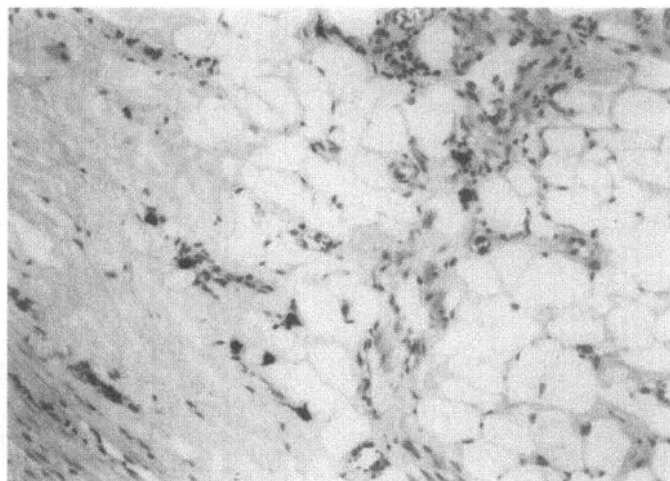
FIG. 2. Immunoperoxidase localization of TcR Vβ6⁺ lymphocytes in a cryostat section of retroorbital connective tissue derived from patient 1 with GD, severe ophthalmopathy, and pretibial dermopathy. Infiltration of retroorbital connective tissue by TcR Vβ6⁺ lymphocytes (*arrows*) is demonstrated. Original magnification, ×250.

identified (10). In a recent study, CD8⁺ CD45RO⁺ orbit-infiltrating T lymphocytes from patients with GO were demonstrated to selectively recognize autologous fibroblasts derived from retroocular connective tissue and, to a lesser degree, other anatomical locations (14). These results support the concept that connective tissue fibroblasts represent major targets for T cells that infiltrate the extrathyroidal manifestations associated with GD (26). Recent data from several laboratories have demonstrated TcR V gene restriction in both animal models and human autoimmune diseases, supporting a central role for T cells in the initiation and propagation of autoimmunity (17–20). In support of this concept, evidence of limited variability of intrathyroidal TcR V genes in GD has been demonstrated (21–23), although conflicting results have also been reported (27, 28). We have recently detected restriction of TcR V gene usage in retroorbital connective tissue of patients with active GO of recent onset, whereas TcR V gene usage was unrestricted in patients with long standing, inactive GO (24). In addition, analysis of T cell surface markers and TcRs expressed by lymphocytes infiltrating the pretibial space of patients with clinically active PTD revealed a predominance of CD3⁺ T lymphocytes, many of which are CD8⁺ T cells and express TcR α/β-chains (25). Marked restriction of TcR Vα and Vβ gene usage was demonstrated in lymphocytes infiltrating the pretibial tissue of patients with early active PTD, whereas greater diversity of Vβ gene usage and loss of Vα gene restriction were observed in patients with long standing pretibial dermopathy (25). These results suggested that the primary target antigen in GO and PTD may initiate a restricted T cell V gene response, and that later in the natural history of the disease, tissue destruction and cytokine-induced antigen expression may promote the recruitment of a more diverse spectrum of T cells that use multiple TcR V gene families, and react to a variety of tissue-specific and tissue-nonspecific antigens (29).

We hypothesized that among the inflammatory cells infiltrating thyroid, retroorbital, and pretibial tissues of individual patients with GD, subpopulations of autoantigen-

