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No restriction of intrathyroidal T cell receptor Vα families in the thyroid of Graves' disease

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SUMMARY

Recently it has been reported that the intrathyroidal T cells in Graves' disease display restriction in Vα T cell receptor (TcR) gene family usage, although this is not found with TcR Vβ gene families in the same individuals. We have performed a qualitative analysis of TcR Vα family usage in 12 patients with Graves' disease by reverse transcription and polymerase chain reaction (PCR) amplification of RNA extracted from isolated, unstimulated intrathyroidal lymphocytes and from snap-frozen whole thyroid specimens. No restriction was observed, with 10–15 Vα gene families being amplified in all cases. The pattern of usage was similar to that in peripheral blood lymphocytes derived from normal subjects ($n=3$) and from patients with Graves' disease ($n=3$), as well as that present in the thyroids of patients with non-autoimmune toxic multinodular goitre ($n=4$). These results indicated that there is no marked restriction of the unselected intrathyroidal T cell population in patients with Graves' disease who have been treated with antithyroid drugs.

Keywords T cell receptor thyroid autoimmunity Graves' disease

INTRODUCTION

Tolerance to self antigens is thought to result from clonal deletion and anergy of autoreactive T cells: autoimmune disease may ensue if there is failure to maintain this state [1]. Recently, there has been considerable interest in the possibility that only one or, at most, a few T cell clones may be involved in initiating autoimmune disorders, as demonstrated by the restricted usage of T cell receptor (TcR) V genes by autoreactive T cells. For instance, in experimental allergic encephalomyelitis (EAE) produced in B10.PL and PL/J mice and Lewis rats, the encephalitogenic T cells utilize homologous TcR V genes to recognize heterogeneous MHC class II and myelin basic protein epitopes [2–4]. Evidence for restricted TcR V gene usage has also been found in other animal models of autoimmune disease, such as murine collagen-induced arthritis, but not in the islet-infiltrating T cells in the diabetic non-obese diabetic (NOD) mouse [5,6]. Such observations have resulted in the hypothesis that specific combinations of TcR Vα and Vβ sequences are essential for the development of autoimmune disease, and have led to the development of immunomodulatory treatments directed against the disease-associated TcR [7–10].

However, evidence for restricted TcR usage in human autoimmune diseases is incomplete. For instance, some studies have shown that restriction, as demonstrated by dominant TcR rearrangements on Southern blotting, is only rarely found in the synovial lymphocytes isolated from patients with rheumatoid arthritis [11–14], whereas others have found that this is a frequent occurrence [15,16]. It seems likely that these discrepancies are related in part to the techniques used to isolate the T cells, which were expanded by culture with IL-2 in the latter two studies. An alternative technique to assess restriction has been to extract RNA from the lymphocytes infiltrating the target organ of an autoimmune disease, synthesize cDNA and amplify appropriate TcR V gene sequences using specific primers. By this approach, usage of a restricted number of Vα families was found in areas of demyelination in the brains of patients with multiple sclerosis [17]. More recently, restricted Vα but not Vβ gene usage was identified with this technique in the thyroid lymphocytic infiltrate in patients with Graves' disease and Hashimoto's thyroiditis [18,19]. These results are particularly noteworthy given the diversity of thyroid antigens recognized in both disorders and the heterogeneous response to multiple autoantigenic epitopes even within a single patient [20–23]. In view of the limited number of thyroid samples so far examined, we have studied a further 12 patients with Graves' disease using a qualitative cDNA amplification technique and found in these subjects that there was widespread usage of Vα families.

