Human Self-reactive T Cell Clones Expressing Identical T Cell Receptor β Chains Differ in Their Ability to Recognize a Cryptic Self-Epitope

By Sonia Quaratino, Marc Feldmann, Colin M. Dayan, Oreste Acuto,* and Marco Londei

*From the Mathilda & Terence Kennedy Institute of Rheumatology, Sunley Division, 1, London W6 8LW, United Kingdom; and *Laboratory of Molecular Immunology, Pasteur Institute, 75724 Paris, France*

Summary

Recognition of self-antigens by T lymphocytes is a central event in autoimmunity. Understanding of the molecular interactions between T cell receptors (TCR) and self-epitopes may explain how T cells escape thymic education and initiate an autoimmune reaction. We have studied five human in vivo activated T cell clones specific for the region 535-551 of human thyroid peroxidase (TPO) established from a Graves' patient. Three clones (37, 72, and 73) expressed identical TCR β and α chains rearranging Vβ 1.1 and Vα 15.1, and were considered sister clones. Clone 43 differed from clone 37 and its sisters in the Jα region only. Clone NP-7 expressed Vβ 6.5 but rearranged two in-frame TCR α chain, both using the Vα 22.1 segment. Fine epitope mapping using nested peptides showed that clones using identical TCR β chains, identical Vα, but a different Jα recognized distinct, nonoverlapping epitopes in the TPO 535-551 region. This finding shows that a different Jα region alone leads to a heterogeneous pattern of recognition. This indicates that the "restricted" TCR V region usage sometimes found in autoimmune diseases may not always correspond to identical epitope recognition. To confirm that clones 37 (and its sisters) and 43 recognize different epitopes, the T cell clones were stimulated with a TPO-transfected autologous Epstein-Barr virus (EBV) cell line (TPO-EBV) that presents TPO epitopes after endogenous processing. Only clone 37 and its sisters recognizes the TPO-EBV cell line, suggesting that the epitope recognized by clone 43 is not presented upon endogenous processing. We have shown that thyroid epithelial cells (TEC), the only cells that produce TPO, express HLA class II molecules in Graves' disease and can act as antigen-presenting cells, presenting TPO after endogenous processing to autoantigen-reactive T cell clones. We tested, therefore, whether autologous TEC induced the same pattern of stimulation as TPO-EBV; T cell clone 37 recognizes the TEC, whereas it is stimulated poorly by the TPO loaded to autologous peripheral blood mononuclear cells (PBMC). Clone 43, which fails to recognize the TPO-EBV, also fails to recognize the TEC, but is activated by exogenous TPO presented by autologous PBMC. These results show that exogenous versus endogenous processing in vivo generates a different TPO epitope repertoire, producing a "cryptic" epitope (epitope not always available for recognition). Our findings define a route by which human self-reactive T cells may escape thymic selection and become activated in vivo, thus possibly leading to autoimmunity.

A major unsolved question is how self-reactive T cells escape thymic selection to reach the periphery, and how under certain conditions they become activated, leading to autoimmune diseases. A number of hypotheses have been proposed to explain these events, and recently it has become apparent that the overall affinity/avidity of the TCR–peptide–MHC complex dictates thymic selection (1). For instance, such affinity/avidity may be reduced by two TCR expressed on the same T cell surface (2, 3). Alternatively, self-peptides with extremely low affinity for their genetic restriction elements might not form a stable enough complex with MHC in the thymus, and thus permit self-reactive T cells to escape negative selection (4, and Quaratino et al., manuscript in preparation). Another hypothesis is based on the concept that different epitope repertoires may be processed and presented under different conditions or by distinct APC (5–7).

Thus, the immune system may not usually see some important self-epitopes, which are considered to be cryptic since they are hidden during the essential phases of thymic

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education. If these cryptic epitopes are displayed in the periphery, then autoimmunity could ensue (7, 8). The factors leading to this differential display are not totally defined, but it is now known that different arrays of epitopes can be generated if proteins (such as viral proteins) are processed by the endogenous or exogenous pathway (9, 10). However, this has not yet been shown in human autoimmunity.

Ultimately, whatever is the processing pathway, the initiating event of an immune response is the recognition of the peptide fragment exposed in the MHC binding groove by the TCR. Many studies have attempted to characterize the TCR usage, epitope recognition, and HLA restriction of human autoreactive T cells. It has been reported that myelin basic protein (MBP)[1]–specific T cells have biased TCR usage in humans (11, 12) and in mice (13), and may dominantly recognize a specific region of MBP in the context of the same HLA molecule (14). In these studies, however, there is no evidence that the T cells studied were either activated in vivo, or were involved in the disease process. This is because of the technical limitations of work with human pathologic material. In Graves' disease (GD) we have been able to overcome some of these limitations, as the cells studied were activated in vivo expressing the IL-2 receptor, and were cloned without exposure to antigen (15, 16). Hence, these cells may have greater relevance to disease than T cells cloned with antigen from PBMC, for which there is no conclusive evidence of disease relevance, since similar clones can be generated from healthy individuals. Using this approach we found that a region of thyroid peroxidase (TPO) amino acids 535-551 was recognized by a significant proportion (16%) of in vivo–activated T cell clones (16). Thus we focused our attention on these clones. We describe below the TCR sequence expressed by these T cell clones, the core epitope recognized, and the HLA element of genetic restriction. Since thyroid epithelial cells (TEC) can act as APC in GD and present autoantigens to T cells, after endogenous processing (15–17), we used a TPO-transfected EBV cell line as well as the autologous TEC to stimulate the T cell clones and compared them with TPO presented after exogenous processing by PBMC. The differences observed between exogenous and endogenous processing demonstrate the existence of T cells recognizing a cryptic epitope, an epitope otherwise hidden, which can be a strong T cell stimulator. The results described here suggest that cryptic self-determinants generated by atypical APC may play an important role in the pathogenesis of autoimmune diseases.

