T CELL REPERTOIRES IN ANIMAL AND HUMAN GLOMERULONEPHRITIS

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by

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STATEMENT

The research contained in this thesis was performed in the Centre for Kidney Research, part of the Royal Alexandra Hospital for Children, Westmead, Sydney, Australia. Whilst in Australia I was supervised by Dr John Knight, the director of the Centre. My laboratory work was overseen by Dr Huiling Wu, who taught me all the laboratory techniques required for the work presented here.

All the work within this thesis is my own except where acknowledged within the text. Notably, chapters 5 (p. 71) and 6 (p.88) are collaborations with other researchers. Statements on each of these are included in each chapter. Chapters 3, 4, 5 and 6 are the respective publications, reproduced almost verbatim, listed on page iv.

I would like to acknowledge with gratitude the input of each of my supervisors within this project; Dr John Knight for his leadership, vision and wisdom in directing me and those around me; Dr Huiling Wu for her quite extraordinary patience in teaching me at every stage and for her example in hard work and dedication; Professor John Feehally for enabling my placement and for his continued support throughout. I regard it as a major privilege to have an association with each of these people.

ABSTRACT

This thesis aims to study the role of T cells in glomerulonephritis and to take a first step towards design of novel T cell based treatments of these conditions. Two animal models, Heymann nephritis (HN) and experimental autoimmune glomerulonephritis (EAG), are studied. Glomerular T cell receptor (TCR) repertoires from each of these models are analysed using polymerase chain reaction, CDR3 spectratyping and DNA sequencing of their CDR3 regions. In HN, a restricted set of T cells defined by their Vβ Jβ and CDR3 regions are identified as potentially pathogenic cells. In EAG, infiltrating T cells are shown to be clearly oligoclonal with restricted TCR repertoires in each animal. The repertoire restriction differs in each animal studied but the entire population carries multiple TCR CDR3 motifs which are absent in control cells.

The data from HN is utilised to design a DNA vaccination based upon the identified PCR products of infiltrating T cells. Vaccinated rats have significantly improved disease with reduced proteinuria, $CD8^+$ and macrophage infiltration and IFN-γ expression. The mechanism of action of DNA vaccination is explored, demonstrating specific anti-TCR antibodies in vaccinated rats. The antibodies appear to reduce specific T cell infiltration to the kidneys and to reduce IFN-γ expression.

Oligoclonal T cells are also identified in archival human renal biopsy tissue. Restricted TCR repertoires are variable but CDR3 spectratypes clearly show oligoclonality within multiple TCR Vβ families. The data suggests that the most frequently represented T cell mRNA species may account for a large proportion of total T cell receptor mRNA signal.

This thesis demonstrates that infiltrating oligoclonal T cells can be identified in renal tissue and that specific anti T cell therapy may have an important place in future developments.

PUBLICATIONS

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Walters G, Alexander SI. T cell receptor BV repertoires using real time PCR: a comparison of SYBR green and a dual-labelled HuTrec™ fluorescent probe. *J Immunol Methods.* 2004;294:43-52.

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Chapter 1. Introduction and Literature Review.

1.1 αβ T cell receptor

1.1.1. Introduction

The αβ T cell receptor (TCR) is a heterodimer unique to the T cell. Displayed on the cell surface, it mediates antigen recognition by binding to major histocompatibility complex (MHC) molecules complexed with self or foreign peptides on the surface of other cells in the body. The TCR has a highly variable structure to enable a huge diversity of antigens to be bound and thus recognised by the acquired immune system. Each T cell has a single TCR sequence which does not change after maturation of the cell. Thus a single cell has a limited range of peptide:MHC complexes that it is able to bind. On cell division, a T cell produces progeny with an identical receptor. Thus a clone of T cells can easily be identified by their TCR since, due to the enormous variability of the receptor, it is extremely unlikely that two identical T cell clones could exist. T cells have no other way of recognising antigen. The TCR therefore represents the cornerstone of the acquired immune response. Without a T cell response to antigen, even an effective antibody response is rarely possible since T cell help is required by B cells under most conditions.

1.1.2. The nomenclature and organisation of αβ TCR genes

1.1.2.1. Rat TCR Vβ genes

Rat TCR Vβ gene sequences were identified using anchored PCR and named by comparison with the previously defined mouse TCR Vβ genes (1). There are 17 single member and 3 multimember families.

1.1.2.2. Human TCR Vβ genes

Multiple nomenclatures of human αβ TCR genes have been published over a number of

years. The nomenclature used throughout this work for human Vβ TCR genes is described by the ImMunoGeneTics (IMGT) database (2). Genes are named according to their position on the chromosome and are designated BV rather than Vβ. Members of subfamilies have greater than 75% identity. The TCR BV locus is found on chromosome 7 and consists of 52 functional V gene segments, one D gene segment, up to seven J gene segments and a single C gene (3).

Where work from other authors is quoted, TCR are referred to by the names given by the authors of that work. Many workers continue to use previous nomenclatures labelled Vβ, there are therefore mixed names in the text. Though clearly unsatisfactory, this reflects the current situation in the literature. In my own work, I have utilised the IMGT names for human work only since they are not yet available for the rat.

1.1.3. The rearrangement and diversity of αβ TCR genes

Each TCR Vβ chain is made up of single V, D, J and C regions arranged in series. Germline sequences are retained with important exceptions at the VD and DJ junctions. Each developing T cell undergoes rearrangement of its germline genes to select a combination of V, D, J and C genes. Whilst developing in the thymus, each T cell alters its germline DNA to approximate a V region to a D and J region, generating a functional VDJβ exon. This exon is subsequently spliced onto a Cβ region once transcribed as mRNA.

Alterations in the DNA sequence during recombination of the gene segments generates a highly diverse VDJ region which is otherwise known as the Complementarity Determining Region 3 (CDR3). This region corresponds to the antigen binding domain of the TCR.

Diversity in the TCR is generated by several mechanisms (4). First, somatic recombination as described above combines different gene segments in varying combinations in different T cell progenitors. This mechanism is complemented by alterations to germline DNA sequences at the junctions of gene segments. Several processes take place. P (or palindromic)

nucleotides are added to gene segments via the action of RAG-1 (Recombination Activation Gene 1) enzyme. N nucleotides are non-template encoded and added at random to the end of the segment by terminal deoxynucleotidyl transferase (TdT). Nucleotides are also removed by as yet unknown exonucleases. The final CDR3 region has a significantly different DNA sequence to the original set of gene segments that make it up. This process generates an enormous repertoire of unique TCRs. Each individual developing T cell utilises a single TCR. All progeny from a T cell carries the identical TCR. A clone of T cells can therefore be identified by a shared TCR. Because of the enormous repertoire generated by this process of combinatorial and junctional diversity, it is highly unlikely that two T cells clones could develop with an identical TCR.

1.1.4. The structure of the αβ TCR

The αβ TCR is made up of an α and β chain folded together into the final structure. It is highly similar to the Fab portion of an antibody molecule with the α and β chains corresponding with the light and heavy chains respectively. The chains are folded together such that the CDR3 regions of each chain approximate to form an antigen binding domain which has been shown to align with the peptide binding groove of MHC molecules (5). The receptor is displayed on the surface of the T cell. A transmembrane domain in the constant region of each chain anchors the receptor to the cell and a disulphide bond between the constant regions links the two chains. The variable regions lie outermost on the receptor and come into contact with MHC peptide complexes.

There are several important differences between immunoglobulin (Ig) and TCR structure. TCR do not have effector functions in themselves. They serve only as antigen detection systems. Effector functions are defined by the co-receptors expressed alongside the TCR like the CD4 and CD8 molecules. TCR are designed to pass signals to the T cell not to promote any particular effector reaction. Thus a TCR may be used to promote or suppress

inflammation depending on the T cell upon which it is expressed.

1.2 Antigen recognition by the TCR

Unlike Ig, TCR do not recognise native antigen in isolation. Instead peptide fragments are presented to the TCR bound to molecules of the MHC or major histocompatibility complex.

1.2.1. The major histocompatibility complex

The major histocompatibility complex (MHC) is an area of the genome first described (and named) because of its association with rejection of transplanted tissue. MHC molecules are cell surface glycoproteins encoded by MHC genes. There are two main classes, class I and II. Class I present antigens to T cells bearing the CD8 coreceptor, class II to those bearing the CD4 coreceptor. These coreceptors bind to the MHC molecule alongside the TCR and to a some extent dictate the effector function of the T cell. Without the coreceptors, binding of a TCR would be ineffective. These coreceptor molecules bind to invariant regions of their respective MHC molecules leaving the top surface free for simultaneous TCR interaction. Binding of CD4 and CD8 increases the intracellular signalling of the associated TCR via alterations in their cytoplasmic tails. This increases the sensitivity of the receptor by at least 100 fold. Less antigen is therefore required to stimulate the T cell.

1.2.2. The recognition of antigen by the αβ TCR

The available crystal evidence is limited but suggests that TCR bind to MHC molecules in a similar fashion regardless of antigen specificity with the Vα domain approximating with the class I MHC α2 helix and the Vβ domain with the MHC α1 helix. The position occupied by the Vα domain is currently thought to be invariant overlying the N-terminus of the bound peptide and the α2 domain of the MHC. The binding of the Vβ domain is more variable suggesting that the Vα chain is mainly responsible for anchoring the TCR in place over the MHC.

The CDR3 α and β regions are brought into close proximity to the centre of the peptide

binding groove of the respective MHC molecule and appear to recognise critical residues of the peptide.

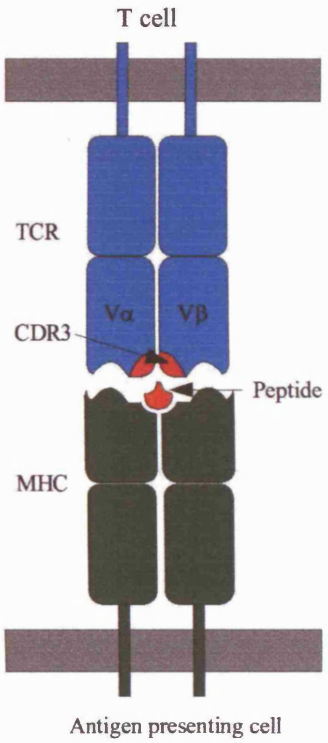


Figure 1.1 TCR binding to a peptide MHC complex. The CDR3 regions bind to peptide and adjacent areas of MHC. The variable chains bind the helices of the MHC chains.

1.3 TCR repertoire studies in animal and human diseases.

TCR repertoire studies are based on the premise that T cells infiltrating a diseased tissue represent a subset of all T cells that can be defined by their TCR expression, usually by the V gene segments of their β chain. Many studies have confirmed this finding showing restricted expression of Vβ genes in the mRNA extracted from tissue and demonstrating clonal expansion of potentially pathogenic T cell clones.

1.3.1. Techniques for analysis of TCR repertoires and oligoclonality

1.3.1.1. Monoclonal antibodies

Monoclonal antibodies raised against specific Vβ proteins has been extensively utilised to

analyse the repertoire of T cells in several situations (6) (7). Antibodies bind to the TCR in a Vβ specific manner. These are subsequently labelled with a fluorescent-labelled second antibody and analysed by fluorescence activated cell sorting (FACS). The readout provides a measure of the number of T cells bearing a specific Vβ gene in the sample tested.

There are several disadvantages to this method. First, there has been a limited number of monoclonal antibodies available such that the analyses have been unable to reflect the entire TCR repertoire, for example, Kotzin (7) used only five available antibodies. As such the analyses are incomplete and may have missed important alterations. Second, the use of this method necessitates obtaining T cells that can be physically isolated from surrounding tissue such that they can be mixed in solution with the monoclonal antibodies and then analysed by FACS. This is clearly difficult when working with diseased tissue samples where extraction of lymphocytes is a time-consuming and inefficient process.

Monoclonal antibodies can also be used in histological study of tissue. This is not, however, part of TCR repertoire analysis but rather simple detection of a particular Vβ gene expression within the specimen.

1.3.1.2. Anchor polymerase chain reaction (PCR)

Anchor PCR involves the isolation of mRNA from tissue and conversion to cDNA. This is followed by ligation of a known sequence tag (the anchor) on the 5' end of all cDNA. PCR is then performed using a primer for the anchor sequence and a standard primer for the Cβ gene. Under these conditions, all Vβ genes are amplified with equal efficiency. The results are then thought to reflect relative quantities of Vβ gene expression than standard semiquantitative PCR (8). Another advantage of this method is the amplification of all Vβ genes irrespective of whether their sequence has already been defined. It has therefore been used to detect all expressed alleles in an animal as a definitive study (9).

1.3.1.3. Northern blot

Northern blotting is now, except under exceptional circumstances, an outmoded method of RNA analysis involving the isolation of RNA from a sample and electrophoresis on a formaldehyde agarose gel. RNA is then transferred onto nylon membranes and crosslinked. The membrane must then be probed with radiolabelled Vβ specific probes. The membranes are exposed to x-ray films and the results assessed by densitometry (10). It is very time consuming and presents multiple opportunities for error.

1.3.1.4. Semiquantitative PCR

Total or mRNA is extracted and reverse transcribed to cDNA. The Vβ repertoire is then assessed with multiple PCR reactions each primed with a Cβ and single Vβ primers. The resulting PCR products are electrophoresed usually on an agarose gel to demonstrate the size and relative quantity of the specific product in each reaction. Further analysis may then be carried out either on the gel using southern blotting, or on the PCR product remaining. Preliminary reactions with two or more Vβ primers must be first carried out to assess the optimum number of cycles that are required to interrupt the reaction whilst all PCR products are detectable and likely to be undergoing exponential amplification. This compromise can difficult to carry out. Standard PCR requires all reactions to stop at a particular cycle. Some may have no detectable product whilst others are overamplified having reached the plateau phase of amplification. Multiple problems need to be overcome to ensure results are meaningful.