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PATIENTS AND METHODS

Preparation of tissue and lymphocytes

Samples of thyroid tissue were obtained from patients undergoing partial thyroidectomy for Graves' disease ($n=12$) or toxic

multinodular goitre ($n=4$). All patients had been treated with antithyroid drugs (carbimazole plus thyroxine) for at least 2 months before surgery. Samples were processed within 1 h of removal. Sections of approximately 0·5 cm^3 were snap frozen directly in liquid nitrogen or used immediately to prepare RNA. If sufficient material remained, this was minced and digested with dispase (4 mg/ml, Boehringer Mannheim) and collagenase (1 mg/ml, Sigma) for 15–30 min at 37°C with stirring. The cells were filtered through sterile gauze, washed, and incubated overnight at 37°C in M199 medium supplemented with 10% fetal calf serum (FCS). After incubation, the lymphocyte-rich supernatant was decanted from the adherent thyroid follicular cells, debris removed by density gradient centrifugation on Ficoll–Hypaque and the purified intrathyroidal lymphocytes (ITL) washed and frozen for storage in liquid nitrogen. In one of the 12 patients no whole tissue was frozen and only ITL were studied; in seven patients, both whole tissue and ITL were available for analysis. Peripheral blood lymphocytes (PBL) were prepared by Ficoll–Hypaque density gradient centrifugation from three patients with Graves' disease and three healthy controls.

Preparation of total RNA and cDNA synthesis

RNA was prepared from ITL and PBL using RNAzol B (Biogenesis Ltd) following the manufacturer's protocol. First-strand cDNA was prepared from 2–4 μg of this total RNA using random priming. RNA was incubated with primer (500 pmol $pd(N)_6$, Pharmacia), RNAguard ribonuclease inhibitor (70 units, Pharmacia), 1 mM each dNTP and 1000 units Moloney murine leukaemia virus reverse transcriptase (MMLV-RT, GIBCO/BRL) with the supplied buffer in a 60 μl final volume for 10 min at room temperature, followed by 60 min at 37°C. The cDNA was then denatured by heating to 99°C for 2 min, and polymerase chain reaction (PCR) amplification carried out on 1 μl aliquots.

RNA was prepared from whole thyroid tissue by homogenization in 4 M guanidinium isothiocyanate, 5 g/*l* sodium lauryl sarcosine, 25 mM sodium citrate, pH 7·1, 100 mM 2-mercaptoethanol, 0·1% (v/v) antifoam A, 5 ml per gram of tissue. CsCl was added (1 g per 2·5 ml of homogenate) and RNA pelleted by centrifugation for 16 h at 190 000 *g* through CsCl underlay (5·7 M CsCl, 100 mM EDTA, pH 8·0). The RNA pellet was washed with 70% ethanol, and resuspended in TE/SDS (10 mM Tris/HCl, pH 7·4, 5 mM EDTA, 1% SDS). RNA was extracted once each with phenol/chloroform (1:1 v/v) and chloroform and ethanol precipitated. First-strand cDNA was prepared from 2–10 μg of this total RNA both using random- and dT-priming methods. RNA was incubated with primer (7·5 μg dT_{12-18} or 250 pmol $pd(N)_6$), 10 μg bovine serum albumin (BSA; nuclease free, Pharmacia), RNAguard ribonuclease inhibitor (70 units), 1 mM dNTPs, and 1600 units of MMLV-RT with the supplied buffer in a 200 μl reaction volume for 10 min at room temperature followed by 60 min at 37°C. The cDNA was ethanol precipitated and resuspended in 100 μl of 10 mM Tris/HCl, pH 8·0, 0·1 mM EDTA; 2·5 μl aliquots of cDNA were used for PCR amplification.

PCR amplification

Aliquots of the bulk cDNA preparations were amplified with each Vα gene primer (18 Vα primers in total) and a common Cα primer (primer sequences from reference 18). All primers were synthesized using an Applied Biosystems DNA synthesizer. Each reaction consisted of 0·6 μg of each primer (one Vα primer and the Cα primer), 100 μM each dNTP, buffer (final concentrations of 1·5 mM $MgCl_2$, 10 mM Tris/HCl, pH 8·3, 0·01% (w/v) gelatin, 50 mM KCl, 0·1% (v/v) Tween 20, 0·1% (v/v) Nonidet NP40) and 2·5 units *Taq* polymerase (Perkin Elmer Cetus and Amersham) in a final volume of 50 μl. This was amplified using 35 cycles of 95°C for 1 min, 56°C for 2 min, 72°C for 3 min, with a final 10-min extension at 72°C, using a Perkin Elmer Cetus thermal cycler. Control reactions with no cDNA template were carried out regularly to ensure that reagents were not contaminated and these were consistently negative. Amplified products were resolved by agarose gel electrophoresis on ethidium bromide-stained 2% agarose gels, with molecular weight determined by comparison with a 123 bp ladder. Gels were visualized by ultraviolet illumination and recorded.