Materials and Methods

T Cell Clones. T cell clones were established from Graves' thyroid infiltrate in absence of antigen, according to our published procedure (15). Briefly, activated thyroid-infiltrating T lymphocytes were cultured in the presence of IL-2 (20 ng/ml, kindly donated by Hoffmann-La Roche, Nutley, NJ) for 1 wk in RPMI 1640/10% human serum and then expanded for a further week with irradiated (4,500 rad) autologous peripheral blood leukocytes, OKT3 anti-CD3 mAb (30 ng/ml), and IL-2. Cells were cloned by limiting dilution in absence of any antigen as described (15, 16). The T cell clones were stimulated with irradiated feeder cells and PHA or with OKT3 coated to plastic and cultured in media consisting of RPMI 1640, 10% pooled human serum, 100 U/ml penicillin, and 50 μg/ml streptomycin. Every 4–5 d, expanding T cell clones were fed with 10 ng/ml of IL-2.

Antigens. Peptide TPO 535-551, corresponding to amino acids 535-551 of the human TPO molecule (LDPLIRGLLARPAKLQ), and eight overlapping "nested" synthetic peptides of 12 amino acids spanning from residues 534 to 552 of human TPO were gifts from Anergen (Redwood City, CA) and Chiron Mimotopes (Clayton, Australia).

T Cell Proliferation Assay. The TPO peptides were used to define the minimum epitope specificity of each T cell clone. The T cell clones were tested at least 2 wk after the last TCR stimulation and 5 d after the last addition of IL-2. The reactivity to the TPO 534-552 synthetic overlapping peptides was assessed by coculturing 10^4 T cells from each T cell clone with 3×10^4 glutaraldehyde-fixed, autologous EBV-transformed PBMC as APC (16). The autologous EBV-transformed cell line was pulsed with or without 10 μg/ml of each of the peptides used in the assay for 1 h at 37°C before incubation with T cells. All T cell clones were tested at least twice, and all the experiments showed a similar profile of responsiveness.

T cell clones were also tested using autologous PBMC as APC. 3×10^4 PBMC isolated from heparinized whole blood by buoyant density centrifugation (Lymphoprep; Nycomed Pharma, Oslo, Norway) were irradiated (4,500 rad) and incubated with 25 μg/ml of recombinant human TPO expressed in Chinese hamster ovary (CHO) cells for 2 h before adding the T cell clones.

When autologous TEC were used, they were obtained after enzymatic digestion of the thyroid and used in proliferative assays without addition of antigen as described (15). Briefly, TEC were irradiated as above and plated at 3×10^4/well and used to stimulate the T cell clones plated at 10^4/well. All the proliferative assays were performed in triplicate for 72 h in a flat- or round-bottom 96-well microtiter plate and the cells were pulsed with [^{3}H]thymidine (1 μCi) during the last 8 h of culture.

TCR Genetic Restriction. Two homozygous EBV-transformed B cell lines expressing identical DP2 or DQ6 with the autologous APC were selected from the 10th HLA Workshop to define whether the element of genetic restriction was DP2 or DQ6. EBV cell line 9036 expressed DPB1*02012 and DQB1*0502/DQA*0102, whereas cell line 9013 expressed DPB1*0402 and DQB1*0602/DQA*0102. 3×10^4 gluteraldehyde-fixed EBVs were challenged with or without the TPO peptide 535-551 for 1 h before adding the T cell clones. The experiment was performed in triplicate, and the cells were pulsed with [^{3}H]thymidine during the last 8 h of the 72-h culture.

PCR Amplification and Sequence Analysis of TCR-α and -β cDNA. Total RNA was extracted from $3–5 \times 10^6$ cells by a modified guanidine-isothiocyanate-phenol-chloroform method and first-strand cDNA was synthesized by a standard oligo(dT) method, using ~5 μg of total RNA (18). To elicit maximum TCR expression before RNA extraction and eliminate possible contamination by feeder cell RNA, resting T cells (more than 14 d after last stimulation) were stimulated with immobilized OKT3 for 24 h before harvesting for RNA preparation. cDNA encoding the TCR α and β chains were then amplified using the anchored

[1] *Abbreviations used in this paper:* AnPCR, anchored PCR; GD, Graves' disease; MBP, myelin basic protein; TEC, thyroid epithelial cell; TPO, human thyroid peroxidase.

