

## Glomerular T cells in Heymann nephritis

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### SUMMARY

Active Heymann nephritis (HN) is a rat model of human membranous nephropathy. The appearance of T cells within the glomeruli of HN rats suggests a role for these cells in the pathogenesis of the disease. The aims of this study were to investigate T cells infiltrating the glomerulus in HN in Lewis rats by polymerase chain reaction (PCR) of their V $\beta$  chains, CDR3 spectratyping and sequencing. HN was induced in Lewis rats by immunization with renal tubular antigen (Fx1A) in CFA. Kidneys were collected between 4 and 10 weeks. The glomeruli were separated, homogenized and RNA extracted. RT-PCR, CDR3 spectratyping and sequencing were used to further characterize the infiltrating T cells. Multiple V $\beta$  families showed restriction of their CDR3 spectratypes in each animal. Several TCR V $\beta$  families had identical-sized restricted spectratypes across several different animals. Four V $\beta$  families were sequenced. In three of those four families, the dominant clones showed identical sized CDR3 regions and a striking over-expression of J $\beta$ 2.6. Further analysis of the CDR3 regions of the J $\beta$ 2.6 clones showed a significant restriction of the amino acids at four of the six CDR3 positions. Glomerular T cells bearing similar CDR3 sequences, using J $\beta$ 2.6 and expressing at least two, and possibly more, V $\beta$  genes are involved in the pathogenesis of HN.

**Keywords** T-cell receptor T cell Heymann nephritis CDR3 spectratyping

### INTRODUCTION

Active Heymann nephritis (HN) is a rat model of human membranous nephropathy associated with immune deposits in glomeruli, and infiltration of the glomeruli and interstitium by mononuclear cells [1]. The autoantigens thought to be involved in HN are expressed in the glomeruli and have been extensively characterized [2,3]. The appearance of T cells in the glomeruli at 8 weeks, coincident with the development of proteinuria, implies a role for glomerular T cells in the pathogenesis of this disease model [4].

Further evidence for the part played by T cells in HN derives from blocking experiments. Treatment with monoclonal antibodies to CD4 prior to immunization has been shown to completely prevent proteinuria in this model [5], whereas the use of anti-CD8 antibodies abrogates proteinuria. However, the monoclonal antibodies will interfere with T-cell function in the glomeruli, the interstitium and the periphery, so the observation does not provide unequivocal evidence that glomerular T cells in HN recognize and proliferate in response to specific autoantigens.

We have previously described the T-cell receptor (TCR) repertoire in the renal interstitium of HN rats [6]. The aims of this

study were to characterize T cells infiltrating the glomeruli in HN in Lewis rats by polymerase chain reaction (PCR) of their V $\beta$  chains, CDR3 spectratyping and sequencing.

### MATERIALS AND METHODS

#### *Induction of Heymann nephritis*

Extract of renal tubular antigen (Fx1A) was prepared as described previously [7].

Seven-week-old inbred male Lewis rats were obtained from the Animal Resources Centre in Perth, Western Australia. One week after arrival, they were immunized subcutaneously into each of their hind footpads with 15 mg Fx1A emulsified with complete Freund's adjuvant (CFA) containing 1 mg *Mycobacterium tuberculosis* HRA37 (Difco, Detroit, MI, USA), 100 ml incomplete Freund's adjuvant (IFA) and 100 ml phosphate-buffered saline (PBS). Booster injections of 7.5 mg Fx1A in IFA were given subcutaneously at 2 weeks. Control animals were immunized with the appropriate emulsion prepared without Fx1A.

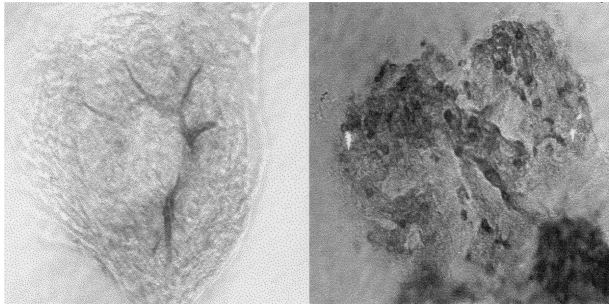
Animals were killed at 4, 6, 8 and 10 weeks under halothane anaesthesia. Both kidneys were perfused with saline and removed. Samples were placed in OCT, liquid nitrogen and PBS.