Hybridization of TcR Vα genes

Gels of PCR products were blotted onto Hybond-N membrane (Amersham, manufacturer's protocol) and the DNA immobilized by UV-cross-linking (UV Stratalinker 1800, Stratagene). Blots were prehybridized for 1 h at 45°C in 0·5% SDS, 6 × SSPE, 1 × Denhardt's solution, before hybridization overnight at 45°C with gamma-^{32}P-ATP-labelled Cα oligonucleotide complementary to an internal Cα site [18] ($0·4 \times 10^6$–$1·9 \times 10^6$ ct/min per ml probe). After hybridization, filters were washed in 1 × SSC, 1% SDS for 1 h each at 60°C, 66°C and 72°C. After each wash, blots were exposed to X-ray film (Hyperfilm-MP, Amersham) at −70°C for 15 min to 2 h. Densitometry of hybridized bands was carried out using the Bio-Profile Image Analysis System (Version 4·01, Vilber-Lourmat). No hybridization signals were present on film after prolonged exposure to blotted PCR-amplified β-actin products when hybridized as above.

RESULTS

TcR Vα gene amplification from normal PBL and cell lines

Amplification was initially carried out on cDNA prepared from the T lymphoblastoid cell lines MOLT4 and Jurkat and from normal PBL. There was consistent amplification of a single Vα gene family product (Vα7 from MOLT4; Vα1 from Jurkat) with both random-primed and dT-primed cDNA prepared from total RNA from the cell lines (data not shown). Analysis of primer function was carried out on cDNA synthesized from individual and pooled normal PBL samples to ensure all primers were capable of amplifying products of the predicted sizes. All products were of the expected sizes (Fig. 1) with variation in the intensity of products as previously reported [17].

TcR Vα gene expression in ITL and whole thyroid tissue

Analysis was carried out on eight ITL samples and 11 samples of fresh or snap frozen thyroid tissue from Graves' patients. Seven of the ITL and tissue samples were from the same individuals, allowing comparison of TcR Vα gene expression from the two sources. In assessing the autoradiographs of hybridized TcR Vα genes, an arbitrary cut off point of 0·5% of the total density (produced by all 18 Vα amplifications) was used to assign the presence or absence of amplification. Signals produced by the amplified products of individual families at this cut off point were clearly visible to the naked eye and could readily be distinguished from background: this arbitrary lower limit

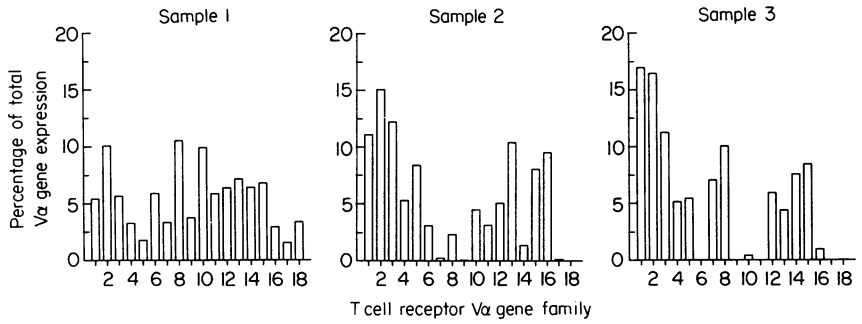

Fig. 1. Amplification of TcR Vα gene families from normal peripheral blood lymphocytes (PBL). Polymerase chain reaction (PCR) amplification and Southern blot analysis of rearranged TcR α-chain transcripts expressed in PBL from three normal controls. Blots were hybridized with an internal Cα-specific oligonucleotide probe, and computerized densitometry carried out on the resulting autoradiographs.