PCR method (AnPCR) (19). A poly(dG) tail sequence was added to the single-strand cDNA with terminal deoxynucleotidyl transferase in cobalt buffer (Boehringer-Mannheim Corp., Indianapolis, IN) and 0.1 mM dGTP for 30 min at 37°C. AnPCR was performed using oligonucleotide primers complementary to the poly(dG) (5′-CACTCGAGCGGCCGCGTCGACCCCCCCCCC-3′) and to either the 5′ end of α constant region (5′-GCGAATTCA-GATCTTAGGCAGACAGACTTG TCACTGG-3′) or the 5′ end of β constant region (5′-GCTCTAGAGTCG ACGGCT-GCTCAGGTCAGTATCTGGAGT-3′). Each DNA reaction mixture containing 15 mM $MgCl_2$ and 50 pmol of each of the two oligo primers was subjected to 25 cycles of amplification in a thermal cycler (Perkin-Elmer Cetus Corp., Norwalk, CT) at the following temperatures: 93°C for 15 s, 55°C for 1 min, 60°C for 15 s, and 72°C for 2 min. A final 10-min extension was performed at 72°C to ensure fully duplexed DNA for optimal ligation efficiency. PCR products (~0.6–0.7 kb) were digested with appropriate restriction enzymes and isolated from agarose gels, subcloned into PUC19 vector, and sequenced according to the dideoxynucleotide termination method (20). Sequencing was performed on both strands using the forward and reverse universal M13 primers. Several independent colonies (usually five to eight) were sequenced to confirm the clonal nature of the T cell clones, to exclude base misincorporations due to PCR errors (21), and to detect eventual functional rearrangements of both the α loci in a single clone (19, 22). Sequences obtained were compared to those of EMBL/GenBank/DDBJ using the DNAStar® software.

Different RNA preparations of the tested clones were analyzed for 2 yr. They were also tested using a set of Vβ and Vα family-specific primers (23–25) as described (18). The amplified products were purified from unincorporated primers and nucleotides and directly sequenced using a Dye deoxy terminator cycle sequencing kit (Applied Biosystems, Inc., a division of Perkin-Elmer Cetus Corp., Foster City, CA) in an automatic sequencer (model 373 DNA Sequencing System; Applied Biosystems). Each clone amplified only one Vβ or Vα product, and was sequenced using 5 pmol of the Vβ or Vα primers, respectively. PCR amplification was also performed incorporating 10 μCi of [^{32}P]dCTP, to detect eventual amplified products imperceptible after ethidium bromide staining and UV visualization. The gels were dried and exposed for 24 h on X-omat film (Eastman Kodak Co., Rochester, NY).

TPO Transfection of Autologous EBV. The autologous TPO-transfected EBV-transformed B cell line was a kind gift of Dr. R. Mullins (The Mathilda & Terence Kennedy Institute of Rheumatology). The entire cDNA encoding the human TPO (3.1 kb, gift of Dr. B. Rapaport, University of California, San Francisco, CA) was ligated into the Asp718-HindIII sites of the vector pREP 4 (Invitrogen, San Diego, CA), containing the Rous sarcoma virus enhancer/promoter and the hygromycin-resistance gene. Successfully transfected cells were selected by growth in hygromycin-containing medium at a final concentration of 200 μg/ml in RPMI 1640 containing 10% FCS (26). An autologous EBV-transformed B cell line transfected with the vector pREP-CAT, encoding the CAT protein, was used as control in the proliferative experiments.

Results

A panel of self-reactive T cell clones specific for the 535-551 region of the human TPO antigen was raised from the thyroid infiltrate of a patient with autoimmune thyroiditis (GD). These clones were established in the absence of antigen (15, 16), reducing the bias of in vitro antigen-driven expansion, and enabling analysis of a spontaneous in vivo autoantigen-reactive T cell response. The antigen specificity of all the clones was determined by their responsiveness to exogenous TPO, processed and presented by autologous PBMC (16). The epitope specificity of some of the TPO-specific T cell clones was further characterized, and the TPO region 535-551 was recognized by 6 out of 81 clones (16). Five of these clones have been analyzed in detail in this study.

TCR Analysis. We asked whether the similar peptide specificity of the clones reflected a TCR sequence similarity, and whether there were any conserved amino acids in "motifs" at particular positions of their α or β chains. The cDNA corresponding to the TCR α and β chains of the five T cell clones was amplified by the AnPCR technique and sequenced (19). The nucleotide and predicted protein sequences spanning the junctional V(D)J regions of the TCR β and α chains of the five clones are shown in Fig. 1, *A* and *B*, respectively. Clone NP-7 expressed Vβ 6.5/Dβ 2.1/Jβ2.5, whereas clones 72, 73, 37, and 43 expressed an identical TCR β chain, Vβ 1.1a/Dβ 2.1/Jβ 2.1 (Fig. 1 *A*). A common motif was observed in the CDR3 of these β chains (Fig. 1 *A*): at position 101, a Glu was flanked on both sides by polar uncharged residues (Asn-Gln and Gln-Thr). Alignment of the sequences showed that the overall length of the presumptive TCR CDR3 loops of clones bearing identical specificity differed in length by only one amino acid.

We detected two "productive" α chains in clone NP-7 (Fig. 1 *B*): productive rearrangement of both α chain loci has been observed in up to 30% of both human and murine T cells (19, 22); two α chains can be expressed on the surface of a single cell, pairing with the only β chain available (2, 3). The motif Gly-Asn (residues 95-96) observed in the two CDR3 of clone NP-7 was also detected in the CDR3 of T cell clones 37, 72, and 73. The sequences of the α chains of clones 72, 73, and 37 were identical, rearranging Vα 15.1/Jα AC24. Since clones 37, 72, and 73 express identical TCR α and β chains at the nucleotide level, they are sister clones.

