

# Restrictions of T cell receptor $\beta$ chain repertoire in the peripheral blood of patients with systemic lupus erythematosus

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## Abstract

**Objective**—To investigate whether oligoclonal T cell populations occur in peripheral blood lymphocytes from patients with systemic lupus erythematosus (SLE).

**Methods**—RNA was extracted from the lymphocytes isolated from whole peripheral blood of five female patients fulfilling ARA criteria for SLE and two healthy female controls, and synthesised into cDNA. CDR3 length spectratyping was performed using a polymerase chain reaction (PCR) run to saturation followed by a primer extension with a radioactively labelled primer. The resulting samples, one for each of the 23 V $\beta$  families, were then run on a polyacrylamide sequencing gel to examine the T cell receptor  $\beta$  chain repertoire at the level of VDJ length heterogeneity.

**Results**—The two healthy female controls showed faint oligoclonal bands in only two and three V $\beta$  families respectively. Three of the patients showed a similar degree of oligoclonality to the controls, while the other two, who had active disease as shown by SLAM scores of 17 and 19 and in one case low C4 and raised C3dg levels, showed marked oligoclonality of their T cell  $\beta$  chain repertoire affecting more than 17 of the 23 V $\beta$  families analysed.

**Conclusions**—Using the technique of CDR3 length spectratyping, restriction of T cell receptor  $\beta$  chain usage by peripheral blood T cells in patients with SLE has been demonstrated for the first time.

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The T cell is pivotal in the pathogenesis of a wide variety of autoimmune conditions including systemic lupus erythematosus (SLE). A way of distinguishing disease-relevant clones is through the T cell receptor by which T cells recognise antigen presented by the major histocompatibility complex (MHC).<sup>1-2</sup> The T cell receptor is a heterodimer of either  $\alpha\beta$  or  $\gamma\delta$  chains. The T cell receptor genes have multiple variable (V) region minigenes which undergo clonal rearrangements with joining (J) and, in the case of  $\beta$  and  $\delta$  chains diversity (D) segments during T cell development<sup>3</sup> to generate functional genes in combination with the constant [C] region exon. This imprecise junctional recombination with nucleotide additions results in length and sequence heterogeneity.<sup>4</sup> The recombined V region determines antigen and MHC specificity<sup>5</sup> and the V-D-J (or VJ)

junctional segment is most variable and encodes the third complementarity determining region (CDR3) which has an essential role in antigen recognition.<sup>6</sup>

The  $\beta$  chain of the T cell receptor is thought to be the most important for antigen specificity.<sup>7</sup> The genes which encode the variable region of the  $\beta$  chain can be divided into 24 families based on sequence similarity<sup>8</sup> and therefore provide a potential target for identifying the subsets of T cells which are active in autoimmune disease, where a limited subset of autoantigens is suspected of causing disease initiation and progression.

Several techniques have been used to detect V $\beta$  repertoires including monoclonal antibodies, but the later are not available for all V $\beta$  families. Restriction fragment length polymorphism (RFLP) analyses give only limited information, even when a link with a particular V $\beta$  type has been shown. Other techniques rely on polymerase chain reaction (PCR) amplification of genomic DNA or RNA turned into cDNA. However, this standard technique does not provide a quantitative result to enable determination of oligoclonality of T cell receptor V $\beta$  receptors and although several semiquantitative PCR techniques have been published, they continue to have significant limitations; furthermore, the extensive in vitro manipulation of cells before PCR may cause additional confounding effects. Further development of the anchor polymerase chain reaction,<sup>9</sup> which avoiding such manipulations, has successfully demonstrated V $\beta$  restriction in human endogenous uveitis.<sup>10</sup> However, techniques which estimate V $\beta$  usage may not detect clonal expansions which do not cause significant changes in total V $\beta$  expression.<sup>11</sup>

A technique termed "CDR3 spectratyping", using a PCR run to saturation followed by a primer extension with a labelled primer,<sup>12,13</sup> has been used successfully to examine T cell receptor  $\beta$  chain repertoires at the level of VDJ length,<sup>14</sup> revealing at least 2000 groups of length variants as a result of junctional diversity. This technique has demonstrated the oligoclonal response to vaccination<sup>15</sup> and has shown oligoclonality within lymphocytic infiltrates in tumours.<sup>16,17</sup> Experiments using this technique in humans have revealed stable profiles of CDR3 size<sup>12,18</sup> and have shown that clonal expansions, often of CD8 positive T cells,<sup>18</sup> may occur in healthy individuals,<sup>19,20</sup> and that public responses to antigenic stimuli can occur.<sup>21-23</sup> However, this technique does not detect true polyclonal expansions in which all T cells bearing certain V $\beta$  types are

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expanded proportionally, as may occur with superantigens. However, when this technique does reveal oligoclonality, identified when certain clones bearing a particular V $\beta$  become expanded, it does not allow determination of how many clones are involved in the expansion, as each CDR3 band may represent one or more expanded clonal populations.