1.3.1.5. Real time PCR

Real time PCR is a relatively recent development and has transformed PCR to make it more rapid with a larger dynamic range. The PCR reaction now contains not only two primers but also one or two fluorescent-labelled probes which provide a real time readout of the level of product at each cycle of a reaction. It has therefore become possible to accurately compare

samples of very different signal strength in the same experiment whilst being certain of the level of amplification. In experiments used in this thesis, a single dual-labelled DNA probe is utilised. The method is described in detail in chapter 3 (p.37). At each PCR cycle, primers bind to the DNA template and promote elongation of the DNA. On one of the strands, the probe is also bound. As the Taq polymerase passes along the template, the fluorescent labelled probe is broken down into its constituent parts releasing the two fluorescent molecules. Whilst the probe is intact the fluorescence from its FAM moiety is quenched by the molecule at the opposite end of the probe. When the probe is destroyed, the FAM is released into solution free to emit its fluorescent signal. The number of fluorescing molecules, and therefore the level of fluorescence, is a direct measure of the formation of PCR product.

1.3.1.6. CDR3 spectratyping

CDR3 spectratyping involves a second PCR reaction. Specific Vβ PCR product is fluorescent labelled using a labelled primer for 6 to 10 cycles. The product is then run on a high resolution sequencing gel. This demonstrates small differences in the length of the products in that Vβ family. If a diverse population of TCR is present in the test sample, there will be a Gaussian distribution of cDNA lengths in the PCR product. If an oligoclonal or monoclonal TCR is present, one or two cDNA lengths may be demonstrated. This is a rapid method for screening the diversity of TCR in a particular Vβ family.

1.3.2. T cells and TCR repertoires in animal models

An enormous literature exists on this subject. I have therefore confined myself to a discussion of a very well known autoimmune disease, experimental autoimmune encephalitis, which has been extensively studied followed by the literature on the diseases and models of the work contained in this thesis. In the human section, the parallel human disease to EAE, multiple sclerosis, is also summarised.

1.3.2.1. Experimental Autoimmune Encephalomyelitis (EAE)

EAE is a T cell mediated disease generated in mice and rats characterised clinically by an acute or relapsing, remitting paralysis and histologically by perivascular lymphocytic infiltration and demyelination (11). It bears some similarities to the human demyelinating disease multiple sclerosis and is cited as an animal model for its investigation (12). Acute EAE is generated by injection of the hind foot pads of Lewis rats with an emulsion of guinea pig or rat myelin basic protein (MBP) with complete Freund's adjuvant (13). Over the subsequent two weeks, rats develop a symmetrical ascending paralysis whose clinical stages are divided into four. Stage 1, floppy tail; stage 2, mild paraparesis; stage 3, severe paraparesis; stage 4, tetraparesis or moribund. If the animals survive, week three begins a total recovery. Chronic relapsing EAE is generated using injections of spinal cord homogenate rather than MBP co-administered with intraperitoneal injections of Cyclosporin A (12). A histologically similar disease occurs but in a relapsing remitting form.

The transfer of $CD4^+$ T cells reactive to MBP was shown to transfer disease to naïve rats (14). Several groups identified T cells responding to an immunodominant epitope of MBP as responsible for the majority of disease in this model in rats. T cells utilising Vβ 8.2 responding to an epitope within the region of amino acids 68-89 was identified as the encephalitogenic epitope (15) (16).

Analysis of pathogenic T cells was first performed on *in vitro* manipulated T cells in the form of T cell clones, lines and hybridomas. Burns et al (15) generated a series of T cell clones and hybridomas from the lymph nodes of MBP-immunised Lewis rats. These were tested against Vα and Vβ probes generated from known encephalitogenic $CD4^+$ T cell hybridomas. All encephalitogenic clones bore the same Vβ sequence which was subsequently named Vβ 8.2. Vα usage involved Vα 2 in 7 of 10 encephalitogenic clones. This finding was confirmed and extended to show similarities in the CDR3 sequences of T

cells reactive to MBP 68-88 (17). All MBP reactive T cell clones utilised Vβ 8.2. Twelve of 15 sequences bore closely related CDR3 regions with aspartic acid followed by serine as the first two amino acids.

Studies of *in vivo* T cells mostly confirmed the *in vitro* experiments. Infiltrating T cells studied using monoclonal antibodies to Vβ 8.2, 8.5 and 10 showed a predominance of Vβ 8.2 cells throughout the course of chronic relapsing EAE. Vβ 8.5 and 10 were present more during relapses than remissions (12). Subsequent CDR3 spectratyping demonstrated oligoclonal expansion of Vβ 8.2 at all stages of the relapsing disease and in the acute form. DNA sequencing showed CDR3 sequences identical across the two forms of EAE. Other Vβ families also appeared to be involved but were different in each disease. Oligoclonality was detected in Vβ 17 in acute disease and Vβ 12 in chronic relapsing EAE. Vβ 12 expansions could only be demonstrated during disease relapse whereas Vβ 8.2 expansions persisted throughout the disease.

Other T cell studies have focused on cells responding to secondary epitopes on the MBP molecule and other proteins thought to have a potential role in pathogenicity. Encephalitogenic clones specific for proteolipid protein (PLP), and bearing Vβ 2, transferred to a naïve Lewis rat caused a form of chronic relapsing EAE. The clonal cells were predominant throughout the acute disease but appeared to play a minor role in subsequent relapses (18). Vβ 10 cells may be specific for the secondary epitope MBP 44-67 and are encephalitogenic. Depletion of T cell lines of both Vβ 8.2 and 10 still leaves an encephalitogenic population of cells which use mainly Vβ 3 and Vα 2 (19).

1.3.2.2. Heymann Nephritis

Active Heymann Nephritis (HN) is a rat model of human membranous nephropathy generated by injection of susceptible strains of rats, such as Lewis or Fisher, with crude rat renal tubular antigen (RTA/Fx1A) emulsified in complete Freund's adjuvant (CFA) (20).

Antibodies to Fx1A bind to autoantigens expressed on glomerular epithelial cells (GEC). Subepithelial deposits of IgG form and result in activation of complement producing severe glomerular injury, proteinuria and renal failure (21) (22). Proteinuria develops in 80% of rats within 8 to 10 weeks of immunisation (23).

Histologically there are similarities between HN and human membranous nephropathy with subepithelial electron dense deposits detectable on electron microscopy. Interstitial infiltration by mononuclear cells including αβ TCR^+ $CD4^+$ and $CD8^+$ populations (24) occurs early in the disease. Monoclonal antibody studies have demonstrated glomerular infiltration by macrophages, $CD4^+$ and $CD8^+$ cells with associated mRNA expression of IFN-γ, granzyme A, perforin and TNFα. Production of anti-Fx1A antibodies is dependent on $CD4^+$ T cells (25). Blocking experiments using anti-CD4 antibodies showed a marked reduction in immunoglobulin deposition, proteinuria and mononuclear cell infiltration (24). The use of anti-CD8 monoclonal antibodies was also shown to be effective in the prevention of active HN. This method depleted $CD8^+$ cells temporarily and reduced proteinuria and mononuclear cell infiltration. This strongly suggested that the role of T cells is not confined to help for antibody production but may involve the direct mediation of glomerular or interstitial injury.

The appearance of glomerular T cells was shown to be correlated with the development of proteinuria by Penny et al (26). In their experiments, immunoperoxidase staining with monoclonal antibodies was used to demonstrate the influx of mononuclear cells into the glomeruli at 8 weeks. This was associated with the development of proteinuria which was not correlated with the deposition of immunoglobulin, as might have been expected. Ig deposition was present at 4 and 8 weeks. Cells infiltrating the glomeruli were $CD4^+$ Th1 T cells, $CD8^+$ T cells and macrophages. This was confirmed using RT-PCR to detect cytokines associated with each cell type. $CD4^+$ Th2 cells could not be demonstrated in the absence of mRNA expression of IL-4, IL-5 or IL-6. A previous needle aspiration study had shown the

presence of mainly $CD8^+$ T cells (27). The authors had suggested linkage between the presence of T cells and tubulointersitial lesions.

T cell receptor gene usage in active HN is addressed by a single study and showed preferential expression of Vβ 2 and Vβ 16 families in the mRNA extracted from whole kidney tissue (28). This could only be detected at the 8 week stage of pathogenesis. Later in the course of the disease the relative expression of Vβ families returned to normal. It was suggested that these cells infiltrating the kidney early in the disease represented antigen specific T cells which were responsible for the interstitial damage and which later gave way to a more severe, non-specific infiltrate. The non-specific nature of the subsequent infiltrate may explain the difficulty in identifying preferential expression of Vβ families at that stage. CDR3 spectratyping was performed on overexpressed Vβ families and showed conserved usage of particular regions and the over-representation of arginine residues in the CDR3 region. The restricted Vβ gene usage and CDR3 regions were purported to indicate clonal expansion of antigen specific T cells within the kidney during the pathogenesis of HN.

1.3.2.3. Experimental Autoimmune Glomerulonephritis

Experimental autoimmune glomerulonephritis (EAG) is a model of human Goodpasture's disease. Initially described in 1962, it was induced initially in sheep by injection of heterologous GBM antigens in CFA. Subsequent experiments demonstrated that EAG could be generated in susceptible strains of rat by a single injection of collagenase digested homologous or autologous GBM in CFA (29) (30) (31). It is characterised in the WKY rat by sustained anti-GBM antibody production, linear deposition of antibody on the GBM and severe focal necrotising glomerulonephritis with crescent formation.

T cell involvement in this model has been demonstrated by a reduction in severity of the disease with various therapeutic manoeuvres. Cyclosporin A was used both in preventive and therapeutic regimens (32). High dose treatment both prior to and at the time of

immunisation with GBM produced a significant reduction in circulating anti-GBM antibodies and almost complete abrogation of proteinuria. Treatment of established disease was also successful. Levels of anti-GBM antibodies were less markedly affected by treatment but, in animals treated from day 14 from immunisation, levels of proteinuria were virtually undetectable.

Monoclonal antibody therapy has supported these observations. Intraperitoneal injection of anti-CD4 monoclonal antibody (W3/25) was performed three times a week at 5 and 10 mg/kg doses for 2 and 4 weeks on induction of disease (33). This was highly effective if given for 4 weeks with only a single animal developing low levels of anti-GBM antibody. A further experiment explored the use of higher dose antibody (30 mg/kg) given three times in the first week only. Three of 5 animals developed a low titre of antibody in this group suggesting that profound depletion of $CD4^+$ T cells within the first week after immunisation. All animals in the treatment groups had negligible proteinuria and were protected from disease.

The use of anti-CD8 monoclonal antibody (OX8) showed similar efficacy (34). Rats were treated with 5 mg/kg doses injected intraperitoneally three times a week. Therapy began either at disease induction for 4 weeks (prevention) or from weeks 2 to 4 (treatment). The preventive therapy completed prevented proteinuria, fibrin deposition, crescent formation, glomerular and interstitial damage and the infiltration of mononuclear cells into the kidney. Treatment from week 2 was almost as effective. Some T cells and macrophages were detectable in the renal tissue and a small percentage of glomeruli had detectable abnormalities on histology. There were no glomerular crescents. Circulating antibody concentrations, however, were entirely unaffected by either treatment protocol, demonstrating a clear distinction between the functions of $CD4^+$ and $CD8^+$ cells in the pathogenesis of this disease.

Adoptive transfer of $CD4^+$ T cells from immunised animals primed naïve animals to produce an enhanced anti-GBM antibody response to subsequent immunisation, suggesting $CD4^+$ T cell involvement in antibody production but not directly in renal damage (35). More recently, it has been shown in a similar rat model of EAG that disease can be transferred using autoreactive $CD4^+$ T cells (36). This second model has some differences to that used by Reynolds et al. The α3 chain of type IV collagen (Col4α3NC1) is used as the antigen by Wu et al. This has been shown to be the main antigen responsible for disease in EAG (37) (38) (39) and T cells from GBM immunised animals have been shown to proliferate in response to Col4α3NC1. In spite of this similarity, Wu's model has some significant differences to EAG. Rats were immunised with 300 μg of Col4α3NC1 in a single hind foot pad and at the base of the tail. Disease generated does not involve the deposition of antibody in the glomeruli (40). The adoptive transfer of $CD4^+$ cells is also performed differently to Reynolds. Primed T cells are extracted from lymph nodes and stimulated *in vitro*. They are then transferred into pertussis primed rats rather than naïve rats used by Reynolds et al. Thus, both the disease and the transfer of $CD4^+$ cells are different. The experiments do, however, demonstrate that different roles played by $CD4^+$ cells in the pathogenesis of EAG may include help for antibody production or more direct involvement in renal damage. Other investigators have shown in a mouse model of EAG, also utilising Col4α3NC1 as the immunising antigen, that MHC class II genes are critical for the development of crescentic nephritis (41). By implication this suggests the involvement of $CD4^+$ cells since they bind to class II MHC. This model does generate circulating and deposited anti-GBM antibodies but passive transfer of these alone is not sufficient to generate disease. Also crescentic nephritis is dependent upon MHC genes and $CD4^+$ T cells rather than the presence of anti-GBM antibodies.

T cells are therefore critically important in the development of EAG. However, no studies of TCR repertoires had been carried out until the work presented in this thesis.

1.3.3. T cell involvement and TCR repertoires in human diseases

1.3.3.1. Multiple Sclerosis

Multiple Sclerosis (MS) is a neurological disease widely believed to represent an autoimmune reaction to myelin components. It is characterised by infiltration of T cells macrophages and B cells, demyelination and to some extent degradation of axons. Extensive study of T cell involvement has taken place based upon previous work on EAE in the rat, mouse and other animals. The remarkable findings in EAE began the search for similarly restricted Vβ TCR repertoires in MS in humans. The investigation has been hampered by the fact that the organ of interest is the CNS; biopsies are rarely taken from patients, therefore T cell studies have mainly focused on PBMC, cerebrospinal fluid (CSF) T cells and postmortem studies of brain tissue.