#### *Isolation of glomeruli*

Glomeruli were isolated as previously described [4,8]. Cortical slices were pressed through a 250  $\mu$ m stainless steel sieve. The

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**Fig. 1.** Immunoperoxidase staining using monoclonal anti CD4 antibody followed by an HRP-labelled goat anti-mouse IgG antibody. HN glomerulus (right) shows a heavy infiltrate of cells. Control glomerulus shown on the left. Magnification  $\times 400$ .

filtrate was then washed through a  $150\ \mu\text{m}$  sieve and the glomeruli collected on a  $75\ \mu\text{m}$  sieve. They were then rinsed in PBS and purified further by gravity sedimentation. Microscopic examination confirmed purity of over 90% compared with non-glomerular fragments. Glomeruli were processed for RNA extraction and immunoperoxidase staining.

#### Immunoperoxidase staining

Isolated glomeruli were enzymatically permeabilized for immunoperoxidase staining by incubation at  $37^\circ\text{C}$  with a solution of  $0.1\ \text{mg/ml}$  Collagenase D and  $10\ \text{mg/ml}$  Soybean Trypsin inhibitor in PBS. The slides were then washed in PBS for 15 min and stained with monoclonal antibodies, followed by HRP-labelled goat anti-mouse IgG (Pharmingen, San Diego, CA, USA). The monoclonal antibodies used were R73 for the  $\alpha\beta$  TCR, W3/25 for  $\text{CD4}^+$ , OX-8 for  $\text{CD8}^+$  (Serotec, Oxford, UK) and OX-12 for IgG (Zymed Laboratories, CA, USA).

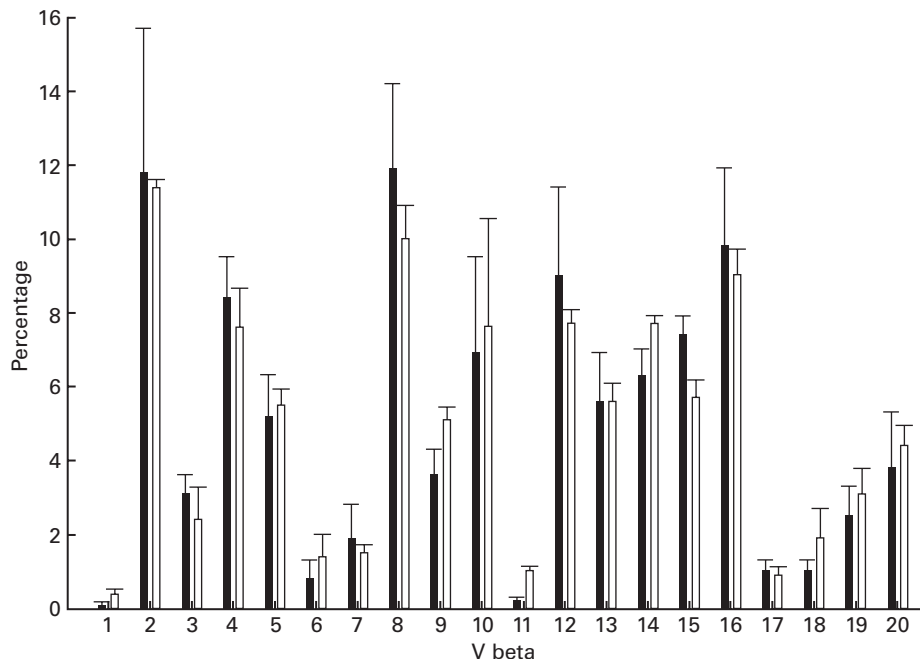
#### Extraction of RNA and reverse transcription

Total RNA was extracted from isolated glomeruli using a modification of the method of Chomczynski and Saachi [9]. Samples of glomeruli were dissociated in RNazol B (Cinna/Biotech, Houston, TX, USA). RNA was then extracted following the standard protocol. The final product was then air dried, dissolved in DEPC-treated water and stored at  $-80^\circ\text{C}$ . First strand complementary DNA was synthesized using the M-MLV Reverse Transcription kit (GIBCO BRL, Invitrogen Corporation, Carlsbad, CA, USA). A  $1\ \mu\text{g}$  aliquot of RNA and random hexamer primer was used to prime the reaction.