## Methods

### PATIENT SELECTION AND DISEASE ACTIVITY ASSESSMENT

Five patients meeting ARA criteria for SLE<sup>24</sup> without other coexisting autoimmune disease, past history of malignancy, or concurrent infection, and not on cytotoxic drugs for the previous three months, were selected, informed consent obtained, and their disease activity assessed by the standardised published scoring systems SLAM<sup>25</sup> and BILAG.<sup>26</sup> SLAM scores total disease activity on a numerical score from 0 to a theoretical maximum of 106, based on addition of scores for individual symptoms, with higher scores representing greater disease activity. Inactive patients usually having SLAM scores of under 10. BILAG scores different organ systems individually, giving each a letter score, with C, B, and A representing mild, moderate, and severe disease activity, and D and E, no current disease activity—E indicating that the organ system has not previously been involved by SLE. Blood was also taken for tests of disease activity and lymphocyte extraction. Two healthy control subjects without evidence of infection or previous malignancy and with negative autoantibody screens were also selected, gave their consent, and had blood taken for lymphocyte extraction.

### LYMPHOCYTE SEPARATION, RNA EXTRACTION, AND CDNA SYNTHESIS

Peripheral blood mononuclear cells were isolated from 5 ml EDTA blood samples by density cushion centrifugation on histopaque-1077 (Sigma). Using diethylpyrocarbonate (DEPC; Sigma) treated and autoclaved solutions, total RNA was extracted from  $1 \times 10^6$  cells with 100  $\mu$ l of Trizol (Gibco BRL) using the manufacturer's protocol. The RNA was heated at 65°C for 5 min and cDNA synthesised using a Ready-To-Go T primed first strand kit (Pharmacia Biotech).

### V $\beta$ FAMILY PCRS AND EXTENSION REACTIONS

Aliquots of cDNA were used for DNA amplification of T cell receptor  $\beta$  chain sequences using the polymerase chain reaction<sup>27</sup>; 23 separate reactions, one per V $\beta$  family, were performed per sample using an established set of C $\beta$  mid and V $\beta$  primers using 50  $\mu$ l reactions and 40 cycles<sup>17</sup>; V $\beta$  20 was not analysed as consistent results could not be obtained with the primer. Ten microlitre extension reactions using 0.1  $\mu$ l of a C $\beta$  mid primer radiolabelled with phosphorus-32, 2  $\mu$ l of 5 $\times$  buffer B (Invitrogen), 0.2  $\mu$ l of 10 mM deoxynucleotide triphosphate (dNTPs; Invitrogen), 1  $\mu$ l of 0.1 U  $\mu$ l<sup>-1</sup> red hot Taq (Applied Biotechnologies) and 0.1  $\mu$ l of 1% Tween 20 (Sigma) were over-

laid with oil and run at 94°C for 2 min, cycled five times at 94°C for 1 min, 64°C for 1 min, and 72°C for 1.5 min, then run at 72°C for 5 min

### CDR3 SPECTRATYPING

The resulting run off reactions were diluted 1:1 with stopping mix (Sigma) heated at 80°C for 2 min and 5  $\mu$ l of each sample run on a standard 20 cm  $\times$  40 cm 0.4 mm thick 6% acrylamide sequencing gel (Scotlabs, Easigel) at 35 W for 2 h. The gel was fixed with a 10% methanol, 10% acetic acid mix for 1 h, dried, and visualised by autoradiography with a 24 h exposure at room temperature. Elongated primer sequences show length heterogeneity due to variation in the CDR3 size in the original T cell receptor mRNA sequences. These vary by multiple of three nucleotides around a mean size dependent on the V $\beta$  primer used. The resulting gel shows 7 to 11 bands per V $\beta$ , band intensity showing a normal distribution around the mean size. However, abnormally expanded populations due to the increase in cells expressing the same V $\beta$  with the same VDJ region show up as bands which are abnormally intense (figure) and are taken to be indicative of oligoclonality.