A first AnPCR analysis of clone 43 indicated the presence of a TCR α chain identical to one of the two α chains of clone NP-7. To exclude cross-contaminations that occurred in manipulating clone 43, T cells were subcloned by limiting dilution. We analyzed the TCR of only a single subclone that functionally behaved in an identical way to the original one. The expression of only a single Vβ and Vα was confirmed using a panel of 22 Vβ and 24 Vα family-specific primers (Fig. 2). Direct sequencing of the Vβ amplified product confirmed the AnPCR analysis. The direct sequencing of the only Vα amplified (Vα 15) indicated that Jα IGR Ja08 was used instead of Jα AC24, used by clone 37 and its sisters (Fig. 1 *B*). No Vα 22 product was amplified, indicating that the previous result was due to an AnPCR or cellular contamination. We also used the same panel of Vα and Vβ family-specific primers to test

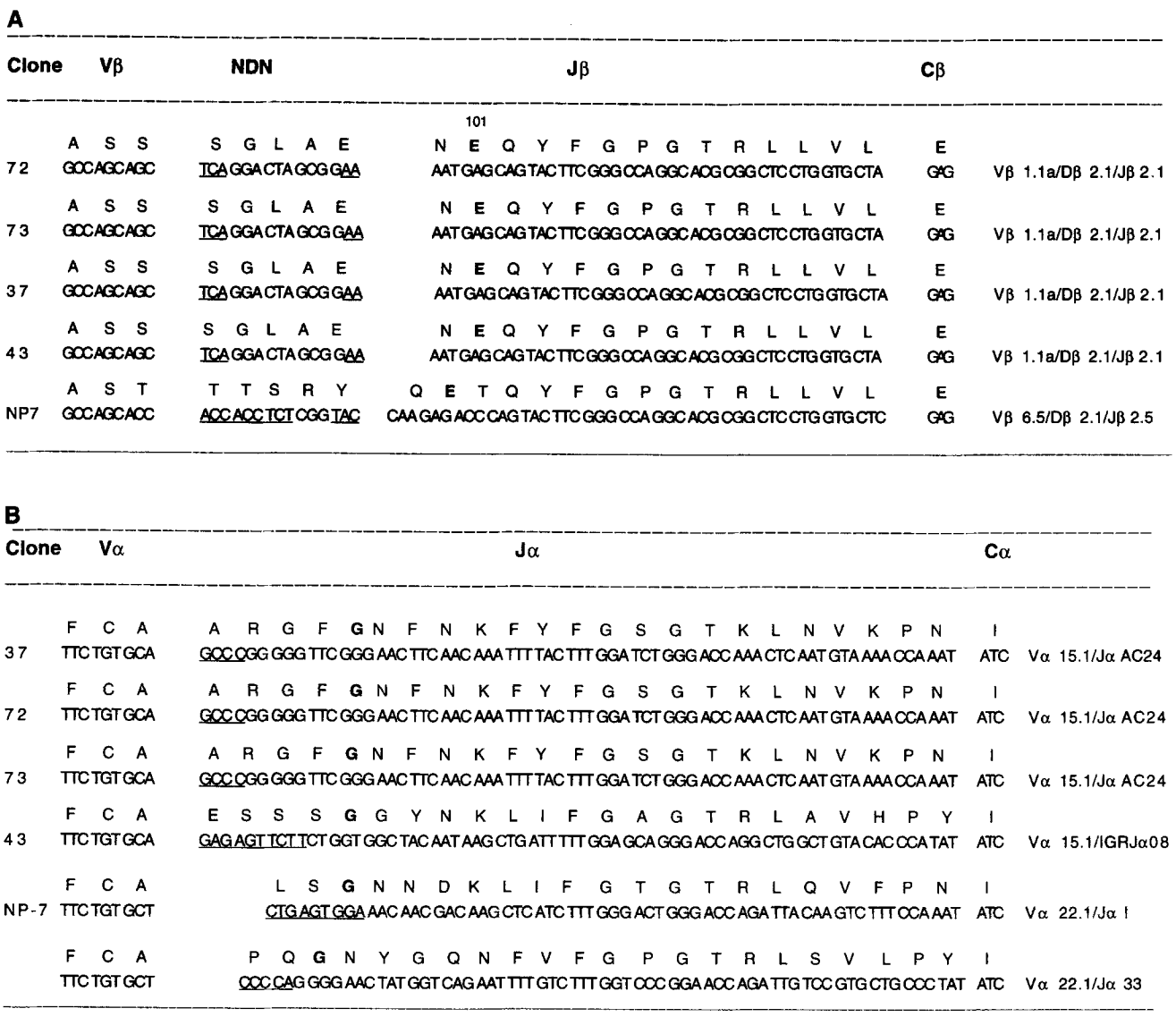

Figure 1. Nucleotide and amino acid sequence and alignment of (*A*) TCR β V-(D)-J regions and (*B*) TCR α chain V-J regions. For each clone are indicated the names of the corresponding Vβ, Jβ, Vα, and Jα genes. Only the last nine nucleotides encoding for the last three amino acid residues of each V segment are shown, followed by the junctional regions. The random TdT addition nucleotides 5′ and 3′ to the diversity region are underlined. Amino acids are indicated by the single letter code, numerated according to Chothia et al. (36). Amino acids in bold are the common motifs in the CDR3 region. Several sequences from 5 to 10 bacterial colonies were performed for each chain, and repeated analysis using mRNA obtained several months apart gave identical results. (*A*) In the CDR3 of all chains at position 101 corresponds a Glu residue (*E*), which is flanked at positions 100 and 102 by polar but uncharged amino acids Asn (*N*), Gln (*Q*), or Thr (*T*). Jβ 2.1 was used by all the clones, except clone NP-7, which encoded for the full germline Jβ 2.5. (*B*) The CDR3 of the α chains of clone NP-7 revealed a common motif Gly-Asn (*GN*) entirely template dependent in Vα22/Jα33, whereas the gly was encoded by the N-nucleotide addition and the Asn was template dependent in Vα22/JαI. Similar GN motif was also shared by the CDR3 of the sister clones 37, 72, and 73. J regions were named accordingly to reported sequences; JαC24 (50), IGR Ja08 (25), JαI (51), and Jα33 (52). These sequence data are available from EMBL/GenBank/DDBJ under accession numbers: X84687, X89751, X89752, X89753, X89754, X89809, and X89860.