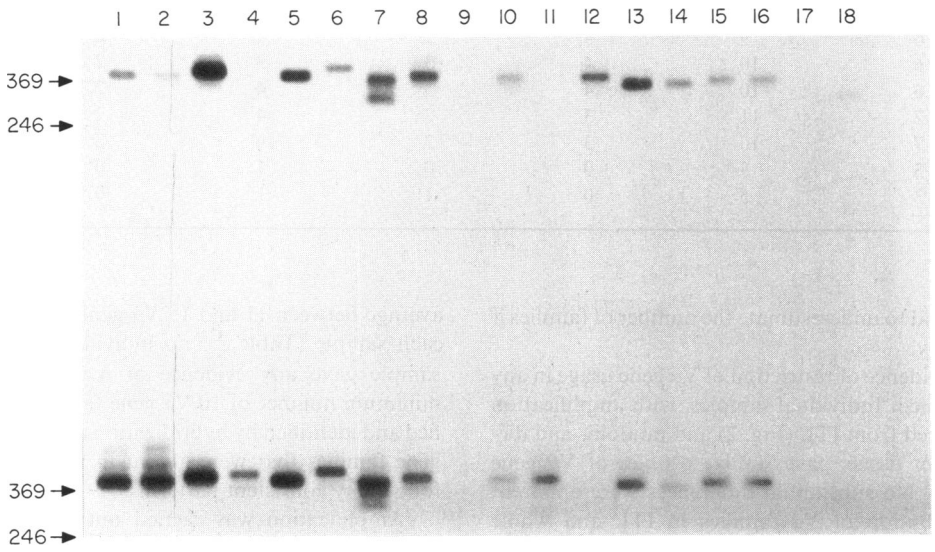

Fig. 2. Amplification of TcR Vα gene families from Graves' ITL. PCR amplification and Southern blot analysis of rearranged TcR α-chain transcripts expressed in two independent samples of Graves' ITL. Blots were hybridized and exposed to film for 20 min. Molecular weight markers are indicated.

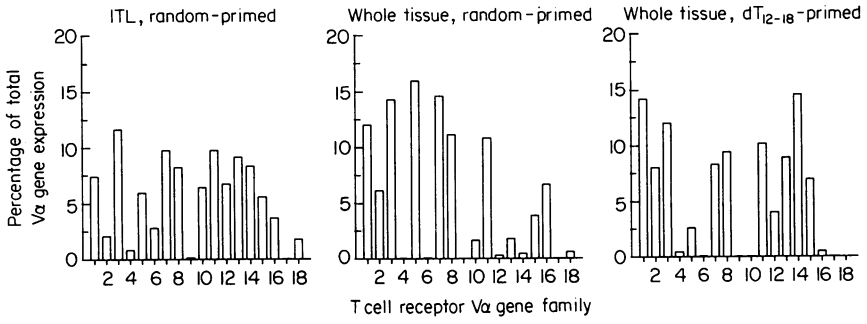

Fig. 3. Amplification of TcR Vα gene families from ITL and whole thyroid tissue from an individual with Graves' disease. PCR amplification and Southern blot analysis of rearranged TcR α-chain transcripts expressed in Graves' ITL and tissue from a typical patient. cDNA was prepared from thyroid tissue by two different techniques. Blots were hybridized and computerized densitometry carried out on the resulting autoradiographs.