## Results

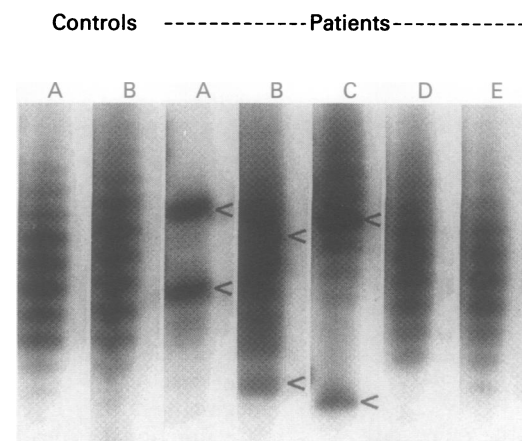
### NORMAL CONTROLS

Both female controls revealed faint oligoclonal bands: control A, aged 31, showed single bands in V $\beta$  families 6, 7, and 21; control B, aged 29, showed single bands in V $\beta$  families 1 and 6 (table 1).

### PATIENTS

Disease activity in the patients, as assessed by SLAM and BILAG scores and blood activity markers, is shown in table 2.

*Patient A* was aged 53 with a 16 year history of SLE characterised by arthralgia, Raynaud's phenomenon, pleurisy, malar rash, and neuropsychiatric symptoms. Investigations had revealed lymphopenia, thrombocytopenia, antinuclear antibodies, anti-double-stranded DNA [anti-ds DNA] antibodies, anti-Ro



T cell receptor V $\beta$  11 CDR3 spectratypes in two healthy controls and five patients with systemic lupus erythematosus. Both controls and patients D and E show a normal distribution of CDR3 size bands, while in patients A, B, and C there are two intense bands (arrows) indicating expanded V $\beta$  11 populations.

Table 1 Number of expanded CDR3 bands detected in SLE patients and normal controls by V $\beta$  family

V $\beta$	Controls		Patients				
	A	B	A	B	C	D	E
1	—	1	3	—	3	1	—
2	—	—	4	—	1	—	1
3	—	—	4	—	2	—	—
4	—	—	3	—	1	—	—
5	—	—	2	—	1	—	—
6	1	1	2	1	2	1	—
7	1	—	3	—	1	—	—
8	—	—	3	—	—	—	—
9	—	—	4	—	1	—	—
10	—	—	3	—	2	—	—
11	—	—	2	2	2	—	—
12	—	—	2	—	1	1	—
13	—	—	2	—	2	—	—
14	—	—	2	1	2	—	—
15	—	—	1	—	1	—	—
16	—	—	2	—	2	—	—
17	—	—	2	1	—	—	—
18	—	—	4	1	—	—	—
19	—	—	4	—	—	—	—
21	1	—	2	—	1	—	—
22	—	—	1	—	—	—	—
23	—	—	1	1	—	—	—
24	—	—	1	—	1	ND	—

—, No oligoclonal bands detected.

ND, CDR3 length spectratyping bands not detectable.

Table 2 Disease activity in SLE patients as assessed by SLAM, BILAG and blood activity markers

Test	Patient				
	A	B	C	D	E
SLAM	19	12	17	7	18
BILAG					
General	C	E	C	E	C
Mucocutaneous	E	B	E	D	E
CNS	E	E	E	D	E
Musculoskeletal	C	C	B	C	C
Cardiorespiratory	D	E	E	D	E
Vascular	C	C	C	C	E
Renal	E	E	E	E	E
Haematological	C	C	C	C	D
ESR (mm h <sup>-1</sup> ) [age in years/2]	11	3	15	5	42
CRP (mg l <sup>-1</sup> ) [ $<10$ ]	11	6	$<6$	$<6$	8
C3 (g l <sup>-1</sup> ) [0.63–1.19]	0.96	0.69	1.06	0.80	0.87
C4 (g l <sup>-1</sup> ) [0.11–0.43]	0.096	0.283	0.205	0.235	0.214
C3dg (units ml <sup>-1</sup> ) [5–12]	38	12	7	7	9
ds DNA antibody (IU ml <sup>-1</sup> ) [ $<30$ ]	56	5	6	14	75

ESR, erythrocyte sedimentation rate; CRP, C reactive protein; normal ranges given in square brackets.

antibodies, and anticardiolipin antibodies. At the time of the study she was only taking Distalgesic, and had increasing arthralgia, myalgia, and fatigue with thrombocytopenia. Moderate disease activity was confirmed by a SLAM score of 19 and complement activation with low C4 and raised C3dg. T cell receptor analysis by CDR3 length spectratyping showed oligoclonality in all 23 V $\beta$  families analysed, with multiple oligoclonal bands in 19 of these.

*Patient B* was aged 70 with a 10 year history of SLE characterised by arthritis, Raynaud's phenomenon, and mouth ulcers. Investigations had revealed lymphopenia, antinuclear antibodies, and anti-Ro antibodies. At the time of the study she was only taking hydroxychloroquine and Feldene, and had a maculopapular rash, lymphopenia, and myalgia. Clinical impression of mild disease activity was confirmed by a SLAM score of 12 and a score of B in the mucocutaneous BILAG category. T

cell receptor analysis by CDR3 length spectratyping showed seven oligoclonal bands in six of the 23 V $\beta$  families analysed. Such a spectratyping profile may be within normal limits.