reactive T cells may be responsible for initiating and perpetuating the immune process. To identify such disease-associated T cells, we have analyzed the patterns of TcR-αβ gene expression and sequenced the junctional regions of dominating TcR Vβ genes in thyroid, retroorbital, and pretibial tissue samples derived from two patients with GD, GO and PTD. Although the spectrum of actively transcribed TcR Vα and Vβ genes varied among the various samples derived from either patient, several striking features were noted. Marked restriction of the TcR V gene repertoire and overexpression of certain TcR V gene segments were detected in lymphocytes infiltrating thyroid, orbital, and pretibial skin. Heterogeneity of TcR V gene usage may be explained by the fact that the TcR recognizes similar antigenic peptides in the context of HLA molecules, which were different in the two patients studied. Individual HLA gene endowment may play an important role in shaping the TcR V gene repertoire (30, 31), and this may influence TcR V gene usage in lymphocytes capable of recognizing common antigenic epitopes presented at distinct involved sites in GD. Given the marked similarity in individual TcR V genes expressed by lymphocytes infiltrating distinct anatomical sites, our results suggest that infiltrating T cells might recognize a limited range of antigenic epitopes shared by the thyroid gland, retroorbital connective tissue, and pretibial skin. Furthermore, our data indicate that analogous mechanisms may act to control T cell selection and expansion within both the thyroid gland and the involved extrathyroidal compartments.

To define further the significance of dominating Vβ2 and Vβ6 gene families in thyroid, retroorbital, and pretibial tissue of patients 1 and 2, we next examined the clonotypic heterogeneity of these T cells. Sequence analysis of the junctional regions of Vβ6 and Vβ2 genes revealed a limited number of independently rearranged TcR β-chain genes, the presence of conserved amino acid residues within the CDR3 region, and selective usage of related Jβ gene family members by Vβ6⁺ and Vβ2⁺ T cells infiltrating distinct tissues in the two patients studied. By contrast, limited clonotypic heterogeneity and biased Jβ gene usage were not detected among patients' PBL or in tissue controls obtained from healthy individuals. The CDR3 region is commonly associated with fine antigenic specificity, and TcRs selected by defined antigenic peptides often show homologous amino acid motifs in the junctional region (16). Accordingly, detection of conserved CDR3 motifs in clonally expanded TcR from distinct anatomical sites is consistent with T cell activation by antigens bearing shared antigenic determinants. Given the similarity of certain HLA alleles in the two patients studied, detection of conserved junctional motifs may reflect the influence of common HLA alleles on TcR selection. The presence of conserved junctional CDR3 motifs joined to different Vβ segments has been reported in several HLA class I-restricted T cell lines and T cell clones specific for the same peptide/MHC complex or in tissues affected by autoimmune conditions, such as giant cell arteritis (32, 33). Thus, homologous junctional regions appear to confer specificity for the same antigenic determinant even if associated with different Vβ segments. Our detection of a limited number of independently rearranged TcR β-chain genes together with selective usage of related TcR Jβ gene

Table 1. Summary of junctional sequence analysis of TcR Vβ genes in T lymphocytes rescued from thyroid, orbital, pretibial tissue, and peripheral blood of two patients with Graves' disease, ophthalmopathy, and pretibial myxedema

Patient no.	Source	TcR Vβ	No. of clones	% of total	TcRβ-V-N(D)N-J junctional sequences		
					Vβ	N-Dβ-N region[a]	Jβ
1	Thyroid	Vβ6	8/15	53.3	Vβ6.2-CASS	RGLA	2.1
			3/15	20.0	Vβ6.2 CASSL	GTAS	2.2
			1/15	6.7	Vβ6.2 CASS	YSGIT	2.1
	Orbit	Vβ6	5/17	29.4	Vβ6.1 CASSL	GQG	2.1
			2/17	11.8	Vβ6.2 CASS	DGLA	2.3
	Pretibium	Vβ6	7/19	36.8	Vβ6.2 CASSL	PGGGR	2.1
			2/19	10.5	Vβ6.2 CASS	LAGGLG	2.3
			2/19	10.5	Vβ6.2 CASSL	DGLA	1.6
	Blood	Vβ6	0/19	0			
2	Orbit	Vβ2	6/16	37.5	Vβ2.1 CSA	TGQELG	1.5
			5/16	31.3	Vβ2.3 CSAR	GERGS	2.3
			2/16	12.5	Vβ2.3 CSAR	DPERGA	2.7
	Pretibium	Vβ2	4/13	30.8	Vβ2.1 CSA	RTAY	2.1
			2/13	15.4	Vβ2.1 CSA	NGQELV	2.1
			1/13	7.7	Vβ2.1 CSA	RDN	2.2
	Blood	Vβ2	0/19	0			