1.3.3.1.1 T cell studies in MS

Peripheral blood mononuclear cells were taken from MS patients and controls and stimulated with either whole myelin basic protein or various peptides from putative epitopes identified in EAE work. Responding T cells were then cloned and examined for Vβ TCR repertoire and other characteristics. Kotzin et al cultured T cells from 7 patients with MS with whole human myelin and found preferential use of Vβ 5.2 and 6.1. This was not reflected in alterations in the peripheral TCR repertoire (7). Other workers have shown that different Vβ genes are used in different patients, suggesting individual immunotherapy would have to be the way forward (42). In 1995, Richert et al examined 54 MBP specific clones from the blood of 15 MS patients and 3 normal controls. Multiple Vα and Vβ genes were used in conjunction with 23 $CDR3_{\alpha}$ and 30 $CDR3_{\beta}$ regions. Some CDR3 motifs were shared across T cells which also shared epitope specificity, others were detected in T cells of differing

epitope specificity (43). Hong et al stimulated patient PBMC with the presumed dominant epitope of MBP (MBP 83-99) and demonstrated an identical CDR3 motif in Vβ 13.1 cells across different individuals (44). A CDR3 probe was generated to detect the T cell clone in the PBMC of 15 out of 48 randomly selected MS patients and in 36 of 44 T cell lines. The majority of work has not shown shared motifs across different individuals but does strongly support the oligoclonality of T cells responding to MBP. Wucherpfennig found that the immune response to MBP was dominated by expanded clones in some patients but not others (45).

Another group examined T cells from peripheral blood responsive to IL-2 from MS patients and controls. Without IL-2, the numbers of T cells responsive to MBP was similar in controls and patients. With primary recombinant IL-2 stimulation, numbers increased in patients. CSF was also examined in MS patients where up to 7% of IL-2 responsive T cells were MBP reactive, a ten fold increase over results from peripheral blood (46). These cells recognised MBP peptides 84-102 and 143-168 and were $CD4^+$. A further study compared Vβ 5 and 17 expression in CSF and peripheral blood. Reduced diversity was found in both Vβ families but recurrent clonal expansion was found only in Vβ 17. T cell clones utilising Vβ 17 Jβ 1.6 were found with identical CDR3 length in 3 patients (47). Wilson et al examined CSF T cells and showed over representation of several TCR Vβ families including 2, 4, 5, 6.5 and 6.7. Two thirds of patients showed clear evidence of clonal dominance with over 50% of sequences from a single Vβ family originating from a single clone (48). This formed the basis of a later therapeutic trial of Vβ 6 CDR2 peptide immunisation.

Examination of brain tissue from MS patients has been performed by a number of groups and has produced a range of results showing both TCR restriction and diversity. Oksenberg published an initial study showing restricted usage of Vα genes with 2 to 4 genes used per patient. Jβ usage was also restricted on subsequent DNA sequencing of Vα 12 CDR3

sequences (49). Two years later, the same group studied TCR Vβ rearrangements in brain tissue and showed the presence of Vβ 5.2 TCR in lesions of patients with particular HLA genes. One of the CDR3 sequences detected was identical to a T cell clone previously identified reactive to MBP 89-106. It carried a CDR3 motif that had also been demonstrated in rat EAE lesions (50).

Another group, however, have failed to detect restriction in infiltrating T cells in the CNS. Wucherpfennig et al studied postmortem specimens from both acute and chronic MS plaques. Samples were small, taken from small frozen sections. They found diverse Vα and Vβ usage in all plaques (51).

1.3.3.1.2 T cell based therapies for MS

In spite of the lack of a clear consensus on the immunological reaction involved in MS there are multiple studies of T cell based interventions in its treatment. Some of these involve therapy based upon targeting the TCR of implicated T cells. Other treatments under study include oral tolerance with bovine myelin, infusion of synthetic MBP peptide regions 82-98 and 85-96, solubilized HLA DR2:MBP84-102, altered peptide ligand and glatiramer acetate combined with several other agents including natalizumab, IFNβ-1b albuterol and mitoxantrone.

The use of a monoclonal antibody against Vβ 5.2 was a logical extension of the studies by Oksenberg, above. Monthly injections of a humanized monoclonal antibody to 47 relapsing-remitting MS patients reduced target cell numbers effectively but failed to demonstrate any significant clinical benefit (52).

In 1997, Gold et al reported the results of vaccination with a Vβ 6 CDR2 peptide. 10 patients were vaccinated with low (100μg; n=5) or high (300μg; n=5) doses with incomplete Freund's adjuvant (IFA). The study had to be terminated due to the development of delayed type hypersensitivity (DTH) reactions in 3 of the 10 patients. The results did however show a

reduction in CSF cellularity in those treated with high dose peptide along with a reduction in growth of CSF cells in expansion culture *in vitro* and reduced levels of Vβ 6 in these cultures (53). A further study using BV6S2 and BV6S5 peptides reported the vaccine was safe and well tolerated, resulting in cell-mediated immunity to the peptide in 8 of 10 patients (54). No efficacy results were reported. Further results from Vandenbark et al suggest that they have found TCR CDR2 peptides to be safe and immunogenic in over 170 MS patients but with highly variable efficacy (55). They propose that peptide vaccination generates Th2 cells which act to regulate the activity of autoimmune $CD4^+$ Th1 cells.

Vaccination with autologous $CD4^+$ T cells activated with a combination of MBP with or without Myelin Oligodendrocyte Glycoprotein (MOG) has been investigated as an alternative to TCR directed therapies. These studies have all been reported as phase I trials. Medaer and colleagues vaccinated patients with MBP reactive T cell and showed an improvement in 5 of 8 patients with a reduced number of relapses. Brain lesion size increased by 8% in vaccinated patients as compared with a 39.5% increase in control patients (56). Reactive T cells were reduced in vaccinated patients but reappeared in 5 patients. Revaccination abolished these T cells again (57). Extension of this study to 49 MS patients suggested that vaccination was safe and produced a complex cellular reaction (58).

Zhang et al injected irradiated autologous $CD4^+$ T cells into 28 relapsing-remitting (RR) MS patients and 26 secondary progressive MS patients. They documented a 40% reduction in relapse rate compared to the same patients in the 2 years prior to the study. There was no control group. MRI lesions got no worse and there was no change in the patients' EDSS (59).

Achiron reported this year on the treatment with T cell vaccination of 20 RR MS patients who had not responded to other forms of treatment. T cells were activated with both MBP and MOG peptides prior to injection. Relapse rate was cut from 2.6 to 1.1 per year and disability stabilised. There was no significant reduction in the number and volume of MRI

detected CNS lesions (60).

A further encouraging study used vaccination with $CD4^+$ T cells taken from CSF and stimulated in recombinant IL-2 (61). Only 5 MS patients were included (4 RR, 1 chronic progressive) but none suffered a relapse during the study. EDSS scores improved during the study. The $CD4^+$ T cells infused showed reactivity to multiple myelin epitopes. A placebo controlled trial is under way. This study overcomes some of the problems with others. Several of the studies above stimulate autologous cells with either whole MBP or particular peptides of MBP or MOG. In contrast this study merely expands activated cells found in the CSF of each patient. The method therefore does not prejudge the epitopes used by each patient.

There have clearly been significant difficulties in exploring the basis of T cell involvement in MS. After the encouraging initial results from EAE, transfer of techniques used in rodents to humans has been fraught with problems. Humans are an outbred population carrying a huge diversity of HLA genes and therefore generating a large array of T cells and subsequent immune reactions. However some of the data has shown clear indications that MS has an immunological basis with evidence of clonal expansions of T cells. These T cell populations are highly likely to be different in each individual even if particular Vβ genes may take a major role in some patients. It is clearly early days for the use of T cell directed therapy in MS but the above data does suggest that there are significant possibilities for its use.

1.3.3.2. Goodpasture's disease and renal vasculitis in humans

1.3.3.2.1 Goodpasture's disease

Human Goodpasture's disease is characterised by rapidly progressive glomerulonephritis often accompanied by pulmonary haemorrhage and associated with the presence of autoantibodies directed against the glomerular basement membrane (GBM). These

antibodies have been shown to recognise epitopes on the non-collagenous domain of the alpha 3 chain of type IV collagen (α3(IV) NC1) (62) (63) (64). Passive transfer studies have demonstrated the pathogenicity of these antibodies (65). The severity of nephritis has also been correlated with the level of circulating antibodies (66). The disease has therefore been considered an antibody-mediated disease.

There have, however, been several studies that have looked at the role of cell mediated immunity in general (67) (68) and T cells in particular in glomerulonephritis. In a mouse model of anti-GBM glomerulonephritis, Tipping et al showed that $CD4^+$ cells were crucial for development of crescentic nephritis. Absence of $CD8^+$ cells made this disease model worse (69). In 1983, analysis of T cell subsets in patients suffering from Goodpasture's disease showed a persistent deficiency of $CD8^+$ suppressor cells during active disease (70). Subsequently, T cells taken from patients with Goodpasture's disease showed significant responses to affinity-purified human GBM *in vitro* (71). There was no response from control T cells from normal patients. In 1996, autoreactive T cells from two patients with Goodpasture's disease were found to recognise the N-terminal part of the NC1 domain of α3 type IV collagen (64). The three T cell clones in this experiment were all $CD8^+$. Only one of the clones was characterised further. It was HLA class I restricted (HLA A11) and expressed T cell receptor Vβ 5.1 chain.

Recent experiments in the mouse have described a new model of Goodpasture's syndrome utilising the same antigen as that involved in the human. In these experiments, several strains of inbred mice were injected with α3(IV) NC1 collagen subcutaneously. All mice developed autoantibodies detectable in serum and bound to the GBM. However development of crescentic nephritis was dependent on MHC class II genes and the presence of T cells (41).

Human Goodpasture's disease has also been shown to be dependent on MHC class II genes with up to 80% of patients inheriting an HLA-DR2 haplotype. Further studies have

suggested a hierarchy of association of DRB1 alleles, some inducing protection, others susceptibility (72).

The evidence therefore suggests that T cells are involved in Goodpasture's disease and that there are a limited number of epitopes triggering the T cell reaction. This would suggest the possibility that, early in the disease at least, there may an oligoclonal T cell response. This is what we would hope to detect in studying human renal biopsy material from patients with this disease.

1.3.3.2.2 Pauci-immune vasculitis

Pauci-immune vasculitis is a collection of diseases the most well known of which are Wegener's Granulomatosis and Microscopic Polyangiitis. These are characterised by inflammation of small and medium sized blood vessels. Wegener's Granulomatosis also includes granulomatous inflammation of the upper and lower respiratory tract. Both diseases characteristically affect the kidneys with a crescentic glomerulonephritis, infiltration of mononuclear cells and a notable absence of immune deposits in the affected glomeruli (73).

Serum antibodies against neutrophil cytoplasmic antigens have been described since the early 1980s and their use as markers for diagnosis and disease activity is well established (74) (75). Antibodies have been detected against various constituents of the neutrophil intracellular granules the most well known of which are proteinase 3 (PR3) and myeloperoxidase (MPO). Anti-proteinase 3 antibodies are 99% specific in patients with Wegener's Granulomatosis (76) though occasionally present in other disorders. ANCA directed against proteins such as myeloperoxidase, lysozyme, cathepsin G and others are far less specific and are found in a range of diseases, including microscopic polyangiitis. The epitope specificities of antibodies to PR3 have been described (77).

Much attention has been focused on the role of ANCA and their interaction with neutrophils and endothelial cells. ANCA have been shown to activate neutrophils causing degranulation

and release of reactive oxygen species. The effect is much greater in neutrophils primed by cytokines such as TNFα (78). This priming causes expression of antigen such as PR3 and MPO on the surface of the neutrophil (79). Levels of TNFα are increased in the serum of patients with Wegener's Granulomatosis.

Involvement of the endothelium follows the same lines. PR3 expression occurs in the cytoplasm of human endothelial cells with time dependent translocation to the membrane under the influence of cytokines TNFα and IFNγ. ANCA have been shown to bind to cytokine pre-treated endothelial cells in vitro but not to naïve cells (80). Cytokine primed neutrophils also bind to damaged endothelial cells in vitro and cause further damage (81).

The pathogenicity of ANCA has been supported by recent data in an animal model. Xiao and co-workers immunised MPO knockout mice with MPO in order to generate both anti-MPO ANCA and splenocytes. The transfer of splenocytes caused a severe necrotizing crescentic glomerulonephritis associated with other features of a systemic vasculitis including pulmonary haemorrhage. The cells transferred included both T and B cells. To investigate the pathogenicity of anti-MPO antibodies alone, these were transferred alone into Recombination Activating Gene 2 knockout ($RAG2^{-/-}$) mice. This was sufficient to cause a necrotizing crescentic glomerulonephritis albeit milder than that caused by the splenocytes (82).

In spite of the above work, the potential mechanism of action of ANCA is still unclear. Some attention has been focused on the fact that the absolute level of ANCA in a single patient does not necessarily reflect disease activity but a rise or fall in the antibody titre is often far more relevant. Thus two patients with the same total ANCA titre can have different disease activity. One particular study has found a significant correlation between the percentage of IgG3 subclass of total IgG ANCA and disease activity. A higher percentage of IgG3 ANCA was correlated with increased disease activity and relapse (83). The authors suggested that

the IgG3 subclass of c-ANCA was mainly responsible for binding and activation of neutrophils through the FcγII receptors. This subclass of Ig binds to these receptors approximately three times faster than IgG1 antibodies and much faster than IgG2 and 4. The same authors had previously shown that blockade of the FcγII receptor had inhibited neutrophil activation induced by ANCA (84). An earlier study had found only IgG1 and IgG4 antibodies (85).

1.3.3.2.3 Evidence for involvement of T cells

Though much work has focused on ANCA, neutrophils and endothelial cells, there is much evidence to suggest that T cells play an important role in vasculitic diseases. As already stated, renal biopsies of patients show no evidence of immunoglobulin deposition but have dense cellular infiltrates with predominant T cells (86) and CD4:CD8 ratios of 5:1 (87).