*Patient C* was aged 46 with a history of SLE for under one year characterised by Raynaud's phenomenon, arthritis, and a photosensitive rash. Investigations had revealed lymphopenia, antinuclear antibodies, and anti-Ro antibodies. At the time of the study she was only taking Prempak C, but had frank arthritis, myalgia, and fatigue. Moderate disease activity resulted in a SLAM score of 17 and a score of B in the musculoskeletal BILAG category. T cell receptor analysis by CDR3 length spectratyping showed oligoclonality in 17 of the 23 V $\beta$  families analysed, there being multiple oligoclonal bands within individual families in eight cases.

*Patient D* was aged 51 with a 17 year history of SLE characterised by a deforming arthritis, pleurisy, Raynaud's phenomenon, mouth ulcers, epilepsy, and a photosensitive rash. Investigations had revealed lymphopenia, neutropenia, antinuclear antibodies, anti-ds DNA antibodies, anti-Ro antibodies, and anti-RNP antibodies. At the time of the study she was taking hydroxychloroquine, dothiepin, and phenobarbitone. Her disease was clinically inactive and this was supported by a SLAM score of 7 and normal levels of all serological disease markers. T cell receptor analysis by CDR3 length spectratyping showed single oligoclonal bands in only three of the 23 V $\beta$  families analysed.

*Patient E* was aged 46 with a three year history of SLE characterised by arthritis, alopecia, mouth ulcers, livedo reticularis, and discoid and photosensitive rashes. Investigations had revealed lymphopenia, antinuclear antibodies, anti-ds DNA antibodies, and anti-La antibodies. At the time of the study she was taking hydroxychloroquine, prothiaden, and coproxamol, and had myalgia, arthralgia, fatigue, and discoid lesions. Moderate disease activity was registered by a SLAM score of 18 and a raised erythrocyte sedimentation rate. T cell receptor analysis by CDR3 length spectratyping showed a single oligoclonal band in only one of the 23 V $\beta$  families analysed.

## Conclusions

Published attempts to show T cell oligoclonality in SLE through the T cell receptor have up to now been largely inconclusive. RFLP studies have shown polymorphisms in the  $\alpha$  chain region<sup>26</sup> and C $\beta$  region,<sup>29</sup> the latter being associated with anti-Ro antibodies.<sup>30–31</sup> Other studies, however, have failed to show C $\beta$  polymorphisms specific to lupus.<sup>32</sup> Studies have also shown T cell oligoclonality in lymph nodes of patients but not peripheral blood<sup>33</sup> and increased usage of V $\alpha$ 8 in potentially pathogenic helper T cells.<sup>34</sup>

Our results show detectable but minor T cell population expansions in normal individuals which agree with data previously published.<sup>18–19</sup> Such expansions in normal healthy individuals are usually of CD8-positive T cells<sup>20</sup> and may represent the response to past infections or vaccinations.

We have also shown marked T cell oligoclonality in the peripheral blood of some patients with SLE. In two cases of active lupus with SLAM scores of 17 and 19 (patients A and C) CDR3 length spectratyping showed oligoclonality in 23 and 17 of the 23 V $\beta$  families analysed respectively, while one inactive patient (D), one mildly active patient (B), and one other active patient (E) showed similar levels of oligoclonality to healthy controls, although there could be genuine polyclonal expansions present in the patients which this technique would not detect. It is of particular note that the patient with the most obvious oligoclonality affecting all 23 families analysed was sufficiently active to have a low C4 and markedly raised C3dg level.

Studies to address the association of oligoclonality with organ involvement, disease activity, and MHC type will need a larger study cohort than this preliminary study. Clearly, in a more comprehensive study, examination of both peripheral blood lymphocytes and T cells infiltrating tissue lesions would be important where possible as work with other autoimmune diseases indicates that T cell restrictions in lesions may be markedly different to the peripheral blood repertoire.<sup>35-36</sup> Such comparisons in SLE, where possible, may give insight into the relevance of the peripheral blood skewing seen in this study. Further analysis of each V $\beta$  family at the level of J region usage<sup>37</sup> may also help to define the composition of the spectratyping bands seen by the analysis performed here.

This skewing and expansion of T cells in the peripheral blood in lupus that we have documented may be a result of activation and expansion of disease relevant clones and lymphocyte trafficking. The fact that different V $\beta$  families showed oligoclonality in different patients may be a result of a combination of both public and private responses to the autoantigen as well as different MHC types and even different autoantigens.

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