clones 37, 72, 73, and NP-7; the direct sequencing of the only amplified Vα and Vβ products gave identical results to the AnPCR analysis.

Fine Epitope Mapping of TPO 535-551–specific T Cell Clones. The fine specificity of antigen recognition among the T cell clones was examined using a nested set of eight overlapping 12–amino acid synthetic peptides spanning residues 534-552 of the TPO molecule (GLDPLIRGLLARRPAKLQV), each advancing one amino acid in the sequence. We expected that the unusual and exceptionally restricted TCR usage (that is, TCR chain sharing) might produce an identical or very similar pattern of recognition. On the contrary, as shown in Fig. 3, three distinct profiles of recognition of the TPO 535-551 region were identified. TPO 536-547 and 539-550 peptides were recognized by clones 37, 72, and 73, but were not recognized by clone 43, which was specific for the 541-552 region of the TPO molecule. Clone NP-7 recognized the region TPO 537-

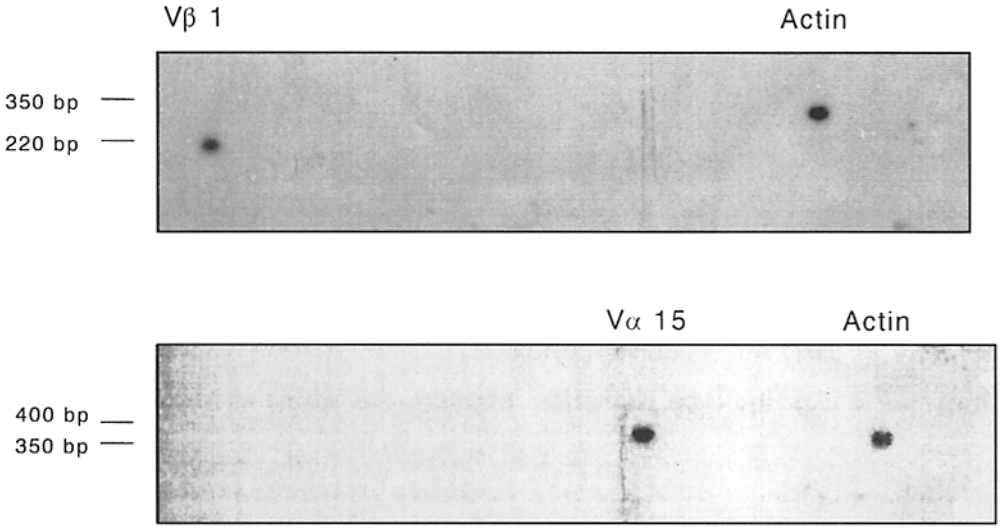

Figure 2. T cell clone 43 was analyzed by RT-PCR incorporating [^{32}P]dCTP, with a panel of Vβ and Vα family-specific primers. PCR products ranged from ~170 to 300 bp and from 300 to 500 bp for the β and α chain, respectively. Products were run on 2% agarose gel and visualized at UV light. The gel was then dried and exposed for 24 h on autoradiograph. This result is representative of three different experiments.

552, but unlike clones 37, 72, and 73, failed to recognize TPO 536-547.

T Cell Clones Recognizing TPO 535-551 in Association with HLA-DQ. In a previous report (16) we excluded the DR antigens as the restricting element, but could not define whether DQ6 and/or DP2 molecules were active genetic restriction elements. Since it has been reported that nonself as well as self-epitopes may be recognized in the context of two different HLA molecules in the same individual (27–29), it was of interest to fully characterize the MHC restriction of the clones. The T cell clones were cocultured with fixed EBV-transformed B cell lines of known MHC haplotypes, with or without exogenous peptide 535-551. The EBV-transformed B cell lines were selected to be homozygous either for DQ6 or for DP2, differing for all the other HLA antigens with the autologous APC. The results (Fig. 4) indicate that none of the clones responded to the TPO peptide 535-551 when presented on EBV 9036, which expressed DPB1*02012, whereas all clones recognized the TPO peptide 535-551 in the context of the DQB1*0602/DQA*0102 molecule (EBV 9013). Further studies of biotinylated peptide binding have shown that DQB1*0602/DQA1*0102 binds the TPO peptide 535-551 whereas DR2 and DR3 do not (Quaratino, S., unpublished observations). Inhibitory experiments performed using anti-DQ, (SVLB4) anti-DP (B7/21), and anti-DR (Tal14.1) mAbs confirmed that all the T cell clones are restricted by DQ molecules (data not shown). Thus, the epitope microheterogeneity we observed was not due to presentation of the same peptide in the context of different HLA molecules.