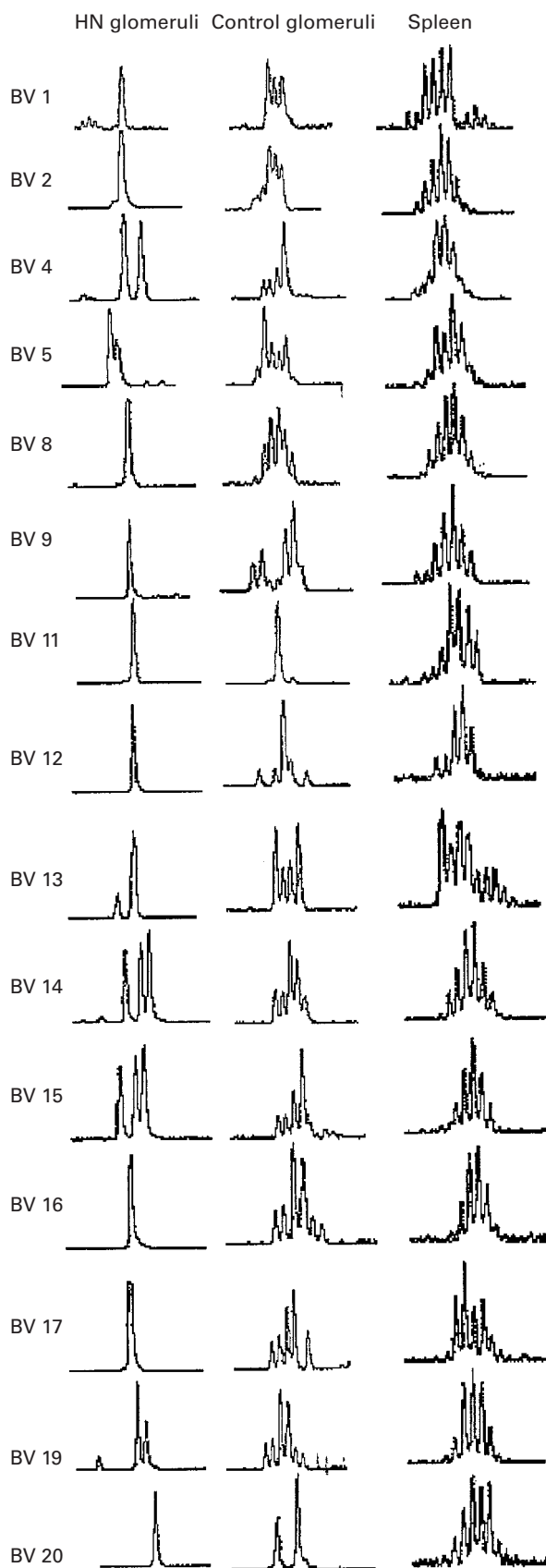
Primers for rat TCR  $\text{V}\beta$  genes were published previously [10]. Amplification of the house-keeping gene GAPDH was used as a positive control for intact RNA and efficiency of RT. PCR amplification was performed using a thermal cycler (Perkin Elmer 9600, Applied Biosystems, Foster City, CA, USA). Products were then analysed on a 2% agarose gel. Standard curves of product signal were generated at different PCR cycle numbers in triplicate, and subsequent  $\text{V}\beta$  repertoires were run at appropriate cycles to ensure no over-amplification of product. The specificity of PCR products was confirmed using QPCR, and individual  $\text{V}\beta$  gene expression was expressed as a percentage of total TCR signal.

#### Detection of PCR products by QPCR System 5000

The specificity of each PCR product was verified by separate hybridization with a tris (2,2-bipyridine) ruthenium (II) chelate (TBR)-labelled, sequence-specific, oligonucleotide probe directed at a segment internal to the amplified PCR segment as previously described [11–13]. The electrochemiluminescent signal of the hybridized probe was detected with a QPCR 5000 system (Perkin Elmer) according to manufacturer's recommendations. The relative luminosity of each  $\text{V}\beta$  family member was expressed as a percentage of the total luminosity in all of the  $\text{V}\beta$  regions for a given sample.