Studies of cytokines have also suggested widespread T cell activation. Serum IL-2 receptor levels are elevated in patients with generalised disease and, in general, correlate with disease activity (88) (89). T cells from peripheral blood of patients suffering from Wegener's Granulomatosis have high levels of HLA DR expression and increased secretion of IFNγ and TNFα (90).

The search for a T cell antigen logically started with proteinase 3 as the most common target for anti-neutrophil cytoplasmic antibodies. An early paper taking PBMC from vasculitis patients failed to show any T or B cell response to neutrophil cytoplasmic antigens (91). Subsequent papers showed T cells reactive to PR3 and MPO present in patients and, in much lower levels, in normal controls (92) (93) (94). The levels appear to be unrelated to clinical course or ANCA titre. One further study found that T cells from patients with Wegener's were more likely to react to unfractionated neutrophil azurophilic granule contents than to purified PR3 and suggested that possibly T cells are reacting to other proteins from the granules as well as PR3 (95).

1.3.3.2.4 T cell receptor repertoires

Various attempts have been made to show oligoclonal expansions of T cells in patients with different forms of vasculitis. Simpson et al (96) found increased expression of Vβ 2.1 in the peripheral blood of vasculitis patients, mainly those with microscopic polyangiitis. They also suggested smaller expansions of Vβ 3, 9, 13, 14 and 15 in various combinations of polyangiitis, Wegener's granulomatosis and non-specific vasculitis. Further study of the Vβ 2.1 expansion showed no evidence of oligoclonality, in fact more oligoclones were found in controls than patients. They raised the possibility of involvement of a superantigen causing polyclonal activation of Vβ 2.1 bearing cells. An alternative explanation was that the increased level of Vβ 2.1 could reflect a genetic predisposition to vasculitis. Normal patients have previously been shown to have a bimodal distribution of Vβ 2.1 expression with the suggestion that low Vβ 2 expression may be protective against superantigen induced disease (97)

Giscombe et al (98) studied T cell receptor V gene usage in vasculitis using monoclonal antibodies against several Vβ and Vα chains and found increased T cell expansions in patients over controls. No attempt was made to look at oligoclonality of these expansions. Over their whole population of patients the significant changes were a reduction in the expression of various Vβ chains on either $CD4^+$ or $CD8^+$ cells or both. The T cell expansions found were observed in longitudinal studies. Some were stable, some decreased in size, others increased. There was no correlation to disease activity.

More recently, Grunewald et al (99) have shown oligoclonal T cell expansions in the blood of vasculitis patients. They identified conserved β chain motifs in 4 unrelated vasculitis patients all carrying the HLA DRB*0401 allele. These expansions were Vβ8 $CD4^+$. This suggests strongly that T cells from these patients have encountered an identical antigen presented on the same HLA DR molecule stimulating Vβ8 $CD4^+$ cells with the same CDR3

motif.

All of the above studies have looked for changes in Vβ TCR repertoire in peripheral blood in these diseases. Though this may on the face of it be logical, the significant background signal from normal PBMC makes it highly unlikely that significant results would be found. There are so far no studies of TCR repertoires of cells infiltrating renal tissue.

1.4 Hypothesis

The work in this study is based on the hypothesis that pathogenic antigen specific T cells can be identified by their TCR expression within renal tissue in immune mediated glomerulonephritis. The design of novel TCR based immunotherapy will be able to interrupt these cells without the side effects associated with broad immunosuppression.

Chapter 2. Materials and Methods.

2.1 Ethics approval

Ethics approval was obtained from the joint Animal Ethics committee of the Royal Alexandra Hospital and the Children's Medical Research Institute, Westmead, Australia.

2.2 Induction of Heymann Nephritis

Extract of renal tubular antigen (Fx1A) was prepared as described previously (100). Seven week old inbred male Lewis rats were obtained from the Animal Resources Centre in Perth, Western Australia. One week after arrival, they were immunised 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), 100 µl incomplete Freund's adjuvant (IFA) and 100 µl phosphate buffered saline (PBS) . Booster injections of 7.5 mg Fx1A in IFA were given subcutaneously at two weeks. Control animals were immunised 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.

2.3 Isolation of glomeruli

Glomeruli were isolated as previously described (101). Cortical slices were pressed through a 250 µm stainless steel sieve. The filtrate was then washed through a 150-micron sieve with phosphate buffered saline (PBS) at 4 °C and the glomeruli collected on a 75 µm sieve. They were rinsed in PBS. The tube was incubated at 4 °C for 10 minutes. The top 43 ml was removed and the tube filled with PBS again. After 30 seconds, the bottom 1 ml was removed by pipette. The tube was centrifuged at 150 x g for 7 minutes at 4 °C and the supernatant discarded. The resulting pellet was either resuspended in 2 ml PBS to be used for histology or counting or it was used for RNA extraction immediately. Microscopic examination confirmed purity of over 90% compared to non-glomerular fragments. Glomeruli were

processed for RNA extraction and immunoperoxidase staining.

2.4 Immunoperoxidase Staining

Isolated glomeruli were enzymatically permeabilised for immunoperoxidase staining by incubation at 37 °C with 100 µl 0.1 mg/ml Collagenase D and 10 mg/ml Soybean Trypsin inhibitor in PBS for 25 minutes. The slides were washed in PBS for 20 minutes to remove calcium ions and the enzymes. Monoclonal antibodies were applied to the prepared slides followed by HRP labelled goat anti-mouse IgG (Pharmingen, San Diego, CA). The monoclonal antibodies used were R73 for the αβ TCR, W3/25 for $CD4^+$, OX-8 for $CD8^+$ (Serotec, Oxford, UK) and OX-12 for IgG (Zymed Laboratories, CA). 50 µl of each antibody diluted 1:80 were used for 30 minutes at 37 °C. The secondary antibody was diluted 1:50 and applied for 30 minutes at 37 °C. The slides were developed using 50 µl diaminobenzidine solution consisting of: 3,3-Diaminobenzidine, Urea Hydrogen peroxide, Water 15 ml and 200 µl 18% sodium nitrite (NaN_3). Each slide was passed through 3 baths 70% ethanol, 3 baths 100% ethanol, 2 baths xylol for 30 seconds in each bath.

2.5 Extraction of RNA and reverse transcription

Total RNA was extracted from isolated glomeruli using a modification of the method of Chomczynski and Saachi (102). Samples of glomeruli were dissociated in RNAzol B (Cinna/Biotec, Houston, TX.). RNA was extracted following the standard protocol. Tissue was dissociated in 1 ml RNAzol B, vortexed with 100 µl chloroform and placed on ice for 10 minutes. After centrifuging for 15 minutes at 12,000 rpm at 4 °C, the upper aqueous phase was carefully removed and pipetted into a separate tube. An equal volume of isopropanol (approximately 0.5 ml) was added, the sample vortexed and incubated overnight at -20 °C. Samples were then centrifuged at 12,000 rpm for 15 minutes at 4 °C and the supernatant removed. The RNA pellet was washed with 1 ml 75% ethanol, vortexed and centrifuged again. The supernatant was removed. The final product was then air dried, dissolved in

DEPC treated water and stored at -80 °C.

2.6 First strand cDNA synthesis.

First strand complementary DNA was synthesised using the M-MLV Reverse Transcription kit (Gibco BRL). Approximately one microgram of total RNA was heated at 70 °C for 5 minutes in a 10 µl reaction containing 100 ng of random hexamer primers then placed on ice. This was then added to a final 20 µl reaction containing first strand synthesis buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$), 10 mM dithiothreitol, 1 mM of each deoxynucleotide triphosphate (dNTPs), 10U human placental ribonuclease inhibitor and 200U Moloney Murine Leukaemia virus reverse transcriptase and was incubated at 37 °C for one hour. The enzyme was then inactivated by heating at 95 °C for 5 minutes and the cDNA stored at -20 °C.

2.7 Rat TCR Vβ Polymerase Chain Reaction

Primers for rat TCR Vβ genes were published previously (103) and are shown in Table 2.2 (p.36). Amplification of the house keeping gene GAPDH was used as a positive control for intact RNA and efficiency of RT.

Master mixes were made up with PCR buffer and other components and aliquoted to tubes. Aliquots contained 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM dNTPs, 0.25 µM reverse antisense Cβ primer, 1.25U Taq DNA polymerase and water to produce a final volume of 25 µl. Control sample aliquots were removed first and cDNA added to the master solution and mixed thoroughly. After aliquoting to PCR tubes, Vβ primers were added to each reaction. PCR amplification was performed using a thermal cycler for a varying number of cycles (Perkin Elmer 9600). After 5 minutes denaturation at 94 °C, cycles consisted of 94 °C for 1 minute, 60 °C for 1 minute, and 72 °C for 1 minute. A final period of 10 minutes extension at 72 °C was proceeded by a 4 °C hold. Products were then analysed on a 2% agarose gel.

Standard curves of product signal were generated at different PCR cycles numbers in triplicate and subsequent Vβ repertoires were run at appropriate cycles to ensure no over-amplification of product. The specificity of PCR products was confirmed using QPCR and individual Vβ gene expression was expressed as a percentage of total TCR signal. For reactions requiring quantification with QPCR, the Cβ primer used was biotin labelled.

2.8 Human TCR Vβ PCR

Human TCR Vβ PCR was performed in a similar manner to the rat experiments. The primer and probes used are documented in Table 2.1 (p.35).

2.9 Detection of PCR products by QPCR System 5000

The specificity of each PCR product was verified by separate hybridisation 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 (104) (105) (106). Two microlitres of each amplified product was denatured in a solution consisting of PCR product, 3 µl water and 5 µl 0.3 M NaOH / 60 mM EDTA buffer at room temperature for at least 5 minutes. After heating to 70 °C for 5 minutes in a thermal cycler, the temperature was held while 40 µl TBR probe solution was added. This solution consisted of 5 µl 10x PCR buffer, 1 µl 10 µM TBR labelled probe and 34 µl water. After a 5 minute hold at 60 °C, the final volume was pipetted into a QPCR tube. Ten microlitres of streptavidin coated magnetic beads were added and the tubes were vortexed on the QPCR machine for 30 minutes.

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 Vβ family member was expressed as a percentage of the total luminosity in all of the Vβ regions for a given sample.

2.10 CDR3 Spectratyping of PCR products

2 µl of PCR product from each Vβ family was used as cDNA for a second round of PCR. Reactions were as before with the replacement of the Cβ primer by a Fam labelled Cβ reverse primer internal to the original Cβ primer. This proceeded for six to ten cycles. 1 µl of PCR product from this reaction was sent to 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.

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 Vβ 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 efficiently screen PCR products 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.

2.11 Cloning of PCR products

PCR products of interest were purified using a Lifetech Concert PCR purification kit (Life Technologies) or using ethanol or isopropanol precipitation. These were cloned into the p-Bluescript SK-T or pGEM-T vector (Promega). This was transformed into JM-109 or XL1 blue competent cells.

For ligation into the vector, 50 ng of PCR product was ligated to vector in a 20 µl reaction consisting of 50 ng vector, 1 µl T4 DNA ligase, 2 µl 10 x T4 DNA ligase buffer and water. Competent cells were thawed in an ice bath for 5 to 10 minutes and gently mixed. Five µl of ligation reaction was mixed with 50 µl competent cells in a sterile microcentrifuge tube on

ice for 20 minutes. The cells were heat shocked at 42 °C for 60 seconds in a water bath and quenched on ice for a further 2 minutes. 950 µl SOC medium (2.0 G Bacto-Tryptone, 0.5G Bacto-Yeast Extract, 1 ml 1M NaCl, 0.25 ml 1 M KCl, 1 ml 1M MgCl2:$MgSO_4$, 1 ml 2 M glucose and distilled water made up to 100 ml) were added to the transformation mixture. This was transferred to a 10 ml Falcon tube and incubated at 37 °C for 1 hour in a shaking incubator at approximately 150 rpm. The cells were pelleted by centrifugation at 1,000 x g for 30 seconds, 800 µl discarded and the pellet resuspended in the final 200 µl. The cultures were plated on ampicillin/X-gal/IPTG LB plates and incubated overnight at 37 °C.

Colonies were screened for PCR inserts. Individual white colonies were replated on Ampicillin plates and at the same time were added to a standard PCR mix using M13 forward and reverse primers. The PCR reaction ran for 30 cycles and was analysed on a 2% agarose gel. Clones with a PCR insert generated a PCR product in this reaction of just over 600 bp as opposed to those without which was approximately 200 bp.

PCR product of clones of interest was purified using ethanol or isopropanol precipitation. Two volumes of 100% ethanol were added to the PCR tubes, vortexed and stood at room temperature for at least 15 minutes. After centrifuging at 13,000 rpm for 15 minutes, the supernatant was discarded and 150 µl 75% ethanol added to wash the pellet. The tubes were vortexed and centrifuged again for 5 minutes. The supernatant was again discarded and the pellets allowed to air dry.

Sequencing was performed using the dye-labelled dideoxy chain termination method with PE Biosystems ABI Prism 377 or 310 Sequencer. 5 ng PCR product was mixed with 2 µl ABI Dye terminator Ready Reaction mix and an appropriate primer (either M13 forward or a specific Vβ primer) to 5 µl and added to 5 µl Ready Reaction buffer (ABI biosystems). The reaction underwent cycle sequencing using 96 °C for 10 seconds, 50 °C for 5 seconds and 60 °C for 4 minutes over 25 cycles. This was then purified using ethanol or isopropanol

precipitation as for PCR product above.