Table 1. Number of samples with TcR Vα gene family expression (in intrathyroidal lymphocytes (ITL), whole thyroid tissue and peripheral blood lymphocytes (PBL) from Graves' patients and controls)

	Graves' patient				Nodular goitre		
Vα gene family	Random-primed ITL ($n=8$)	Random-primed whole thyroid ($n=11$)	dT_{12-18}-primed whole thyroid ($n=4$)	Random primed patient PBL ($n=3$)	Random-primed whole thyroid ($n=4$)	dT_{12-18}-primed whole thyroid ($n=2$)	Normal controls (Random-primed normal PBL ($n=3$))
1	8	10	4	3	4	2	3
2	7	11	4	3	4	2	3
3	8	11	4	3	4	2	3
4	6	5	0	3	2	0	3
5	8	10	2	3	4	2	3
6	8	7	1	3	4	1	2
7	6	10	3	0	4	2	2
8	8	10	4	3	4	2	3
9	0	5	0	0	0	0	1
10	7	9	0	2	4	0	2
11	7	9	4	1	4	2	2
12	6	9	4	2	4	2	3
13	6	10	4	3	3	2	3
14	6	10	4	3	4	2	3
15	7	11	3	3	4	2	3
16	7	10	3	3	4	2	3
17	3	3	0	0	1	0	1
18	3	5	0	1	1	0	1

would, however, tend to underestimate the number of families if these are used rarely.

There was no evidence of restriction of Vα gene usage in any of the samples studied. Individual samples, with amplification from cDNA prepared from ITL (Fig. 2) and random- and dT-primed cDNA from tissue, gave similar profiles of Vα gene expression (Fig. 3). No substantial differences were apparent between the distribution of Vα families in ITL and whole thyroid tissue, and amplification profiles were not obviously different from those obtained from PBL derived from normal controls or patients with Graves' disease (Fig. 1, Table 1). Furthermore, there was no clear difference between the Vα gene amplification pattern seen in the Graves' ITL and tissue samples and the toxic multinodular goitre tissue samples (Table 1). On average between 11 and 15 Vα gene families were amplified in each sample (Table 2): no individual Graves' ITL or tissue sample gave any evidence of marked restriction, with the minimum number of 10 Vα gene family products being amplified and identified by hybridization in only two samples. The Vα gene families that were absent in individual patients did not follow any consistent pattern.

Amplification was carried out from whole thyroid total RNA using first-strand cDNA prepared using both random- and dT-priming to ensure that no bias was introduced during cDNA priming. The results of amplification with the two cDNA types were comparable, but with a slight reduction in the number of families detected following amplification from dT-primed cDNA (Tables 1 and 2). With limiting amounts of cDNA, amplification from dT-primed cDNA might, therefore, give the appearance of restriction of Vα gene transcripts in the thyroid.

Table 2. Average number TcR Vα gene families present in samples

Graves' disease		
Random-primed ITL	($n=8$)	$13{\cdot}9 \pm 1{\cdot}6$
Random-primed whole thyroid	($n=11$)	$14{\cdot}1 \pm 2{\cdot}0$
dT_{12-18}-primed whole thyroid	($n=4$)	$11{\cdot}0 \pm 1{\cdot}4$
Random-primed PBL	($n=3$)	$13{\cdot}0 \pm 1{\cdot}7$
Toxic multinodular goitre		
Random-primed whole thyroid	($n=4$)	$14{\cdot}8 \pm 0{\cdot}5$
dT_{12-18}-primed whole thyroid	($n=2$)	$12{\cdot}5 \pm 0{\cdot}7$
Normal controls		
Random-primed PBL	($n=3$)	$14{\cdot}7 \pm 3{\cdot}1$

PBL, Peripheral blood lymphocytes.

DISCUSSION

The reverse transcription and PCR amplification technique used was qualitative rather than quantitative and therefore we cannot comment on whether there is under- or over-representation of particular Vα gene families, for instance, compared with peripheral blood. However, simply considering the presence or absence of the 18 Vα families, we have shown that in 12 patients with Graves' disease, the intrathyroidal T cell population does not show any marked restriction in the number of TcR Vα gene families employed by T cells, with 10–15 families being used in all cases. The same results were found whether analysis was made of the isolated ITL population, or of the thyroid as a whole. Little difference was found between the use of random-

and dT-priming for first-strand cDNA synthesis, although the former method appeared slightly more sensitive.