Recognition of the Autologous TPO-transfected EBV Cell Line. Recently we reported that clone 37 recognizes an autologous EBV cell line transfected with the cDNA encoding TPO and expressing TPO at the cell surface (26). We

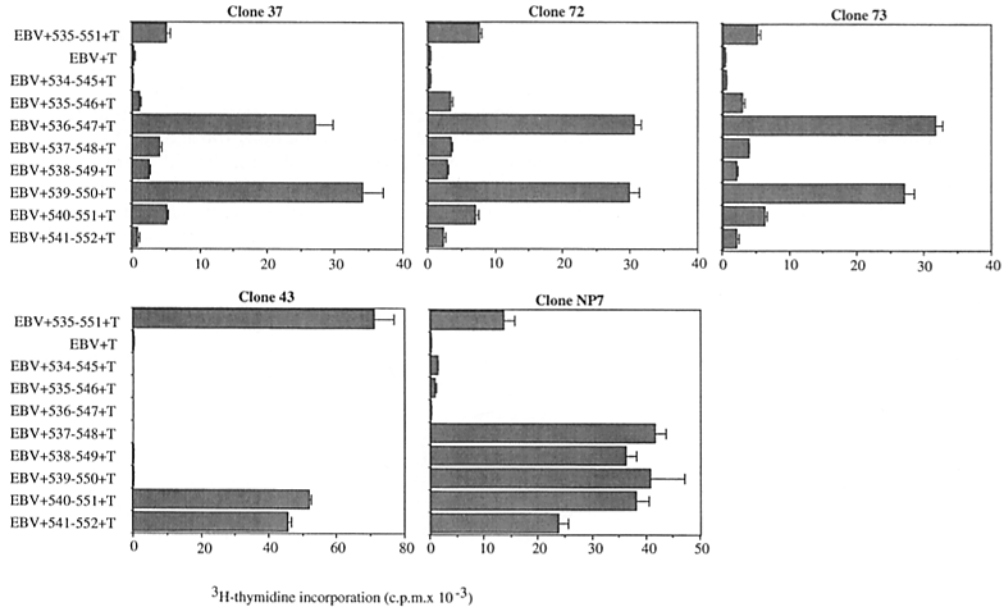

Figure 3. Fine specificity of TPO 535–551–specific T cell clones 37, 72, 73, 43, and NP-7. Eight overlapping synthetic peptides of 12 amino acids spanning from residues 534 to 552 of human TPO were used to define the minimum epitope of each T cell clone. All T cell clones were tested at least twice, and all the experiments showed a similar profile of responsiveness.

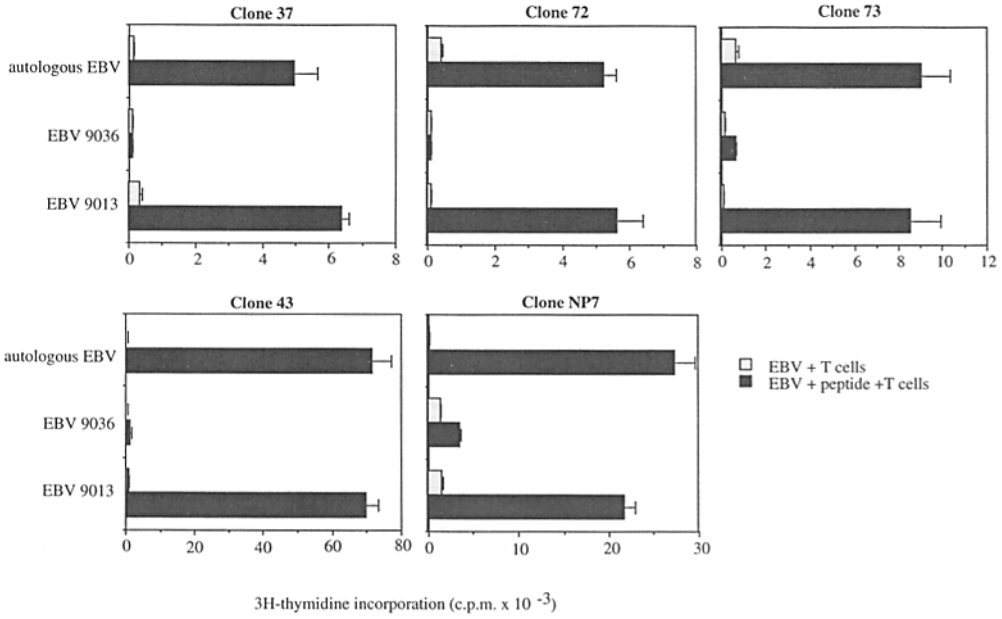

Figure 4. HLA genetic restriction of T cell clones 37, 72, 73, 43, and NP-7. Homozygous EBV-transformed B cell lines 9036 and 9013 expressing identical DP2 or DQ6, respectively, with the autologous APC were chosen to define if the genetic restriction element was DP2 or DQ6. The EBV cell line 9036 expressed DPB1*02012 and DQB*0502/DQA*0102, whereas the 9013 cell line expressed DPB1*0402 and DQB1*0602/DQA*0102. EBV 9036 $DP2^+$ failed to present the TPO 535-551 peptide to all the clones, whereas EBV 9013 $DQ6^+$ presented the peptide to all five clones with comparable efficiency to the autologous EBV cell line.

compared the capacity of this TPO-transfected autologous EBV cell line to stimulate clones 37, 43, and NP-7. Clones 43 and NP-7 did not recognize the autologous TPO-EBV cells, whereas in the same experiment, clone 37 did (Fig. 5). As expected, all the clones responded to the exogenous peptide 535-551 presented by the autologous EBV line (untransfected) and they had previously responded, although to a different extent, to exogenous TPO presented by PBL (16). These results imply that the TPO-transfected EBV cells presented on the cell surface the epitope recognized by clone 37, but not the epitopes recognized by clone 43 or clone NP-7. These data demonstrate that clones 37 and 43, which differ in the TCR Jα region only, recognize two completely different epitopes, only one of which is presented after endogenous processing. Compared with the results shown in Fig. 3, only a peptide identical or very similar to TPO 536-547 was presented, upon endogenous presentation, in the region 535-551.