2.12 DNA vaccination

RNA was isolated from the HN kidney as described (107) and reverse transcribed into cDNA. cDNA was amplified by PCR with primers specific for either

Vβ5 (5'-AAGATGTCTGGGGTGGTCCAGTCT-3'),

Vβ7 (5'-AGC**ATG**TACTGGTATCGACAAGACCCAGATC-3')

or Vβ13 (5'-AGA**ATG**ACTGGAGTCACCCAGTCCC-3)

and a reverse Jβ 2.6 primer (5'-CG**TCA**TAAAACCGTGAGCTTGGTGC-3).

cDNA was also amplified with a Vβ8.2 primer as described (108) and a Cβ primer to generate a control vaccine. All forward primers were designed to include an in-frame ATG. PCR products were cloned into the pTarget plasmid DNA (Promega, Madison. WI) according to the manufacturer's instructions. Plasmid DNA from colonies with an insert were sequenced to confirmed the insertion of the correct gene with the ATG in frame. Large-scale preparation of plasmid DNA was performed using the Mega prep kit (QIAGEN, Hilden Germany).

For DNA vaccination, animals were pretreated with 0.75% bupivacaine (1μl/g bodyweight; Sigma) by injection it into the tibialis anterior muscle 1wk before the first vaccination. This is known to enhance the efficiency of DNA vaccination. 300 μg of DNA was injected into the same site three times at weekly intervals. Two weeks after the last vaccination, rats were challenged with Fx1A in CFA.

2.13 Autoantibody determination

Anti-Fx1A titres (total Ig) were determined by ELISA as described (24). Immulon 1 ELISA microtitre plates (Dynatech Laboratories) were coated overnight with 100 μl coating buffer containing 10-50 μg/ml solubilised Fx1A. The plates were washed twice with 0.05% Tween20 in PBS (PBST) and blocked with 2% BSA in PBS for 1 hour at room temperature

followed by two washes with PBST. 100 µl of a dilution series of known strongly positive serum and 1 in 20 dilution of test sera were added to triplicate samples. A 1 in 20 dilution of normal Lewis rat serum was used as the negative control. These plates were incubated for 1 hour at 37 °C. After the plates were washed 3 times with PBST, 100 µl of alkaline phosphatase conjugated goat anti-rat IgG (Zymed Laboratories, INC) or anti-rat IgG (Boehringer Mannheim) diluted 1:500 in PBS containing 2% BSA was added in each well and incubated for 1 hour at 37 °C. The plates were washed again 3 times with PBST and once with carbonate-bicarbonate buffer and 100 µl p-nitrophenyl phosphate (Sigma, St Louis, MO) in carbonate buffer was added and allowed to stand for 15-30 minutes. Triplicate sample ODs were read at 405nm on an ELISA reader (Dynatech Laboratories).

2.14 Urinary protein estimation

Twenty-four hour urine samples were collected in metabolic cages at 4, 6, 8 and 12 weeks post-Fx1A/CFA inoculation. Urine protein concentrations were determined by colorimetric assay according to manufacturer's recommendations (Biorad, Oakland, CA, USA). A 5 point standard of rat serum albumin (RSA) was prepared of a representative range of protein concentration. Twenty microlitres of each standard and sample solutions were pipetted into 1 ml of diluted dye reagent in a 1.5 ml tube. After vortexing, samples were incubated at room temperature for 5 minutes and absorbance read at 595 nm. All experimental samples were run in duplicate.

2.15 Renal biopsies and PBMC samples

Renal biopsies were obtained from a bank of biopsy tissue at the Department of Nephrology, Leicester General Hospital, UK. At the time of the initiation of this piece of work, ethics permission was not required to utilise tissue stored after histological examination in research work. Arrangements are now in place to obtain consent for use of renal biopsies both prospectively and retrospectively. Diagnoses were either Goodpasture's disease or idiopathic

rapidly progressive glomerulonephritis. Peripheral blood mononuclear cells were obtained by venesection of healthy volunteers followed by density gradient centrifugation. Serial dilutions of subsequent PBMC cDNA were performed for comparison with renal biopsy samples in order to control for dilution in the study of oligoclonality of the infiltrating T cells.

2.16 Real time PCR for CCβ and GAPDH

Limited real time PCR has been used in experiments in Chapter 7. All other material relating to real time PCR is documented in Chapter 3.

Primers and probes to detect Cβ and GAPDH using real time PCR were designed. The Cβ oligonucleotides were designed using Primer Express Software (Applied Biosystems, CA). Primers and probes for detection of GAPDH had been designed by Dr. Alex Bishop of the Centenary Institute, Sydney for use in humans, mice and rats. The sequences are shown in Table 3.1 (p.44). For real time PCR, 1 μl of cDNA was added to a reaction mixture consisting of 10x PCR buffer, dNTPs 0.2 mM, Taq polymerase 0.03 U/ml, Cβ and Vβ primers 0.3 mM and fluorescent probe 0.05 mM. The real time experiments were performed over 40 cycles using an ABI 7700 sequence detection system (Applied Biosystems, CA). Analysis of threshold cycle was performed on the ABI 7700 software. Samples were run in triplicate. Results (mean and standard deviation) are expressed for each Vβ family as percentage of the total Vβ signal.

IMGT Name	Sequence	Product
2	ATGAAATCTC AGAGAAGTCT	234-252
3	CACCTAAATC TCCAGACAAAGCT	194-212
4	CCTGAATGCC CCAACAGCTCTC	190-214
5	CTGATCAAAA CGAGAGGACA GCA	354-375
6	CTCTCCTGTG GGCARGTC	408-425
7	TCAGGTGTGA TCCAATTTC	329-347
9	GCACAACAGT TCCCTGACTTGCAC	195-207
10	GRCATGGGCT GAGGCTGAT	267-290
11	GGCTCAAAGG AGTAGACTCC	185-200
12	GGTGACAGAG ATGGGACAAG A	355-373
13	GATCAAAGAA AAGAGGGAAA C	358-370
14	AAAGAGTCTA AACAGGATGAGTCC	241-256
15	TACCCAGTTT GGAAAGC	353-368
16	CAGGTATGCC CAAGGAAAGA	226-241
18	AGCCCAATGA AAGGACACAGTCAT	325-337
19	TTTCAGAAAG GAGATATAGCT	226-241
20	TCATCAACCA TGCAAGCCTGACCT	195-207
24	AGTGTCTCTC GACAGGCACAG	193-208
25	TGTTCTCAAA CCATGGGCCATGAC	321-333
27	ACCCAAGATA CCTCATCACAG	368-383
28	GTCTCTAGAG AGAAGAAGGAGCGC	190-208
29	ACGATCCAGT GTCAAGTCGAT	334-346
30	CTCTGAGGTG CCCCAGAA	218-227

Table 2.1 Human BV primers used for standard PCR and approximate PCR product length.

TCR	Sequence
1	TCTAGTCATGGGAAGAGCAA
2	CCAAGGTGGCGTCTGGTACC
3	ACTCCACAGCATCTGCTCAG
4	CTGGTGGCAGGTCCAGTCGACCCTA
5	CTGGGGTGGTCCAGTCTCCAAG
6	GGACTTAGACTGATCTACTA
7	GACCCAGATCTGGGGCTACGG
8.2	ACACATGGAAGCTGCAGTCAC
8.5	GTTCAACTGTCACCAGACTG
8.6	ATTGAGCTGTCGCCAGACTA
9	GTTATGCAGAACCCAAGATAC
10	TCTAAGCAATTGCTGAAGGT
11	ACAAATGCTGGTGTCATCCA
12	AGGGCCACAATGATCTTTTC
13	CCACTCCCCCAGATATGCAA
14	GACTATCCATCAATGGCCAG
15	GGAGCTCTCGTCCACCAATC
16	GACCCAGTTTCTAATCACCT
17	GTAACCCAGACTCCAAGATAC
18	CTGTGTTCAGTGTCTTCCTC
19	GAACAGAAAGCCAGAATGCA
20	AGGGCAGCAAGTAGAGATGA
Cβ	TGTTTGTCTGCGATCTCTGC
Cβ probe	AGGTCTCCTTGTTTGAGCCA
C-Cβ for	ACCATGTGGAGCTGAGCTG
C-Cβ rev	CACTACCAGCGTACTGACAAGC
C-Cβ probe	ACAATCCTCGCAACCACTTC

Table 2.2 Sequences for the rat Vβ TCR primers and probes used in QPCR semi quantification.

Chapter 3. T cell receptor BV repertoires using real time PCR: a comparison of SYBR green and a dual-labelled HuTrec™ fluorescent probe.

3.1 Introduction

The real time quantification of PCR product has revolutionised both quantitative and semiquantitative PCR. Other more complex and time-consuming methods of quantification, such as manual construction of standard curves, southern blotting and QPCR (section 2.9, p.29) have been rendered obsolete. The method is generally performed in two hours or less and therefore dramatically reduces the time taken for these experiments which previously have taken several days. Two main systems exist for real time quantification: SYBR Green and fluorogenic probes. SYBR Green is a dsDNA-binding fluorescent green dye which binds to all dsDNA in a PCR reaction. Fluorogenic probes are fluorescent-labelled specific oligonucleotides added to a reaction. These probes either fluoresce on binding their specific target (single-labelled hybridisation probes) or when degraded by Taq Polymerase on amplification of the target (dual-labelled Taqman-style probes). The use of these procedures is now very well established in many areas of research and clinical practice where specific measurement of RNA is required, for example, cytokine measurements and viral load (109) (110).

We have studied TCR BV repertoires in glomerulonephritis for a number of years using the conventional form of the polymerase chain reaction (111) (112). Semiquantitation has been achieved using standard curves generated with multiple samples run at different PCR cycles. These samples have been quantified using QPCR, a process taking approximately two days to perform.

On moving to real time PCR, I have assessed the two available systems, SYBR Green and a HuTrec™ fluorogenic probe. PCR applied to low signal samples such as renal biopsies has been shown to produce non-specific product in many reactions. In the light of this, I wished to test whether the non-specific dye, SYBR Green, would be effective in these

measurements. The current literature on the real time assessment of TCR BV repertoires has mainly reported on the use of SYBR Green (113) (114) (115). The data presented are only a measure of fluorescence without visual confirmation by agarose gel of the PCR product. In the event that there is non-specific PCR product in the reactions, these results would probably be inaccurate. One group have published an elegant paper on the use of a dual-labelled fluorogenic probe for the assessment of TCR BV repertoires (116) but all of their assays were carried out on high signal samples in the form of PBMC or sorted T cells.

The study of renal biopsies and other small tissue samples with low levels of T cell infiltrate has presented particular problems in semiquantitative PCR. Very small quantities of RNA are often retrieved from these samples making the generation of valid standard curves difficult if not impossible and quantitation unreliable. Real time PCR offers the opportunity to obtain far more accurate information on alterations in the BV repertoires in diseased tissue specimens because of the minimal RNA quantities needed to establish a specific repertoire.

I have studied both SYBR Green and HuTrec™ fluorogenic probes in the assessment of BV repertoires in sequential dilutions of PBMC and in a low TCR signal cDNA to mimic the study of renal biopsies.

3.2 Methods

3.2.1. Generation of cDNA

Normal human peripheral blood mononuclear cells (PBMC) were obtained using density gradient centrifugation with Ficoll Paque™ PLUS (Pharmacia Biotech) from a single donor. Blood diluted in phosphate buffered saline (PBS) was layered onto Ficoll Paque™ and centrifuged at 300xg for 30 min. PBMCs were removed and RNA extracted immediately.

RNA extraction was performed using a modification of the method of Chomczynski and Saachi (117). PBMC were dissociated in RNAzol B (Cinna/Biotec, Houston, TX.). RNA was then extracted following the standard protocol. The final product was air dried, dissolved in

DEPC-treated water and stored at -80 °C. First strand complementary DNA was synthesised using the M-MLV Reverse Transcription kit (Gibco BRL). One microgram of RNA and random hexamer primer was used to prime the reaction.

cDNA with no TCR signal was prepared from a 293 cell line in culture. RNA extraction and reverse transcription were performed as above.

Multiple dilutions of PBMC cDNA in sterile water were then prepared at 1/10, 1/100, 1/1000 and 1/10000. These were designated PBMC 10, 100, 1000 and PBMC 10000. A single dilution of PBMC in 293 cell cDNA was prepared mixing 1 volume of 1/100 PBMC with 4 volumes of 293 cell cDNA and was denoted PBMC 500.

3.2.2. Design of real time PCR primers and probes

Primers and probes were designed for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), BC and individual BV genes. GAPDH primers and probe have been designed to work on human, mouse and rat genes. BC forward and reverse primers were designed to cross an intron to exclude the amplification of DNA. BV genes are amplified using a forward BV and a second reverse BC primer. Primer and probe design was performed using Primer Express software version 1.5 (Applied Biosystems, CA)

TCR nomenclature is consistent with the International Immunogenetics database (2) (http://imgt.cines.fr:8104/). A list of primers and probes used in this paper are provided in Table 3.1 (p.44).

3.2.3. Real Time PCR

All experiments were performed in triplicate on an ABI 7700 Sequence Detection System. Reagent concentrations were tested independently for both assay systems. Titrations of primer, probe and magnesium concentrations were performed. Final reaction conditions involved the amplification of 1-2 µl of cDNA in a 25 µl reaction consisting of 2.5 µl 10x PCR buffer containing 1.5 mM magnesium chloride (Roche), 2 µl 2.5 mM dNTPs (final

concentration 0.2 mM), 1.5 µl 5mM BC primer (final concentration 0.3 mM), 5 µl 1.5mM BV primer (final concentration 0.3 mM), 0.125 µl 5 U/ml Taq DNA Polymerase (Roche) and either 0.25 µl 5 mM dual labelled fluorescent probe (final concentration 0.05 mM) or 6.25 µl 0.1 X SYBR Green (final concentration 0.025 X). Reactions were made up to final volume with sterile water. Samples were all run in triplicate. PCR products were run on a 2% agarose gel to determine the presence of non-specific products.

3.2.4. Statistical Methods

Intrasample variation was assessed using the standard deviation of triplicate samples for the fluorogenic BC probe and SYBR green. Intersample reliability was assessed by comparison of matched repertoires using the Pearson correlation coefficient.

