Peripheral CD25 positive T lymphocytes with biased T cell receptor V β gene usage in autoimmune endogenous posterior uveitis

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Abstract

Aims—To determine T cell receptor (TCR) V β gene usage in peripheral blood T lymphocytes of patients with endogenous posterior uveitis (EPU). If biased TCR variable (V) gene usage occurs in this autoimmune disease, it should be detectable in immune activated peripheral blood T cells in vivo.

Methods—Relative proportions of each V β gene family expressed in total peripheral blood lymphocytes (PBL) and in vivo activated (CD25+) T cells from patients with EPU and controls were determined using the anchored polymerase chain reaction (anchored PCR) in conjunction with a novel hybridisation assay. The TCR V β repertoires seen in these cell populations were then compared.

Results—V β 1 usage within the CD25+ lymphocytes of patients with EPU was substantially elevated (mean $\pm$ SD 15 $\pm$ 9%) compared with control CD25+ cells (3.3 $\pm$ 2.4%).

Conclusions—By contrasting the repertoires of these cell populations, biased TCR V β gene usage was detected in patients with EPU, namely increased usage of V β 1 in CD25+ T cells from peripheral blood of these patients. This approach of directly analysing the activated T cells in blood, using bulk PBL as an internal control, has wide applicability where specific T cell subpopulations are thought to play an important aetio-pathological role.

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Keywords: T cell receptor, V β gene, endogenous posterior uveitis.

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Endogenous posterior uveitis (EPU) is thought to be a specific autoimmune disease of the eye.^{1,2} Experimental autoimmune uveitis in several animal species is clearly a T cell mediated disease; its induction, transfer and modulation can all be achieved by manipulation of culpable T cells.³⁻⁵ Immune involvement in human uveitis is further highlighted by the finding in peripheral blood and vitreous of specific T cell mediated immune responses to putative retinal antigens^{6,7}, as well as small numbers of interleukin-2 receptor (CD25) positive activated T cells.^{8,9}

Within rarely studied specimens of human eye lesions, activated T cells also

predominate.^{6,10} Studies of animal models of autoimmune diseases, including experimental autoimmune uveitis, have shown by molecular analysis of the T cell receptor (TCR) variable region (V) β chain repertoire that the auto-aggressive T cells use a restricted range of receptor genes,^{11,12} and are amenable to specific immunotherapy aimed at interfering with specific V β expressing T cells.^{13,14} Consequently, considerable efforts have been made to define the autoreactive T cell repertoire in human diseases such as autoimmune thyroiditis, rheumatoid arthritis, myasthenia gravis, insulin dependent diabetes mellitus, and multiple sclerosis. Human data however, especially based on blood lymphocyte analyses, have been largely negative or non-conclusive and have tended to show significant diversity regarding the usage of TCR V β region genes.¹⁵

We postulated that a barrier against clearly defining in vitro the autoreactive circulating T cell repertoire operative in vivo in human autoimmune disorders may be caused by the confounding effects of the in vitro manipulation of T cells adopted in many studies. Manoeuvres used include analysis of total genomic DNA or reverse transcribed messenger RNA (mRNA) after in vitro culture and generation of T cell lines and clones using combinations of cytokines, peptide antigens, and mitogens. It is likely that these manoeuvres diversify and distort the detected T cell repertoire by selection both for and against certain clonotypes.^{15,16}

As human EPU is such an organ specific intraocular disease, it was reasoned that culpable T cells involved in the induction or maintenance, or both, of pathology, if present in blood by way of trafficking to or from the disease site, should be present within the population of cells expressing generated activation markers in vivo, such as the interleukin-2 receptor (CD25), as a result of clonal selection, activation, and proliferation. We therefore designed the strategy of analysing and comparing the TCR β repertoires of CD25+ cells and the total lymphoid population in blood. To obtain these cells for analysis with minimal manipulation, CD25+ blood lymphocytes were isolated using immunomagnetic affinity techniques.