**Fig. 2.** Expression of  $\text{V}\beta$  TCR genes at 4 weeks after induction of HN shows no significant difference with expression in Lewis rat spleen. (■), HN glomeruli; (□), spleen.



#### CDR3 spectratyping of PCR products

A 2  $\mu$ l volume of PCR product from each V $\beta$  family was then used as cDNA for a second round of PCR. Primers were as before with the addition a Fam-labelled C $\beta$  reverse primer internal to the initial C $\beta$  biotin primer. This proceeded for six to 10 cycles. PCR product (1  $\mu$ l) from this reaction was sent to the University of New South Wales sequencing facility and run on a Perkin Elmer ABI Prism 373 Sequencer. The results were analysed using Genescan and Genotyper software (Applied Biosystems).

CDR3 spectratyping is a well described method used as a measure of oligoclonality of T cells. PCR products are run on a high resolution sequencing gel along with size standards. The length of a particular product can be determined to one or two nucleotides. A normal splenic sample of a single V $\beta$  family gives a Gaussian distribution of 6–11 peaks each separated by three nucleotides. Oligoclonal T cells give fewer peaks in a restricted distribution. Single clones give a single peak. This method can be used to screen PCR products efficiently for the possible presence of T-cell clones of similar CDR3 size across different animals, without the need to sequence all the different products. Sequencing is used later to confirm spectratyping results.

#### Cloning and sequencing of PCR products

PCR products of interest were purified using a Lifetech Concert PCR purification kit (Life Technologies, Invitrogen Corporation) and cloned into the p-Bluescript SK-T vector. Plasmid DNA was recovered using the Lifetech Concert Miniprep kit (Life Technologie, Invitrogen Corporation). Sequencing was performed by the Sydney University Prince Alfred Macromolecular Analysis Centre using the dye-labelled dideoxy chain determination method with the PE Biosystems ABI Prism 377 Sequencer (Applied Biosystems).

## RESULTS

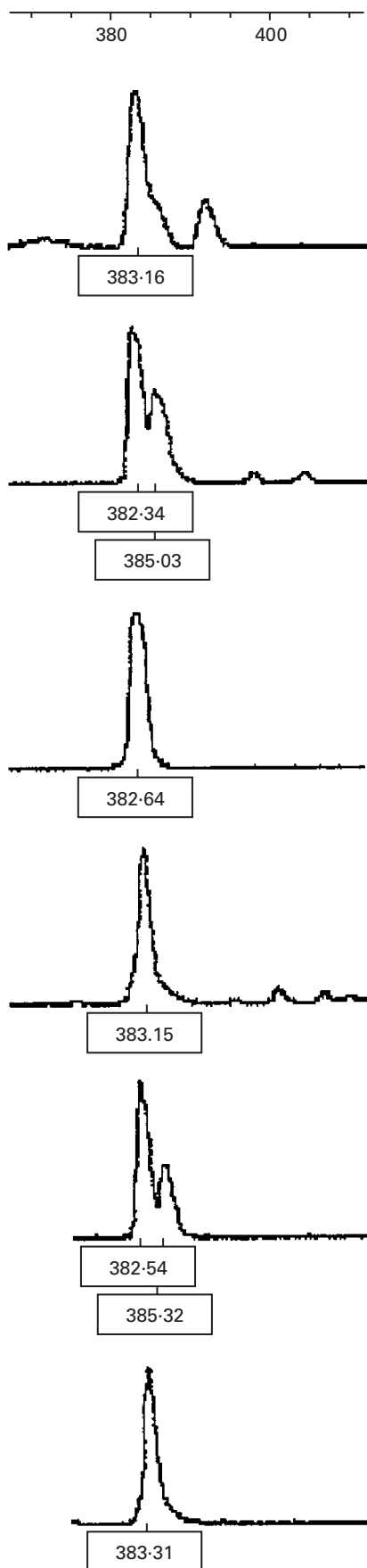
#### Development of Heymann nephritis

All of the rats immunized with Fx1A developed active HN. Proteinuria developed at 8 weeks and increased to 10 weeks in test animals. Control animals did not develop proteinuria. Test animals developed typical changes of active HN, with deposition of IgG along glomerular capillary loops and mononuclear cell infiltration of the renal interstitium. Approximately 30 000 glomeruli were obtained from each animal with a purity of over 90%. Staining with anti-CD4 and anti-CD8 antibodies revealed the presence of T cells within the glomeruli as shown in Fig. 1.