3.3 Results

3.3.1. HuTrec™ fluorogenic probes and SYBR green both show low intrasample variability.

Standard deviations measured on all BV families showed low intrasample variability for both methods. The fluorogenic probe showed errors ranging from 0.02 to 1.53% whilst SYBR green ranged from 0.01 to 3.31%. Standard deviations were calculated on a high signal sample (PBMC 10).

3.3.2. SYBR green and HuTrec™ fluorogenic probes give consistent results in GAPDH and BC assays on serial dilutions of peripheral blood mononuclear cells.

Serial dilutions of PBMCs were assayed for GAPDH and BC genes using both a specific BC dual labelled probe and SYBR Green. Both SYBR Green and the specific probe produced consistent results though the non-specific dye gave lower threshold cycles (Ct) showing earlier detection of product. Table 3.2 (p.45) shows threshold cycles for both SYBR green and specific probe. The difference between Ct of the two genes was constant over serial

dilutions of PBMC in water. Both assays therefore gave consistent results.

3.3.3. TCR BV repertoires measured with a HuTrec™ fluorescent probe.

BV repertoires were performed on each of the dilutions of PBMC used above except PBMC 10000. In order to mimic renal biopsy material, PBMC 100 was diluted 1/5 with cDNA with no TCR signal. This cDNA was prepared from 293 cell culture material and the final mixture was denoted PBMC 500.

Assays using the specific BC probe gave consistent repertoires across all dilutions of PBMC including PBMC 500. These results are shown in Figure 3.1 (p.46). Each series showed very similar results for each BV family. The exceptions were BV5 and BV9 but the differences involved were of the order of 1.5% and are therefore small.

Figure 3.2A (p.47) shows the same results plotted to show the degree of correlation between the results for PBMC 10 and the more dilute samples. A high correlation would be expected if the method were consistent regardless of signal strength and contaminating DNA. The fluorogenic probe showed a correlation of greater than 0.9 over two log dilutions. Additionally, this tight correlation was seen in samples containing contaminating non-TCR cDNA, the conditions that exist with T cell infiltration into tissues.

The results demonstrate that the use of a specific probe for real time PCR gives consistent results across at least 1000 fold dilutions of PBMC and that the level of contaminating non T cell cDNA is unimportant, making this procedure appropriate for the examination of small samples of tissue.

3.3.4. TCR repertoires measured with SYBR Green.

Figure 3.1B (p.46) shows the results from the repertoire experiments performed using SYBR green. Results of high signal, low dilutions of PBMC gave very similar results to the specific probe. However at lower signals, the repertoires showed major alterations. TCR BV families 4, 5, 6, 7-3, 10, 15, 16, 18, 20, 24, 27, 28 and 29 exhibited differences in either or

both the PBMC 1000 and 500 results. Figure 3.2B (p.47) illustrates the loss of correlation between repertoires of either increasingly dilute samples or samples containing contaminating cDNA. The Pearson correlation coefficient was as low as 0.79 with lower dilutions but was further reduced to 0.65 with contaminating cDNA.

3.3.5. Assessment of the cause of SYBR green error with small samples.

The reason for the loss of correlation with increasing dilution or contamination was likely to be due to either variations in primer efficiency or contamination with non-specific product. It is apparent on examination of the agarose gel results shown in Figure 3.3 (p.48) that contamination with non-specific PCR product was a major problem with the SYBR green system. PBMC 1000 and 500 showed increasing quantities of non-specific PCR product which was measured by the SYBR Green in the reaction. Thus as the T cell receptor signal decreased and was diluted by the presence of significant amounts of non-T cell cDNA, the accuracy of the SYBR green measurements reduced dramatically.

3.3.6. Comparing the TCR repertoires produced using the fluorogenic probe and SYBR green.

Repertoires measured with the two different assay systems are compared in Figure 3.4 (p.49). PBMC 10 exhibited similar repertoires in both systems. There were small differences that might be expected since SYBR green is known to affect the efficiency of some PCR reactions. Of note, the BV families which has the highest percentage expression under the HuTrec™ probe system appeared to be overrepresented in the SYBR green system. To some extent the converse applies in that BV families with a lower percentage expression under the fluorogenic probe had an even lower signal in the SYBR green system. When repertoires from PBMC 500 were compared, however, there were large differences. Since all these samples consisted of the same T cell receptor cDNA, they should all produce identical repertoires in an assay system. The matching results at higher concentrations of cDNA from

matched peripheral blood mononuclear cells suggests that at this concentration the SYBR green can adequately assess the repertoire. The loss of correlation at lower concentrations with non-TCR contaminating cDNA again demonstrated the loss of specificity of the SYBR green.

3.3.7. Assessment of the non-specific binding of SYBR green causing elevated results by real time PCR

During the work up of the SYBR green reactions, the non-specific nature of the dye was found to cause several inappropriate results. Figure 3.5 (p.50) shows the real time results and agarose gel of an experiment conducted to titrate the levels of SYBR green used. Three identical samples were run with a no template control. The agarose gel shows that specific product was only produced by two samples in lanes 1 and 3. These samples appear to give the same results on the real time analysis. Lane 2 gave the lowest Ct on the real time curves, while lanes 1, 3 and the non-template control (NTC) all rose together. These results show that non-specific PCR product is produced with the same kinetics as specific product and cannot be assessed simply on the basis of real time results. A separate method is required in the form of either a melting point analysis or some form of gel electrophoresis. Melting point analysis involves the collection of fluorescence data while the temperature of the PCR reaction is gradually raised from annealing temperature to melt all DNA products in the tube. Changes in fluorescence reflect the melting of specific and non-specific products and enable identification of tubes with non-specific products contaminating the reaction.

Melting point analysis would be helpful here in the case of the NTC but lane 3 could not be quantified adequately because there was both specific and non-specific product present on the gel. Other researchers have investigated the use of data collection at higher temperatures above the non-specific product melting point but results proved disappointing especially at low target signals (118).

Gene Name	Oligonucleotide Sequence
BV2	GAAATCTCAGAGAAGTCTGAAATATTCG
BV3	CCTAAATCTCCAGACAAAGCTCACT
BV4	CCTGAATGCCCCAACAGC
BV5	ACCTGATCAAAACGAGAGGACAG
BV6	CTCTCCTGTGGGCAGGTCC
BV7-2	GTTTTTAATTTACTTCCAAGGCAACA
BV7-3	CAAGGCACGGGTGCG
BV7-6	ACTTACTTCAATTATGAAGCCCAACA
BV7-7	GAGTCATGCAACCCTTTATTGGTAT
BV7-8	AGGGGCCAGAGTTTCTGACTTAT
BV7-9	CTCAACTAGAAAAATCAAGGCTGCT
BV9	AACAGTTCCCTGACTTGCACTCT
BV10	TTCTTCTATGTGGCCCTTTGTCT
BV11	GGCTCAAAGGAGTAGACTCCACTCT
BV12	GGTGACAGAGATGGGACAAGAAGT
BV13	CATCTGATCAAAGAAAAGAGGGAAAC
BV14	AGAGTCTAAACAGGATGAGTCCGGTAT
BV15	CCAGGTTACCCAGTTTGGAAAG
BV16	AAACAGGTATGCCCAAGGAAAGA
BV18	CAGCCCAATGAAAGGACACAGT
BV19	GGGCAAGGGCTGAGATTGAT
BV20	AACCATGCAAGCCTGACCTT
BV23	TGTACCCCCGAAAAAGGACATAC
BV24	CAGTGTCTCTCGACAGGCACA
BV25	CTCAAACCATGGGCCATGA
BV27	CCAGAACCCAAGATACCTCATCAC
BV28	GGCTACGGCTGATCTATTTCTCA
BV29	GACGATCCAGTGTCAAGTCGATAG
BV30	CTGAGGTGCCCCAGAATCTCT
BC for BV	CTGCTTCTGATGGCTCAAACA
BC for BV probe	6-FAM CACCCGAGGTCGCT MGB NFQ
BC forward	TCCAGTTCTACGGGCTCTCG
BC reverse	AGGATGGTGGCAGACAGGAC
BC probe	6-FAM ACGAGTGGACCCAGGATAGGGCCAA NFQ
GAPDH for	TGCACCACCAACTGCTTAGC
GAPDH rev	GGAAGGCCATGCCAGTGA
GAPDH probe	VIC CCTGGCCAAGGTCATCCATGACAACTT TAMRA

Table 3.1 Primer and Probe sequences for TCR BV repertoire, BC and GAPDH assays. Abbreviations: MGB Minor Groove Binding Protein. NFQ Non Fluorescent Quencher.

A. Specific Cβ Probe

	Cβ		GAPDH		Cβ GAPDH difference
	Ct	**SD**	**Ct**	**SD**	
PBMC 10	24.53	0.07	24.63	0.13	-0.1
PBMC 100	27.66	0.15	27.74	0.1	-0.09
PBMC 1000	31.73	0.24	31.4	0.2	0.33
PBMC 10000	35.1	0.56	34.67	0.22	0.43

B. SYBR Green

	Cβ		GAPDH		Cβ GAPDH difference
	Ct	**SD**	**Ct**	**SD**	
PBMC 10	17.1	0.14	15.81	0.17	1.29
PBMC 100	19.84	0.32	18.47	0.28	1.37
PBMC 1000	23.15	0.28	21.86	0.32	1.29
PBMC 10000	25.96	0.22	24.55	0.29	1.41

Table 3.2 Real time PCR results for GAPDH and BC genes in serial dilutions of PBMC cDNA measured using fluorogenic probes (A) or SYBR green (B). As sample dilution increases, threshold cycle of detection increases linearly for both detection systems. The difference between GAPDH and BC threshold cycle (last column) remains close to constant for both SYBR green and the fluorogenic probe. This suggests that both systems perform well for these two relatively high signal genes.

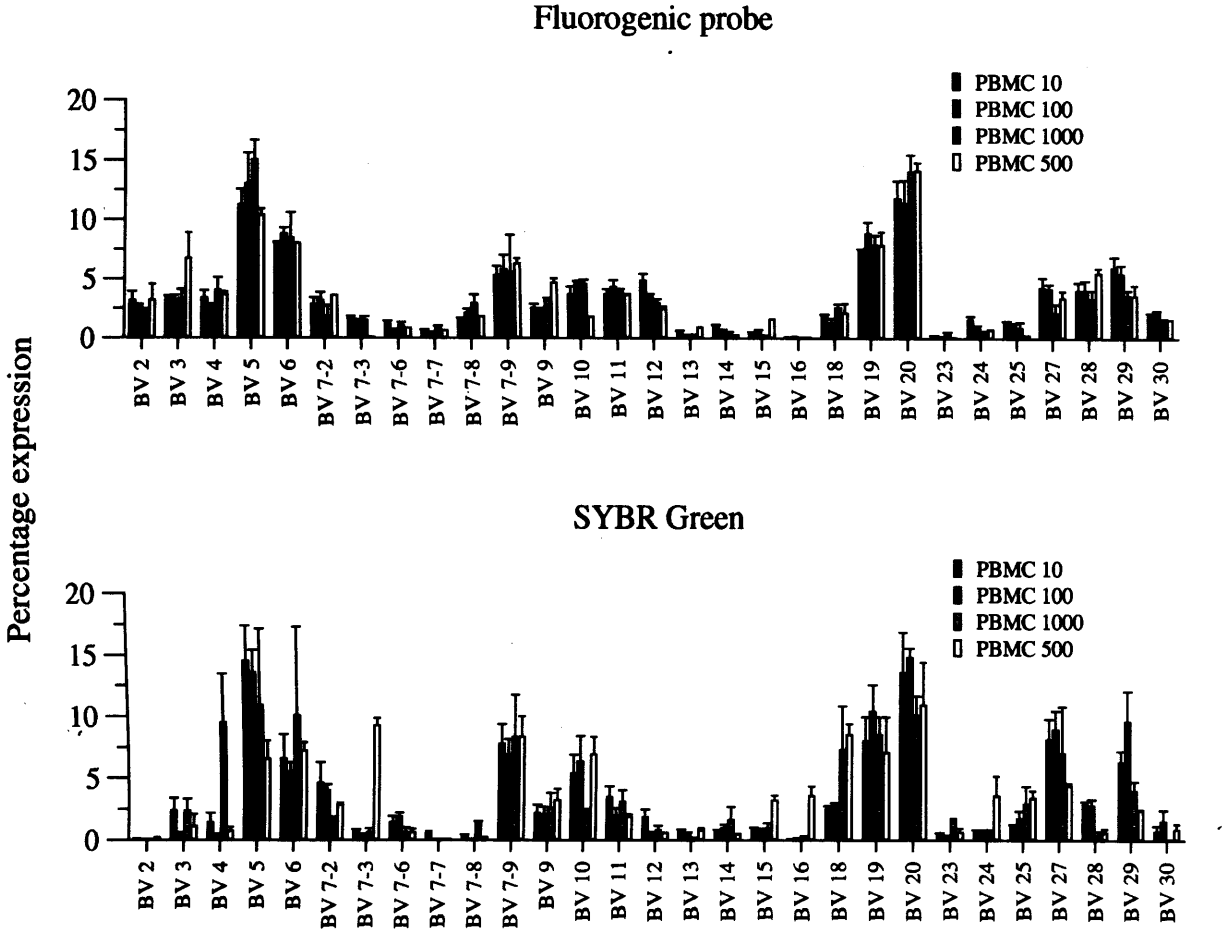
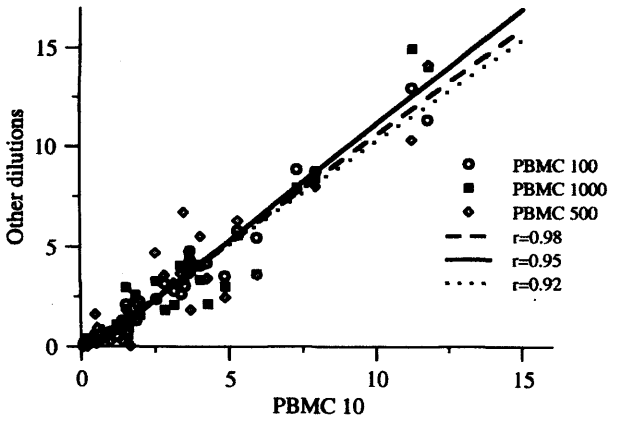


Figure 3.1 Real time PCR results on serial dilutions of PBMC cDNA using a single BC primer and 29 BV primers and with A. HuTrec™ fluorogenic BC probe or B. SYBR green fluorescent reporter dye. PBMC 500 was a 500 fold dilution of PBMC cDNA with non-T cell cDNA, used to mimic a tissue sample with a low TCR signal. PBMC cDNA was otherwise diluted with water.