[a] The single letter amino acid sequences at the 3'-position of TcR Vβ and at the CDR3 region are given. Repetitive amino acid motifs within the junctional regions are underlined.

family members by Vβ2 and Vβ6 T cells infiltrating thyroid, retroorbital, and pretibial tissues suggest clonal restriction of these cells, perhaps in response to local stimulation by one or a limited number of antigenic determinants present in these distinct anatomical locations. Although the significance of this apparent bias remains unclear, it is interesting to speculate that the Vβ6.2-Jβ2.1 clonotype may define a distinct antigenic reactivity, perhaps unique to patient 1. It is likely that Vβ2- and Vβ6-positive T cells proliferate due to antigen-driven stimulation through MHC-antigen-TCR complexes, although the possibility that these cells proliferate in response to a superantigen must be considered.

Involvement of Vβ2⁺ and Vβ6⁺ T lymphocytes has been observed in several other immune responses affecting gut (34, 35), muscle (36), and skin (37, 38). Clonally expanded Vβ6⁺ T cells appear to confer protective immunity to *Mycobacterium leprae* (39), and Vβ2⁺ and Vβ6⁺ T lymphocytes have been attributed antitumor activity against squamous cell carcinomas (40). In addition, Vβ2 and Vβ6 TcRs are highly expressed in the intestinal mucosa (35). In GD and its associated extrathyroidal manifestations, recent evidence suggests that the immune response may be directed against certain antigenic determinants that are present in epithelial (thyroid, skin, and orbit) and mucosal (lacrimal gland) tissue components (41, 42). Thus, in view of our previous and current data, and the proposed functions of Vβ2 and Vβ6 TcRs in conveying immunosurveillance over mucosal and epithelial surfaces, the role of Vβ2 and Vβ6 TcRs in GD warrants further study.

Although the antigens to which T cells infiltrating thyroid, retroorbital, and pretibial tissue in patients with GD respond are unknown, delineation of antigenic reactivities associated with tissue-infiltrating T lymphocytes of patients with GD may help to clarify the significance of the TcR restrictions identified in this study. Analysis of ligands, including self-antigens and HLA alleles, that are recognized by restricted autoreactive T cells should shed light on the mechanisms involved in the evolution of the extrathyroidal manifesta-

tions of GD. Thus, if the recurrent character of TcR rearrangements in T cells infiltrating the involved tissues could be confirmed in a larger number of patients with GD who share certain HLA alleles, this approach might ultimately provide a basis for targeted immunotherapy in patients with thyroidal and extrathyroidal manifestations of GD.

In conclusion, we have demonstrated restriction of TcR Vα and Vβ gene usage and overexpression of certain dominating TcR Vα and Vβ gene families in thyroid, retroorbital, and pretibial tissue of two patients with GD. Furthermore, detection of TcR oligoclonality and marked homology of TcR V genes rearranged and expressed by T cells infiltrating into these anatomically distinct tissues indicate that similar mechanisms may act to locally control T cell recruitment and expansion within thyroid, orbital, and pretibial tissues in GD. These similarities may be considered molecular evidence that some of the clonally expanded intrathyroidal T cells were strongly selected. Thus, our data are consistent with the hypothesis that in GD, common antigenic determinants may drive the immune response against the thyroid gland and certain extrathyroidal sites. Although the nature of the antigen and the mechanisms leading to its recognition by the immune system await further study, the expression of certain antigenic epitopes by a cell type common to the thyroid gland and the affected extrathyroidal tissues remains a strong possibility.

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