These results contrast with those previously reported in which an average of only five of the 18 Vα genes were expressed by T cells from the thyroids of five patients with autoimmune thyroid disease [18]. The range of families identified in that study was two to six and in most cases a single Vα gene predominated. Although some of this apparent restriction may have resulted from culture of ITL with phytohaemagglutinin or IL-2, used to expand certain lymphocyte populations, this cannot be the sole explanation, as restricted usage was also found in non-stimulated cells [18,19]. It is also noteworthy that non-autoimmune thyroid tissue displayed widespread TcR Vα gene usage which was ascribed to contaminating blood-borne lymphocytes [18], in accord with our present findings using toxic multinodular goitre specimens. Since one might expect minor contamination of Graves' thyroids with blood-borne lymphocytes (which are not Vα restricted in this condition), it is difficult to explain the previously described failure to detect any amplification whatever of the broad range of Vα families [18].

The results presented here are compatible with amplification of unrestricted T cells derived from both blood and from the infiltrating population in Graves' disease; if the latter had shown any restriction or predominance of a single Vα gene, one might have expected a low level of hybridization with the majority of Vα families but an exaggerated response with a limited number of families. However, there was no obvious instance of this in any of the specimens studied, although we emphasize that our results are only qualitative. We have tested 12 unselected patients with Graves' disease, in seven of these using paired samples of whole tissue and ITL, and have also used two methods of RNA preparation, with broadly similar results. It is possible that early in disease there may be some restriction of the response; recent work on experimental autoimmune encephalitis suggests that the autoreactive T cell response diversifies with the duration of disease [24]. However, it seems unlikely that the Graves' patients studied previously differed greatly from those presented here as they too had been treated with antithyroid drugs before surgery [18,19]. It would be of interest to study patients as early as possible after diagnosis and before treatment is started. In such studies, it will be important to attempt quantification of the amplification so that comparison with the peripheral blood Vα repertoire can be made: we are currently doing this using amplification of actin mRNA as an internal standard.

Differences from the previous study [18] include the use of random hexanucleotide priming to synthesize cDNA and the avoidance of ITL culture with mitogens or IL-2; both of these, we believe, could have influenced the results. In addition, limiting amounts of sample material or reagents in the stages of reverse transcription or PCR amplification may cause apparent restriction and we have also observed that too much cDNA template may result in failure of amplification of cytokine mRNA from thyroid specimens (unpublished). We therefore performed a series of preliminary experiments to optimize the method and to avoid these problems before committing samples to the assay.

To explain their findings, Davies *et al.* postulated the existence of an unidentified superantigen, uniquely capable of expanding particular Vα families [19], although this would require multiple such antigens as there was no single, common Vα gene family which was predominant in their patients. On the other hand, the spectrum of antigens involved in Graves' disease, with T cell responses demonstrated to thyroglobulin [21,25,26], thyroid peroxidase [21,22,27] and the TSH receptor [28], itself suggests a heterogeneous T cell population active in the thyroid, and studies of T cell gene rearrangements have failed to show oligoclonality in the vast majority of patients with autoimmune thyroid disease [29–31]. Therefore, the lack of Vα gene restriction which we have found, especially when non-restricted usage of Vβ gene families has been demonstrated [19], is perhaps not surprising.

Finally, the use of V gene-specific PCR amplification from large, unselected T cell populations may not be the most suitable approach for defining the presence or absence of TcR V gene restriction in the population. There is an inherent risk that non-productively rearranged transcripts present in T cells, but not functional in thymic selection or the mature T cell response, would nevertheless give positive responses by this method unless further discrimination by sequencing was employed [32]. A more direct assay for the functional protein product using MoAbs or assays involving a smaller population of cells such as those expressing IL-2 receptor *in vivo* [33] would therefore be more suitable material for study. This is illustrated by the recent demonstration of diverse Vα TcR gene usage by T cell clones reactive with a single epitope of myelin basic protein in multiple sclerosis [34]. Given these reservations, demonstration of Vα gene restriction in a large heterogeneous population of T cells would be even more improbable.

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