The HuTrec™ specific BC probe gave consistent results across all cDNA dilutions and also performed well for the PBMC 500 sample. SYBR green gave much greater variability when used for more dilute samples and showed highly inconsistent results in a heterogeneous cDNA (PBMC 500).

A. Fluorogenic probe



B. SYBR Green

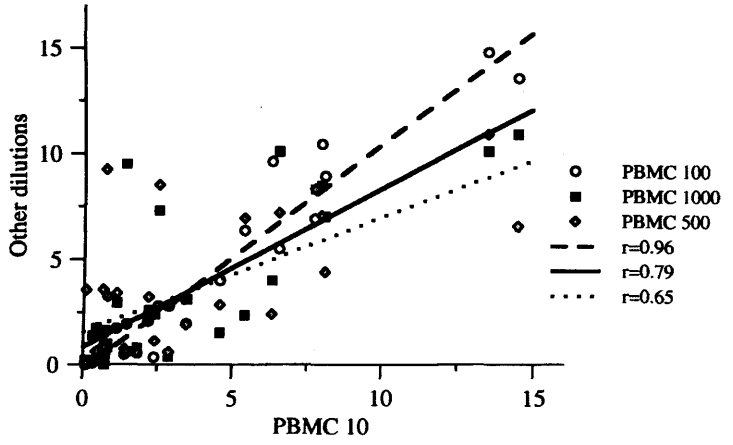


Figure 3.2 Real time PCR results on serial dilutions of PBMC cDNA using a single BC primer and 29 BV primers and with HuTrec™ fluorogenic BC probe or SYBR green fluorescent reporter dye. Results for PBMC cDNA dilutions 100, 1000 and 500 are plotted against the corresponding result for PBMC 10 for each BV family. The resulting line gives a correlation coefficient comparing two BV repertoires from different dilutions of cDNA in a single fluorescence reporting system. A correlation coefficient close to 1.0 suggests the reporting system gives accurate results across different cDNA dilutions. The fluorogenic probe gave high correlation coefficients across all samples. SYBR green showed a gradual decrease in the correlation coefficient with decreasing cDNA concentration and in the presence of high levels of non-T cell cDNA. This suggests SYBR green gives much less accurate results in these samples.

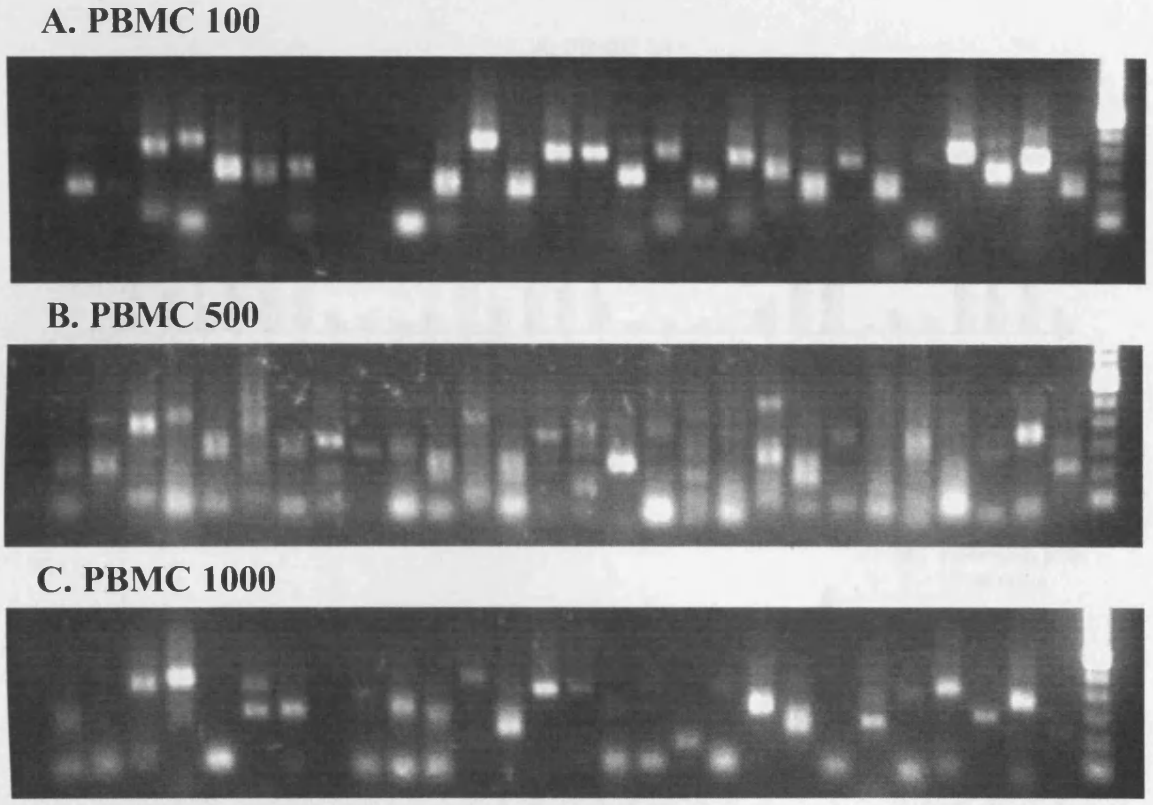


Figure 3.3 Agarose gel electrophoresis of PCR product from experiments in Figure 3.2.

A. PBMC 100 BV repertoire gel shows clear bands with minimal non-specific product.

B. PBMC 500 cDNA is a low TCR signal cDNA with large amounts of non T cell cDNA to mimic a renal biopsy or other small tissue sample. The repertoire gel shows large amounts of non-specific product which will be detected by the SG present in the reaction and reported as specific product by the detection system.

C. PBMC 1000 is low signal but has no contaminating non T cell DNA. The gel shows more non-specific product that PBMC 100 but less than PBMC 500.

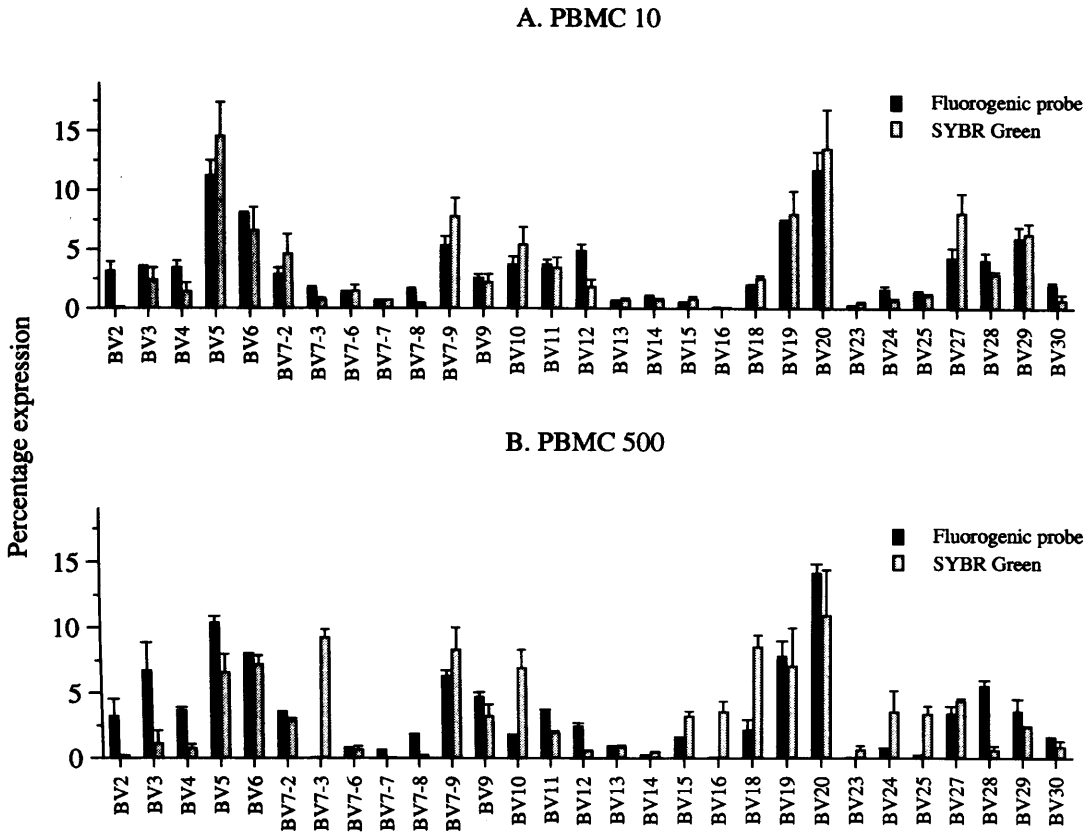
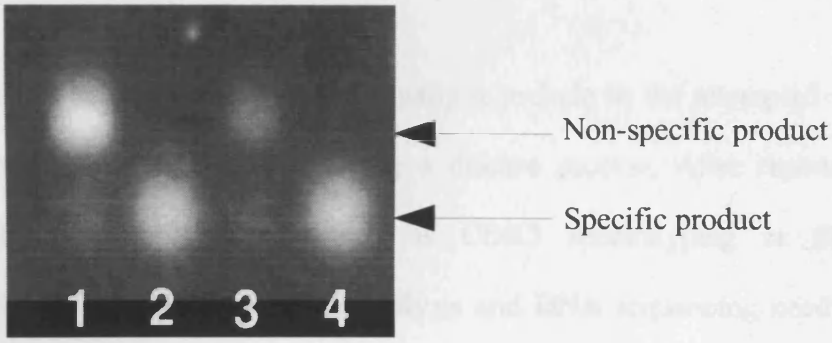


Figure 3.4 Real time PCR results comparing a fluorogenic probe and SYBR green in the measurement of TCR BV repertoires for two dilutions of PBMC cDNA using a single BC primer and 29 BV primers.

A. High signal cDNA. Repertoires generated by specific and non-specific probes are very similar when high signal sample PBMC 10 is studied. There are some moderate differences between individual families that may be accounted for by subtle changes in primer efficiencies under the influence of either the specific probe or SYBR green.

B. Low signal cDNA contaminated with non-T cell cDNA. Low signal samples give very different repertoires when studied with different assay systems. SYBR Green gives a significantly different result under these conditions when compared with the highly consistent result from the HuTrec™ fluorogenic probe. Significant differences between the repertoires are now present in multiple BV families e.g. BV 2, 3, 4, 7-3, 7-8, 10, 12, 16, 18, 28. I have shown previously that the fluorogenic probe results are consistent across samples. Therefore the changes in the repertoire as measured by SG are erroneous.



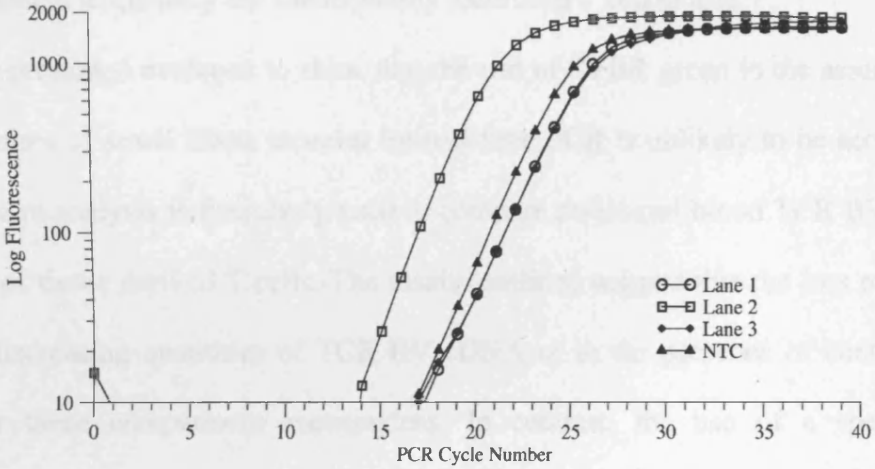


Figure 3.5 Three samples of PBMC cDNA and a single no template control were amplified using two BC primers and SYBR green in a real time PCR reaction. The four lanes on the agarose gel electrophoresis show several different PCR outcomes with SG. Three identical cDNA samples were run with BC primers and SG. Lane 4 is a no template control. Lanes 1, 3 and 4 give very similar results when assessed on the basis of their real time curves. The agarose gel shows that each consists of a different combination of specific and non-specific products. Lane 2 consisting entirely of non-specific product gives the lowest threshold cycle number. In a real time reaction, this would suggest the presence of a large amount of specific product unless further analysis was performed on the final PCR product to validate the result. It is not possible to adequately distinguish between specific and non-specific product using SG without further analysis or post-PCR manipulation.

3.4 Discussion

TCR BV repertoire studies are usually a prelude to the attempted identification of T cell clones infiltrating a tissue during a disease process. After repertoire definition, further studies utilising methods such as CDR3 spectratyping or SSCP (single stranded conformational polymorphism) analysis and DNA sequencing need to be used to identify potentially pathogenic T cell clones. This study has addressed only the first part of this type of investigation. Knowledge of the BV repertoire is an important step which can be used to some extent to quantify the subsequently identified T cell clones.