Recognition of the Autologous TEC. Although the results obtained with the autologous TPO-transfected EBV cell line were intriguing, there was still the possibility that the EBV cells endogenously processing TPO were generating a different epitope repertoire than TEC. To evaluate the potential in vivo situation, we repeated the experiment using the autologous TEC to stimulate the T cell clones in the absence of exogenous antigen. As shown in Fig. 6, clone 43 did not respond to TEC, whereas it did respond to the exogenous TPO presented by PBMC. In the same experiment, clone 37, on the contrary, expressed a marginal (if any) response to exogenous TPO processed and presented by PBMC whereas it was well stimulated by TEC, confirming that different TPO epitopes are displayed upon exogenous versus endogenous processing. This epitope can also be considered as cryptic, since it is an epitope available for recognition only under restricted conditions, in this case by the TEC expressing class II acting as nonclassical APC.

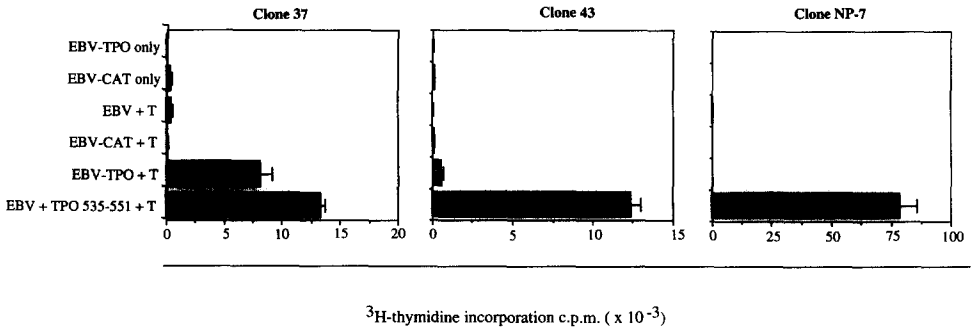

Figure 5. Recognition of autologous TPO-transfected EBV cell line by T cell clones 37, 43, and NP-7. Only clone 37 recognized the endogenously processed TPO. Clones 43 and NP-7 recognized the peptide TPO 535-551 added exogenously, but failed to recognize the endogenously processed TPO. These results suggested that an epitope corresponding to the peptide 536-547 was presented upon endogenous processing. Results shown are from single experiments, and they are representative of those seen in three different assays.

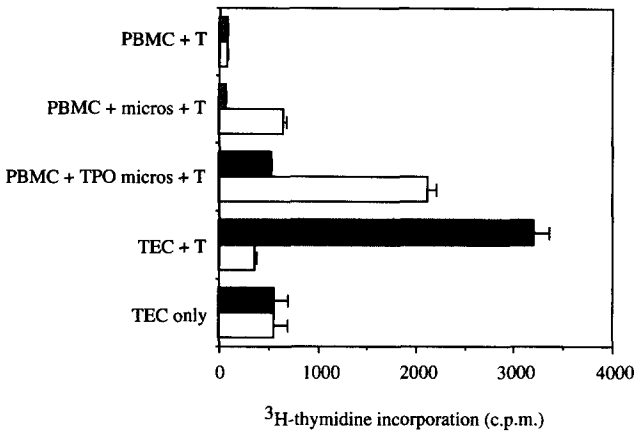

Figure 6. Recognition of endogenously and exogenously processed TPO by T cell clones 37 and 43. (*Striped bars*) Clone 37; (*empty bars*) clone 43. Autologous PBMC were used to present purified microsomes of CHO cells untransfected or transfected with TPO (16). Autologous TEC induced proliferation of clone 37 only, whereas exogenously processed TPO by PBMC was recognized by clone 43 and not by clone 37.

Discussion

The analysis of self-reactive T cells has been one of the major targets of research in autoimmunity during the last few years. These studies have aimed to define the three specific elements involved in self-recognition; much effort has been placed into the characterization of immunodominant self-epitopes and TCR usage (11–13, 30). The aim of these experiments is to understand the general features common to all autoimmune diseases. Many attempts have been made to determine whether self-reactive T cells have a restricted TCR Vα or Vβ usage (11, 24, 31–33), since restricted TCR usage or detection of conserved motifs could suggest dominant self-epitope recognition (34). There is evidence that the CDR3 of the TCR is directly involved in epitope recognition (35–37), and this has led to the notion that T cells expressing conserved CDR3 motifs are prone to recognize identical epitopes (38, 39).