#### No V $\beta$ TCR family was significantly over-expressed

Various individual animals displayed over-representation of single V $\beta$  families, but no single TCR family was over-expressed in multiple animals at any stage of the disease. In Fig. 2, the relative expression of TCR V $\beta$  families at 4 weeks reveals an apparently polyclonal T-cell infiltrate.

**Fig. 3.** CDR3 spectratypes from a single animal with HN compared with those of a single control animal and spleen. The HN glomeruli showed marked restriction of multiple V $\beta$  families. Control glomeruli also showed some restriction in their spectratypes but to a lesser degree than found in HN.

8/8 **CASSLAGDGNVLYFGEGSRLLVV**1/9 **CASSLAGDGNVLYFGEGSRLLVV**8/9 **CASSLSGGDYEQYFGPGTKLTVL**1/6 **CASSLAGDGNVLYFGEGSRLLVV**5/6 **CASSHRERQNTLFFGAGTRLSVL**6/6 **CASSPGTGDQAQYFGEGTRLSVL**9/9 **CASSLDLFSYEQYFGPGTKLTVL**2/2 **CASSLGTDSYEQYFGPGTKLTVL**2/2 **CASSLDRGNTEVFFGKGTRRLTVV**

## Splenic sequences

1/5 **CASSLPTGDEQYFGPGTKLTVL**1/5 **CASSQQGRETQYFGPGTRLLVL**1/5 **CASSQPGQTEVFFGKGTRRLTVV**1/5 **CASSLGSNTEVFFGKGTRRLTVV**1/5 **CASSLTPIGYDYTFPGTRLLVI**

**Fig. 5.** V $\beta$ 5 sequences from seven individual HN animals compared with splenic sequences. The public response is in bold.

*Multiple restricted CDR3 spectratypes present in each animal*  
Spectratyping of glomerular PCR products showed widespread restriction of the normal Gaussian distribution of CDR3 sizes. Across the 20 different V $\beta$  families of the rat, there was an average of 11 abnormal peaks, each within a restricted spectratype. An example of the spectratype results from a single animal are shown in Fig. 3.

*Several V $\beta$  TCR families have identical spectratypes in different animals*

Identical sized CDR3 spectratypes were found in several different animals for eight V $\beta$  families. In V $\beta$ 5, eight animals showed an abnormal peak in their CDR3 spectratype measuring 382/3 base pairs. Other common spectratypes were present in fewer animals (seven animals for V $\beta$ 7, six for V $\beta$ 8, 13, 14, 19 and 20, five for V $\beta$ 6). The spectratypes of interest from V $\beta$ 5, 7, 8 and 13, as the most commonly occurring families, were then sequenced. The spectratypes for V $\beta$ 5 are shown in Fig. 4 and the corresponding sequences of the correct length in Fig. 5.

**Fig. 4.** V $\beta$ 5 CDR3 spectratypes from six different HN rats show a common restricted size peak at 382/3 with little or no other signal present.