In this study the CD25+ cells and total lymphocytes obtained from six patients with EPU with clinically active disease and controls were analysed using the single side specific or anchor polymerase chain reaction (anchored PCR)¹⁷ developed to simultaneously amplify, with equal efficiency, all members of the ex-

pressed TCR Vβ families in one reaction. This, in conjunction with a novel hybridisation based approach for rapid Vβ quantitation, permitted characterisation of Vβ repertoires directly from in vivo activated peripheral blood lymphocytes (PBLs) without in vitro culture or the initial laborious sequencing of cloned anchored PCR products previously used in many studies.

Patient characteristics. Column five gives details of FACS (Becton-Dickinson) analysis of peripheral blood and percentages of T cells (CD3) and "activated" T cells (CD3/25).

Patient No.	Diagnosis	Duration of disease	Therapy	FACS analysis CD3/CD25(%)
1	Panuveitis/retinal vasculitis	Three months	Prednisolone	62.3% CD3 9.2% CD3/25
2	Retinal vasculitis	Eighteen months	Prednisolone and cyclosporin	81.9% CD3 10.4% CD3/25
3	Sympathetic ophthalmia	Over five years	Prednisolone and cyclosporin	82.4% CD3 18.5% CD3/25
4	Vogt-Koyanagi-Harada disease	Over one year	Prednisolone	73% CD3 16% CD3/25
5	Intermediate uveitis/vitritis	Eight years	Prednisolone	84.6% CD3 19.1% CD3/25
6	Peripheral retinal vasculitis	Over one year	Cyclosporin	71.7% CD3 7.76% CD3/25

Control group values mean $\pm$ SD: CD3, 70.7 $\pm$ 4.5%; CD3/CD25, 12.7 $\pm$ 8.9%.

Figure 1 Chemilumogram of reverse dot blot analysis of TCRβ from CD25+ cells from a control normal patient. The panel below indicates the position of each Vβ family specific spot. L=location marks (ink applied to membrane whilst in dot-applicator); B=blank; dT=mock tailing reaction.

Methods

Six patients with active EPU were recruited to the study from the Eye Clinic at Aberdeen Royal Infirmary, Aberdeen, UK. All patients had involvement of the retina/choroid in inflammatory disease. Peripheral blood was obtained from each of these six patients and from six healthy volunteers with no evidence of ocular inflammation. The clinical diagnosis, duration of the disease, and treatments are presented in the table. Peripheral blood lymphocytes were prepared by centrifugation on standard density media (J-prep, TechGen, London, UK) from EDTA treated blood. Flow cytometric analysis for CD3 and CD25 were performed as described by Dick *et al.*⁸

CD25+ T cells were separated from a portion of each PBL preparation using monoclonal mouse antihuman interleukin-2 receptor (Dako), followed by immunomagnetic purification with MACS paramagnetic beads (Miltenyi Biotec GmbH), according to the manufacturer's instructions.

Using an oligo (dT)₁₂₋₁₈ primer, complementary DNA (cDNA) was synthesised (using RNA from 2×10^5 to 2×10^6 cells) and dG tailed according to the method of Loh *et al.*¹⁷ Anchor PCR with label incorporation was performed on tailed cDNA equivalent to between 1×10^5 to 1×10^6 cells in 50 μ l containing $1 \times$ PCR buffer (Boehringer Mannheim, Mannheim, Germany), 1 unit Taq polymerase, 3.5 mM MgCl₂ (final concentration), 0.2 mM each dNTP, 5 μ M digoxigenin-11-dUTP (Boehringer Mannheim), 0.5 μ M ANpolyC primer (CTATCTAGAGAGCTCGCGGCCG[C]₁₃), 0.5 μ M Cβmid primer,¹⁸ and cDNA. Reactions were initiated using a "hot-start" procedure. Amplification of TCR β chain sequences required 35 cycles at 94°C for one minute, at 50°C for one minute, and at 72°C for two minutes followed by 10 minutes at 72°C. PCR products were then cleaned by ethanol precipitation in the presence of 2.5 M ammonium acetate.