Studies of T cell clones specific for MBP (13, 33) and sperm whale myoglobin (40) have demonstrated that multiple combinations of TCR Vα/Jα paired with an identical or similar TCR β chain may yield a nearly identical epitope specificity. It is interesting to note that a conserved CDR3 motif was observed in T cell clones from Lewis rats with experimental acute encephalomyelitis reactive to the MBP peptide 87-99 (38). The finding that this conserved motif was also observed in a large proportion of TCR β transcripts of T lymphocytes infiltrating plaques from multiple sclerosis patients was thus considered to be strong evidence that infiltrating T cells recognized the same MBP peptide (34).

Our report demonstrates that T cell clones sharing the same TCR β and Vα but differing in the Jα are able to recognize contiguous but different epitopes of the same self-molecule. Thus, even with restricted TCR V region usage, multiple epitope recognition still occurs. It is plausible to envisage that T cell clones may share identical β chains at the nucleotide level, since the β locus rearranges before α (41); thus two different clones from a common precursor may rearrange distinct α chains and emerge from the thymus. The TCR sequencing of other TPO-specific T cell clones established from the same individual showed that different α and β chains are used (data not shown).

Regarding clone NP-7 in which we detected two α chain messages, it has been hypothesized that clones expressing two α chains may be involved in the pathogenesis of autoimmune diseases (2, 3). Since the overall expression of the self-reactive TCR is significantly reduced by the presence of a second TCR, such a decreased expression may reduce the specific avidity of these cells for self, thus allowing their positive selection in the thymus (3). Another aspect of double TCR expression is that in clones expressing two functional TCR, one of which may recognize self, the activation of the T cell via engagement of the other receptor may lead to an "unwanted" activation against self, leading to autoimmunity (2, 3). Although we do not know whether the two α chains are expressed at the cell surface, clone NP-7 may be useful to test this hypothesis, by separately cotransfecting the TCR α with the TCR β chain in a suitable TCR-negative cell (42). The marked sequence similarity suggests that antigenic pressure, rather than a random event, selected its two α chains, and indicates that both chains might be involved in self-recognition. If this is the case, and no second specificity is found for clone NP-7, it would suggest that the two expressed TCR, to escape thymic selection, are both of low affinity for self (3).

Different hypotheses have been evoked to explain peripheral activation of self-reactive T cells. It has been suggested that a bystander or unwanted activation of self-reactive T cells could be induced by superantigens engaging T cells in a Vβ-specific fashion (23) or via molecular mimicry (43); recently, it has been demonstrated that T cells specific for a self-epitope may recognize peptides of viral or bacterial origin (44, 45). A theory that can simultaneously address peripheral activation and lack of central tolerance is based on the existence of a "cryptic self-epitope repertoire:" epitopes hidden during thymic education may become evident in the periphery upon restricted conditions such as different antigen loading or type of cells acting as APC (6, 7). That a cryptic repertoire exists has been described in several animal models (46); this theory has also been useful to explain the process of epitope spreading (8), and recently has been shown in a human system (47).

A central finding of our study is that two different types of APC or antigen loading produce functional differences in the self-peptides displayed in their HLA grooves. It indicates that cryptic epitopes of a disease-related autoantigen may be generated, and more importantly recognized by some, but not all, in vivo–activated, antigen-specific T cell clones (Figs. 5 and 6). Our results highlight another remarkable feature of the cryptic (that is, only presented upon endogenous processing) epitope. Indeed, the close analysis of the data reported in Fig. 3 indicates that peptide 536-547, which closely corresponds to the natural cryptic

epitope presented upon endogenous processing is, at equivalent molar concentration of peptide TPO 535-551, extremely efficient in activating the sister clones 37, 72, and 73. The epitope recognized by the T cell clone 37 was studied in detail using truncated peptides at the NH_2 and COOH termini of peptides 536-547 and 539-550, to explain the peculiar pattern of responsiveness observed in Fig. 3; the results are detailed elsewhere (48). It has been suggested that nonprofessional APC such as class II–expressing TEC, could act as APC (5), and via endogenous processing, display a self-epitope profile different than the one displayed after antigen uptake and by reprocessing APC in PBMC (46). A few years ago, we demonstrated that TEC could be specifically recognized by self-reactive T cells (15). Subsequently, we have shown that many TPO-specific T cell clones were also stimulated by autologous thyrocytes (16). More recently, we have demosntrated, using TPO– and thyroid-stimulating hormone receptor–transfected EBV cell lines, that processing and presentation of membrane-associated self-antigens can occur (26, 49). The results reported in this study demonstrate that a cryptic epitope is displayed by TEC and recognized in vivo by autoreactive T cells (Fig. 6). We have also found other T cells at the site of the disease that recognized the autoantigen only when presented by professional APC, as clone 43 recognized TPO presented by PBMC and not by TEC. We do not know which of the two types of self-reactive T cells may initiate the autoimmune process, but we favor the hypothesis that the primary event is the recognition of the self-processed epitope, once the thyroid epithelial cells acquire antigen-presenting capability (5, 15, 17). That recognition of the cryptic epitope may represent a primary event is also supported by the evidence that this epitope is extremely efficient in activating the sister clones 37, 72, and 73, compared to peptide 535-551. Cells such as clones 43 and NP-7 may intervene when the autoantigen, released after tissue damage, is processed by professional APC, and may correspond to a second phase of self-recognition, that may represent epitope spreading in human autoimmunity.

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Address correspondence to Dr. Sonia Quaratino, The Mathilda & Terence Kennedy Institute of Rheumatology, Sunley Division, 1 Lurgan Avenue, London W6 8LW, UK. C. M. Dayan's present address is Department of Medicine, University of Bristol, University Walk, Bristol B581TD, UK.

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