V beta	J beta	Frequency	Sequence	
5	2·6	8/9	97·05·01	<b>CASSLSGGD---YEQYFGPGTKLTVL</b>
7	2·6	7/7	100·07·06	<b>CASSLGGLS---YEQYFGPGTKLTVL</b>
5	2·6	9/9	106·05·02	<b>CASSLDLFS---YEQYFGPGTKLTVL</b>
7	2·6	6/6	107·07·01	<b>CASNAGQVS---YEQYFGPGTKLTVL</b>
7	2·6	8/8	114·07·08	<b>CASSFWGSS---YEQYFGPGTKLTVL</b>
7	2·6	3/8	112·07·04	<b>CASSTGGAS---YEQYFGPGTKLTVL</b>
13	2·6	4/6	116·13·13	<b>CASSLAGGA---YEQYFGPGTKLTVL</b>
5	2·6	2/11	113·05·17	<b>CASSLGTDS---YEQYFGPGTKLTVL</b>
13	2·5	3/20	83·13·06	CASSLELVQ---ETQYFGPGTRLLVL
13	2·4	5/9	113·13·03	CASRAGLAE---NTLFFGAGTRLSVL
5	2·4	5/9	87·05·44	CASSHRERQ---NTLFFGAGTRLSVL
13	2·3	4/9	113·13·04	CASSLQQGV---DKIYFGSGTRLTVL
13	2·3	4/18	89·13·04	CASSLRDMT---DKIYFGSGTRLTVL
13	2·3	2/20	83·13·33	CASSLAQVT---DKIYFGSRTRLTVL
7	2·2	4/9	109·07·05	CASRTGTGT---GQLYFGEGSKLTVL
13	2·1	3/6	101·13·05	CASSLPDRG---AEQFFGSGTRLTVL
7	1·6	9/9	99·07·01	CASSGYDYN---SPLYFAAGTRLTVL
5	1·5	6/7	105·05·15	CASSPGTGD---QAQYFGEGTRLSVL
13	1·5	3/20	89·13·24	CASSQTGEV---QAQYFGEGTRLSVL
13	1·3	6/14	115·13·02	CASSLDRSG---NVLVYFGEGSRLLVV
5	1·3	8/13	91·05·15	CASSLAGDG---NVLVYFGEGSRLLVV
5	1·1	2/3	88·05·15	CASSLDRGN---TEVFFGKGTRLTVV

Fig. 6. Sequences from T-cell clones isolated from HN glomeruli. Clones bearing Jβ2.6 are in bold.

*Small public response*

A small public response was demonstrated in that sequences from three animals were identical. The sequence Vβ5-LAGDG-Jβ1.3 was present as a strong clone (8 of 14) in animal 91. In two other animals, the identical sequence was present but in lower numbers (1 of 9). These sequences are shown in bold in Fig. 5.

*Clones of interest in Vβ5, 7 and 13 had identical CDR3 size*

Sequencing the PCR products of interest demonstrated oligoclonal expansion of T cells, which reflected the restriction in CDR3 length suggested by the spectratyping results; the more peaks

present in the spectratype, the broader the distribution of CDR3 lengths found on sequencing.

In TCR families Vβ5, 7 and 13, the sequences with CDR3 lengths in common across multiple animals were nine amino acids in length. CDR3 lengths are expressed as the number of amino acids from the final serine of the Vβ to the first phenylalanine of the Jβ (exclusive). These sequences, their clonality and Jβ are shown in Fig. 6.