Proportions of each Vβ family present after anchored PCR were determined by hybridisation to a validated panel of 30 mer Vβ family specific oligonucleotides,¹⁸ immobilized on nylon membrane (Hybond-N, Amersham), as described by Saiki *et al.*¹⁹; 4 pmoles of each tailed oligonucleotide were applied at 3 mm diameter dots with a commercially available 96 well "dot blot" microfiltration apparatus.

Location markers for later use where produced by applying permanent red ink to unused wells of each membrane (fig 1). Covalent binding of the tailed molecules to the membrane was achieved using 40 mJ of ultraviolet irradiation in a Bio-rad GS-Genelinker ultraviolet chamber. Membranes were then washed well in 6 $\times$ SSC (standard saline citrate) and dried.

Hybridisation was performed concurrently for each PBL/CD25+ pair. Pre-hybridisation was performed in 4 ml TMACl buffer (3 M tetramethyl ammonium chloride, 0.1% sodium dodecyl sulphate (SDS), 50 mM TRIS.Cl (pH 7.7), including 50 μ g/ml porcine heparin and

100 µg/ml poly (A)) for one hour at 62°C. The PCR product (half of a reaction) was added after being denatured for five minutes at 97°C. Hybridisation took place for one hour at 62°C. Posthybridisation washes were performed for 2 × 15 minutes at 20°C in TMACl buffer without heparin or poly (A) and for 1 × 10 minutes at 73 ± 1°C in the same buffer. The membrane was then rinsed in 6 × SSC at 20°C, and analysed.

Detection of membrane bound digoxigenin labelled DNA was performed using sheep anti-digoxigenin polyclonal antibody (Fab) conjugated to alkaline phosphatase and an AMPPD based chemiluminescent substrate, Lumiphos 530, essentially according to the manufacturer's instructions (from Boehringer Mannheim). Membranes were sealed and left at 37°C for 30 minutes after addition of the substrate to achieve steady state light output.

Light emitted was detected firstly by exposure to x ray film (Fuji RX) to establish that no artefactual marks on uneven background were present. Quantitative determination of light output from each dot was then obtained using a Beckman Topcount liquid scintillation counter in single photon mode. Membrane dampened with Lumiphos 530 was clamped into a custom made black perspex holder designed to copy a 96 well microtitre plate. The upper half being a flat mask, exposing each dot to the photomultipliers and the bottom half a solid block to support the membrane. The very top of the mask was sealed with a clear self-adhesive cover.

Light output was counted for 12 seconds. Care was taken to ensure that all dots on a membrane were counted with one detector to avoid problems associated with imbalances between the Topcount's twin detectors. After background subtraction, data from all Vβ family specific dots on a membrane was summed to give a 100% figure and the percentage contribution of each family to the total was determined.

Results

Anchor PCR efficiency and hybridisation specificity under the conditions stated in the methods was confirmed using T cell lines as RNA sources of single β chain mRNAs. Stringency temperatures were determined empirically, until strong signals were detected on the relevant Vβ probe dot, with low background and minimal cross hybridisation to other dots (typically <1% for single β chain amplifications). TMACl solution permitted hybridisation to be independent of base composition, thus dependent only on the length of the cDNA strands (for the oligonucleotide probes used in this study, this was 26–30 bases allowing for small mismatches) and thus permits high stringency washes to be performed to close tolerances without preferential loss of high A/T content hybrids.²⁰ This, combined with the use of an excess of each immobilised probe (approximately 4 pmoles/dot) ensure that hybridisation is both specific and essentially operating under conditions of (immobilised)

probe excess and therefore with pseudo first order kinetics.²¹ Under such conditions, the amount of labelled PCR product specifically bound to any immobilised oligonucleotide will be proportional to its concentration in the solution and the final light output will also be proportional to the amount of membrane bound PCR product. The results of hybridisation and the semiquantitative nature of the reverse dot blot procedure used is shown by the chemilumogram in fig 1.

As can be seen in fig 2, TCR Vβ usage shows some striking differences to the control samples. Statistical analysis of individual Vβ usage between control and EPU groups was performed using a non-parametric, unpaired method (Mann–Whitney U test). Most values detected are within the range found in control samples but Vβ1 usage is significantly elevated amongst CD25+ cells of EPU samples when compared with control CD25+ cells (mean ± SD 15 ± 9% compared with 3.3 ± 2.4% in PBLs, $p=0.0087$; fig 2B).

Comparing the repertoires the other most obvious differences, relative to the controls, are decreased abundance of Vβ2 in the uveitis PBL samples, which is not statistically significant, and decreased Vβ8 levels in EPU CD25+ samples relative to the control CD25+ samples, which is potentially significant ($p=0.047$).

All of the patients were HLA typed (data not shown) and, as expected from previous data, they have heterogeneous alleles with respect to both class I and class II HLA antigens.²²

Clinical and laboratory examination of the patients at the time of blood sampling excluded any concomitant/intercurrent infection which could account for the significant number of activated T cells present in their blood. Previous work from our laboratory has documented the presence of CD25+ T cells in the blood of patients with EPU and indeed fluctuations of such cells was evident with disease activity. No significant differences were observed in the proportions of CD3+ cells or CD3+/CD25+ cells between the controls and patients in this study (table); however, this may well reflect the treatments to which the patients were subjected.

Discussion

Biased TCR Vβ repertoire usage in patients with EPU, a presumed autoimmune disease without significant HLA association, is compatible with findings reported in experimental animals.²³ Our current findings in humans bear a strong resemblance to a recent report of highly restricted usage of TCR Vβ sequences in autoaggressive T cells in the ocular tissues of mice.¹² Similar Vβ sequences were also detected in peripheral lymphoid cells reacting to the known autoantigens in this mouse model of experimental autoimmune uveitis. In the mouse system restriction was limited mainly to Vβ2, Vβ12, and Vβ15. Interestingly, the patients with EPU represented a clinical spectrum from early postdiagnosis to chronic relapsing disease (table); nevertheless, all had

Figure 2 Comparison of the V β repertoires (% of total light output recorded over all family specific dots on a membrane) of A total PBLs and B CD25+ cells from patients with EPU. Total and CD25+ repertoires shown as mean $\pm$ SD; x axis, V β families as described in the table.

increased numbers of V β 1 $\alpha\beta$ T cells. Recently documented concepts of clonotype and determinant drift associated with disease duration in experimental animals²⁴⁻²⁶ indicate that there can be significant changes in T cell V β repertoire usage depending on disease duration. Our patient group, with varying disease duration, shows little evidence of such drift. Further studies should clarify the importance of the concept of clonotype drift in the pathogenesis of human autoimmune disease.

The degree of biased V gene usage among CD25+ cells, particularly dramatic for the V β 1 family, also raises the possibility that superantigen stimulation²⁷ is involved in the pathogenesis of human EPU. However, we are not aware of currently documented superantigens^{28,29} which could account for the biased usage of V β 1 detected.

In patients with EPU these findings of increased prevalence of particular TCR V β usage within peripheral CD25+ T cells, if proven to be involved in the pathogenesis of EPU, may indicate the feasibility of using selective immunotherapy for the control of this potentially sight threatening disease. Isolation of T cells expressing these defined V β sequences should

permit the elucidation, initially in vitro, of the important in vivo uveitogenic antigens/peptides involved in the induction or maintenance, or both, of the disease.

Finally, the novel approach adopted in this study clearly suggests that re-evaluation of readily accessible activated T cells from blood may prove informative in a wide range of autoimmune and microbial diseases where T cells are thought to play a central role in the pathology.

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