*Over-expression of Jβ2.6 in glomerular clones*

Figure 6 shows that many of the HN clones expressed Jβ2.6. A

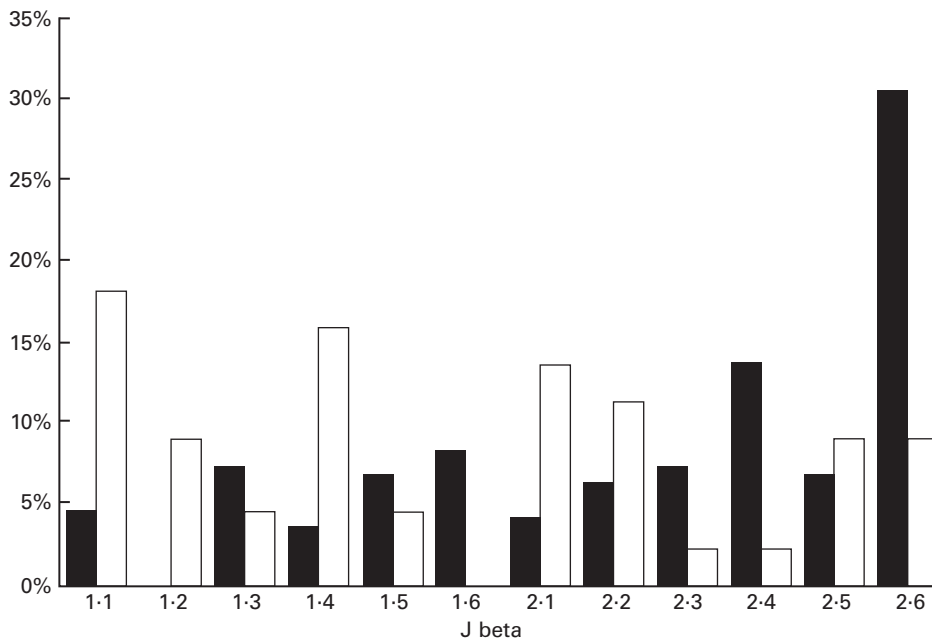
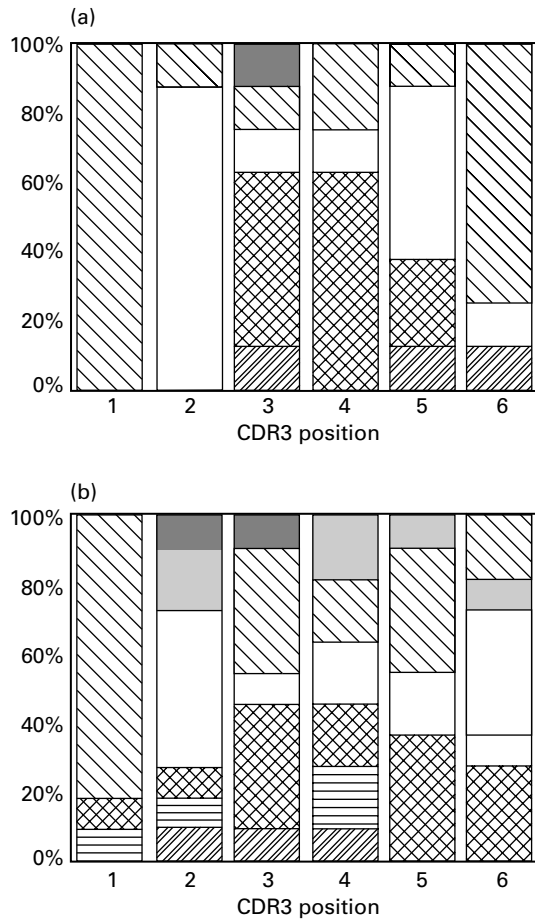


Fig. 7. Jβ repertoire of HN clones (■) show a striking over-expression of Jβ2.6 when compared with splenic sequences (□).



**Fig. 8.** Profiles of six CDR3 positions in HN clones (a) compared with the same positions in splenic sequences (b) of the same length. Amino acids are grouped according to their properties as A acidic (▨), B basic (▤), H hydrophobic (□), L polar (■), G glycine (▩), P proline (■), W tryptophan (▧) and Y tyrosine (▨). Positions 1, 2, 4 and 6 of the CDR3 profile of the HN clones shows clear restriction when compared with splenic sequences of the same length ( $P < 0.05$ ).

comparison with normal  $J\beta 2.6$  expression in Lewis rat spleen is shown in Fig. 7. There was a striking over-expression of  $J\beta 2.6$  in HN glomerular T-cell clones.

Reasoning that these clones may all be responding to the same antigen, we examined their CDR3 sequences in more detail using a profile technique first used by Candeias *et al.* [14]. Amino acid residues are grouped together according to their properties as A, acidic (aspartate, glutamate), B, Basic (lysine, arginine, histidine), H, Hydrophobic (leucine, isoleucine, phenylalanine, methionine, valine, alanine), L, Polar (glutamine, asparagine, threonine, serine), G glycine, P proline, W tryptophan, and Y tyrosine. Each position in the CDR3 sequences from the  $J\beta 2.6$  clones was classified and a profile generated. This profile was compared with that generated by analysis of splenic sequences of the same size. The result is shown in Fig. 8. The profile of the  $J\beta 2.6$  clones is markedly altered when compared with spleen. Positions 1, 2, 4 and 6 are more restricted in the HN clones, and this difference was significant on  $\chi^2$  analysis ( $P < 0.05$ ).

## DISCUSSION

Glomerular T cells are shown here to be present during the

development of HN as reported previously [4]. PCR shows a broad representation of  $V\beta$  TCR families with no specific family over-represented. Subsequent CDR3 spectratyping demonstrated the presence of multiple oligoclonones, many of which shared identical CDR3 sizes across different animals. Sequencing of a subset of these clones found multiple clones with the same sized CDR3 in several  $V\beta$  families expressing  $J\beta 2.6$ . HN clones strikingly over-expressed  $J\beta 2.6$  when compared with spleen. A small public response was demonstrated in three animals.

Further examination of the sequences of the  $J\beta 2.6$  HN clones showed a restricted CDR3 profile compared with splenic sequences of the same size, suggesting that this population of T cells may be stimulated by a small set of peptide:MHC complexes.

These data suggest that T cells responding to antigens in the glomerulus are involved in the pathogenesis of HN. The autoantigens described for HN include the large cell surface glycoprotein receptor, megalin, and a smaller receptor-associated protein (RAP). Megalin is a multiligand-binding endocytic receptor expressed in clathrin-coated pits at the surface of several epithelia, including the proximal tubule and the glomerulus. This antigen contains multiple epitopes linked to the development of HN [2]. We have demonstrated that T cells in HN rats recognize specific peptidic antigens in the RAP [9].

Our data demonstrate that multiple  $V\beta$  genes are involved in the pathogenesis of HN. There could be a number of reasons for this. First, the induction of HN involves reaction to a number of proteins, each of which may contain several T-cell epitopes. Each epitope may also stimulate more than one  $V\beta$  gene. Previous studies of HN have measured involvement of a particular  $V\beta$  gene by its over-expression in semi-quantitative PCR [6,11]. CDR3 spectratyping was a method reserved for only those genes found to be significantly stronger on PCR than others. This study takes a different approach, looking at all  $V\beta$  genes regardless of level of PCR signal, and finds good evidence of oligoclonality in T cells that would previously have been passed over. This suggests that the immune reaction in HN is more complex than previously thought, with multiple  $V\beta$  genes involved in clonal expansion. The presence of multiple epitopes means that the situation is difficult to interpret, and the likelihood of epitope spreading during the course of the disease further compounds the analysis. However, the breadth of the T-cell repertoire at 4 weeks suggests that this picture is due less to epitope spreading than to the extensive network of T-cell responses to the main Heymann antigens.

Studies of other diseases, such as experimental autoimmune encephalomyelitis (the mouse model of multiple sclerosis), have shown over-expression of a particular  $V\beta$  gene, which has subsequently been shown to contain pathogenic T-cell clones [15]. However, this has been difficult to demonstrate in human diseases. More often than not, several  $V\beta$  genes are found to be over-expressed in a disease within a single individual, and different genes are expressed in different patients. This has been shown to be the case for rheumatoid arthritis [16], psoriasis [17], Crohn's disease [18], pulmonary sarcoidosis [19], giant cell arteritis [20] and others. A study on giant cell arteritis even found differing  $V\beta$  expression in discrete inflammatory lesions in a single patient. If the  $V\beta$  gene is not always a critical point of contact with the peptide MHC complex, then the same immune reaction could be generated with different  $V\beta$  genes on the same genetic background or even within the same individual. Thus, in our model, identical animals have the potential to produce diverse  $V\beta$  repertoires from a single antigen.

The nature of the peptide autoantigen being presented may dictate whether single or multiple V $\beta$  genes are expressed in clonally-expanded T cells. Recent work on crystal structures of MHC peptide TCR complexes [21] shows V $\alpha$  to be an anchor to the MHC helices, J $\beta$  to occupy the V $\alpha$ /V $\beta$  interface and V $\beta$  to bind both to the MHC and to the peptide via its CDR3 region. Studies of the human TCR/Tax/HLA A2 complex, however, showed that the CDR3 $\beta$  pushes the TCR away from the MHC, resulting in fewer TCR V $\beta$  chain contacts with the MHC helices. This suggests that the important parts of the  $\beta$  chain are the J $\beta$ , at the V $\alpha$ /V $\beta$  interface, and the CDR3 region, since the rest of the molecule appears to have little contact with the stimulating complex. It is therefore entirely possible to generate the same immune response using different V $\beta$  genes but the same J $\beta$  and similar CDR3 regions. In our model, we have demonstrated a subset of glomerular T cells which shares similar CDR3 properties, uses J $\beta$ 2.6 and expresses different V $\beta$  genes. We speculate that they are all responding to the same antigen.

### CONCLUSION

These data show that glomerular T cells using J $\beta$ 2.6 and a restricted set of CDR3 sequences in the context of at least two (but probably more) V $\beta$  genes are involved in the pathogenesis of HN in the Lewis rat. We intend to use these observations to create new TCR-specific interventions to modify the course of this disease.

### ACKNOWLEDGEMENTS

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