I have presented evidence to show that the use of SYBR green in the assessment of the BV repertoires of small tissue samples by real time PCR is unlikely to be accurate. The use of repertoire analysis is frequently used to compare peripheral blood TCR BV repertoires with those of tissue derived T cells. The results outlined suggest that the loss of correlation seen with decreasing quantities of TCR BV cDNA or in the presence of contaminating cDNA render these comparisons meaningless. In contrast, the use of a specific fluorogenic oligonucleotide probe for BC is a sensitive and reproducible method. This method involves an estimate of the relative percentage of each BV family rather than an attempt at absolute quantification. There are several reasons for this. Firstly the use of a standard curve to quantify each BV family would increase by several magnitudes the time taken to obtain an estimate of the BV repertoire. Secondly, the use of absolute quantification does not provide any further information. This method maximises the yield of small samples of RNA while minimising the work required. Repertoire work is based on the detection of a change between a diseased and a reference tissue thus absolute rather than relative quantity is of no advantage.

These results show that the use of a dual-labelled fluorogenic probe gives consistent BV repertoires in up to 1000 fold dilutions of PBMC. Mixing PBMC with non-T cell cDNA

produces a sample more akin to tissue sample biopsies. The specific probe performed well with this sample giving a nearly identical BV repertoire. SYBR green was shown to perform well at high target signals. It demonstrated an almost matching TCR repertoire to the specific probe when high concentrations of TCR cDNA were present. However when conditions similar to those associated with real tissue samples were used, SYBR green became much less accurate. At a PBMC dilution of 1:1000 SYBR green showed significant errors compared with the fluorogenic probe and when testing a sample with large amounts of non-TCR cDNA mixed with PBMC cDNA, SYBR green gave very poor results.

Previous comparisons of SYBR Green and fluorogenic probes have been conducted by various groups. Hein et al (119) tested both assay systems in a study of the levels of *Staphylococcus Aureus* in cheese. Both the Roche LightCycler and the ABI Prism 7700 SDS were used and results showed a 10 fold reduction in sensitivity using SYBR Green. Taqman chemistry was also found superior to SYBR green by Terry and colleagues in a study to detect levels of genetically modified soya (120). Other groups have either found both assay systems to be equivalent (121) or SYBR green to be more sensitive (122).

These data were generated using peripheral blood cells and pure cell lines not tissue samples. However the errors found with SYBR green analysis are likely to be magnified in tissue specimens where there may be ongoing cell death, artefacts related to homogenisation, variability of T cell number and variability in the non T cell RNA. I would therefore expect that the difficulties found due to non-specific product with SYBR green and low copy numbers is likely to be an even greater problem in tissue samples and that SYBR green is unlikely to be an appropriate tool to examine small tissue samples for TCR repertoire.

Attempts have been made to overcome the quantitation problems with SYBR green but to my knowledge it has not been possible to overcome the difficulty in resolution of specific from non-specific product that low signal samples pose. Yin et al (123) used a method

involving measurement of the melting temperature of non-specific product followed by data collection above that point during the PCR reaction. Though this resulted in some improvement in outcome, it failed to entirely rectify the problem.

In order to produce a reliable estimate of changes in a T cell receptor repertoire, I would suggest the following:

- Use of a dual labelled fluorogenic specific oligonucleotide probe or other specific probe system.
- As an indirect measure of the level of T cell infiltrate in the diseased tissue for comparison with normal tissue, assess the TCR signal level in the sample using real time assays for BC and a housekeeping gene such as GAPDH.
- Samples should be analysed in pairs including the diseased tissue and a control such as PBMC and, for comparison of level of T cell infiltrate, a normal sample of the relevant tissue. These pairs should be diluted to have a similar level of BC in real time assays so that their BV repertoires will be unaffected by any minor differences in primer efficiencies.

I suggest that SYBR Green is an inappropriate method for the real time PCR assessment of TCR BV repertoires in small tissue samples. Any use of SYBR green should be accompanied by a method to detect the presence of non-specific product, either by melting point analysis or agarose gel electrophoresis. Dual labelled fluorogenic probes give consistent results at variable concentrations of T cells and are therefore a more useful and appropriate method.

Chapter 4. Glomerular T cells in Heymann nephritis

4.1 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 (20). The autoantigens thought to be involved in HN are expressed in the glomeruli and have been extensively characterised (124) (125). 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 (26).

Further evidence for the part played by T cells in HN derives from blocking experiments. Treatment with monoclonal antibodies to CD4 prior to immunisation has been shown to completely prevent proteinuria in this model (24) 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 recognise and proliferate in response to specific autoantigens.

We have previously described the T cell receptor (TCR) repertoire in the renal interstitium of HN rats (28). The aims of this study were to characterise T cells infiltrating the glomeruli in HN in Lewis rats by polymerase chain reaction (PCR) of their Vβ chains, CDR3 spectratyping and sequencing.

4.2 Materials and methods

More details on these methods are provided in Chapter 2.

4.2.1. Induction of Heymann Nephritis

Heymann nephritis was induced by standard methods as previously documented in detail in Section 2.2 (p.26)

4.2.2. Isolation of glomeruli

Glomeruli were isolated as previously described (101) and as documented in Section 2.3 (p.26).

4.2.3. Immunoperoxidase Staining

Isolated glomeruli were enzymatically permeabilised for immunoperoxidase staining as described in detail in Section 2.2 (p.27).

4.2.4. Extraction of RNA and reverse transcription

Total RNA was extracted from isolated glomeruli using a modification of the method of Chomczynski and Saachi (102). Samples of glomeruli were dissociated in RNAzol B (Cinna/Biotec, Houston, TX.). RNA was then extracted following the standard protocol as detailed in Section 2.5 (p.27). The final product was then air dried, dissolved in DEPC treated water and stored at -80 °C. First strand complementary DNA was synthesised using the M-MLV Reverse Transcription kit (Gibco BRL). One microgram of RNA and random hexamer primer was used to prime the reaction.

Primers for rat TCR Vβ genes were published previously (103) and are documented in table 2.2 (p.36). Amplification of the housekeeping 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) as detailed in Section 2.7 (p.28). Products were then analysed on a 2% agarose gel. Standard curves of product signal were generated at different PCR cycles numbers in triplicate and subsequent Vβ repertoires were run at appropriate cycles to ensure no over-amplification of product. The specificity of PCR products was confirmed using QPCR and individual Vβ gene expression was expressed as a percentage of total TCR signal.

4.2.5. Detection of PCR products by QPCR System 5000

The specificity of each PCR product was verified by separate hybridisation 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 (104) (105) (106). 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 Vβ family member was expressed as a percentage of the total luminosity in all of the Vβ regions for a given sample.

4.2.6. CDR3 Spectratyping of PCR products

2 μl of PCR product from each Vβ family was then used as cDNA for a second round of PCR. Primers were as before with the addition a Fam labelled Cβ reverse primer internal to the initial Cβ biotin primer. This proceeded for six to ten cycles. 1 μl of PCR product from this reaction was sent to 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.

4.2.7. Cloning and sequencing of PCR products

PCR products of interest were cloned into a T vector and sequenced as detailed in Section 2.11 (p.30). Sequencing was performed by Sydney University Prince Alfred Macromolecular Analysis Centre using the dye-labelled dideoxy chain determination method with PE Biosystems ABI Prism 377 Sequencer.

4.3 Results

4.3.1. Development of Heymann Nephritis

All of the rats immunised with Fx1A developed active HN. Proteinuria developed at eight 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 Figure 4.1 (p.60).

4.3.2. No Vβ TCR family was significantly overexpressed

Various individual animals displayed over-representation of single Vβ families but no single TCR family was overexpressed in multiple animals at any stage of the disease. Figure 4.2 (p.61) shows the relative expression of TCR Vβ families at 4 weeks and reveals an apparently polyclonal T cell infiltrate.

4.3.3. 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 twenty different Vβ 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 Figure 4.3 (p.62).

4.3.4. Several Vβ TCR families have identical spectratypes in different animals

Identical size CDR3 spectratypes were found in several different animals for eight Vβ families. In Vβ 5, eight animals showed an abnormal peak in their CDR3 spectratype measuring 382/3 base pairs. Other common spectratypes were present in fewer animals (7 animals for Vβ 7, 6 for Vβ 8, 13, 14, 19 and 20, 5 for Vβ 6). The spectratypes of interest from Vβ 5, 7, 8 and 13, as the most commonly occurring families, were then sequenced. The spectratypes for Vβ 5 are shown in Figure 4.4 (p.63) and the corresponding sequences of the correct length in Figure 4.5 (p.64).

4.3.5. 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 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 Figure 4.5 (p.64).

4.3.6. Clones of interest in Vβ 5, 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 Vβ 5, 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 Figure 4.6 (p.65).

4.3.7. Overexpression of Jβ 2.6 in glomerular clones

Figure 4.6 (p.65) shows that many of the HN clones expressed Jβ 2.6. A comparison with normal Jβ 2.6 expression in Lewis rat spleen is shown in Figure 4.7 (p.66). There was a striking overexpression of Jβ 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 (126). 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 Jβ 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 Figure 4.8 (p.67). The profile of the Jβ 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 χ^2 analysis ($p<0.05$)

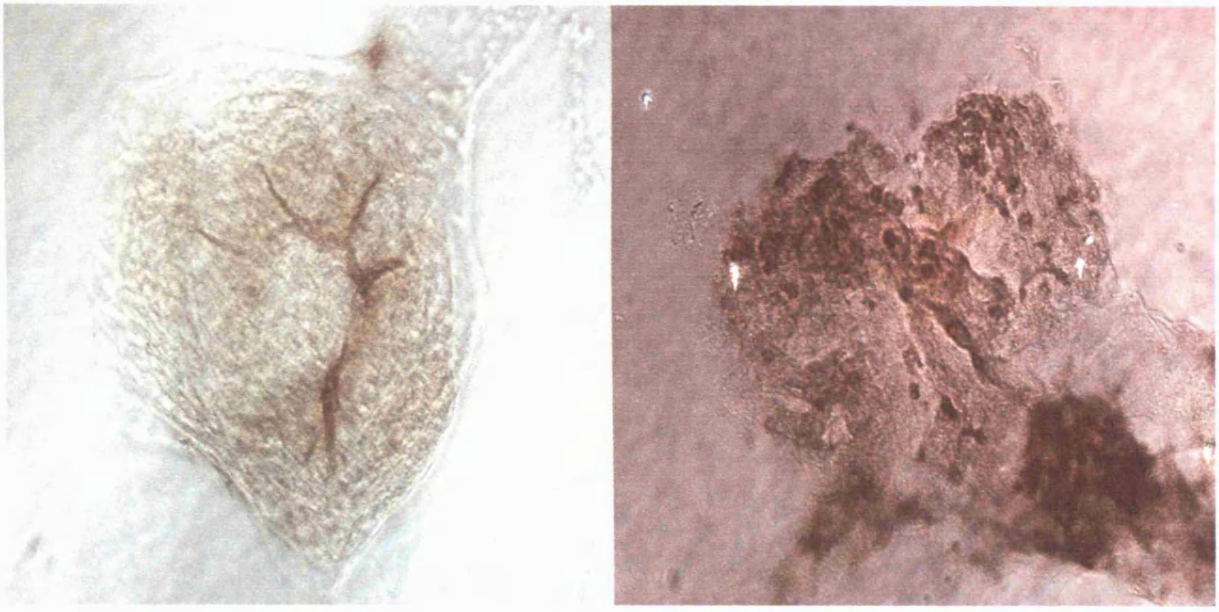


Figure 4.1 Immunoperoxidase staining using monoclonal anti $CD4^+$ antibody followed by an HRP labelled goat anti-mouse IgG antibody. HN glomerulus (right) shows a heavy infiltrate of cells. Control glomerulus shown on the left. Magnification x 400.

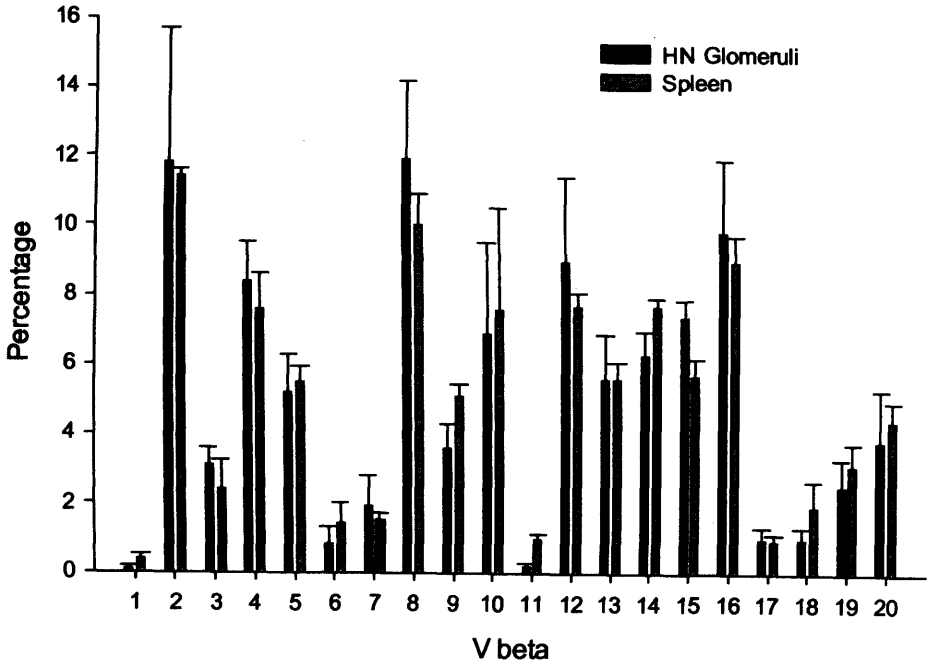


Figure 4.2 Expression of Vβ TCR genes at 4 weeks after induction of HN shows no significant difference with expression in Lewis rat spleen (n=6).

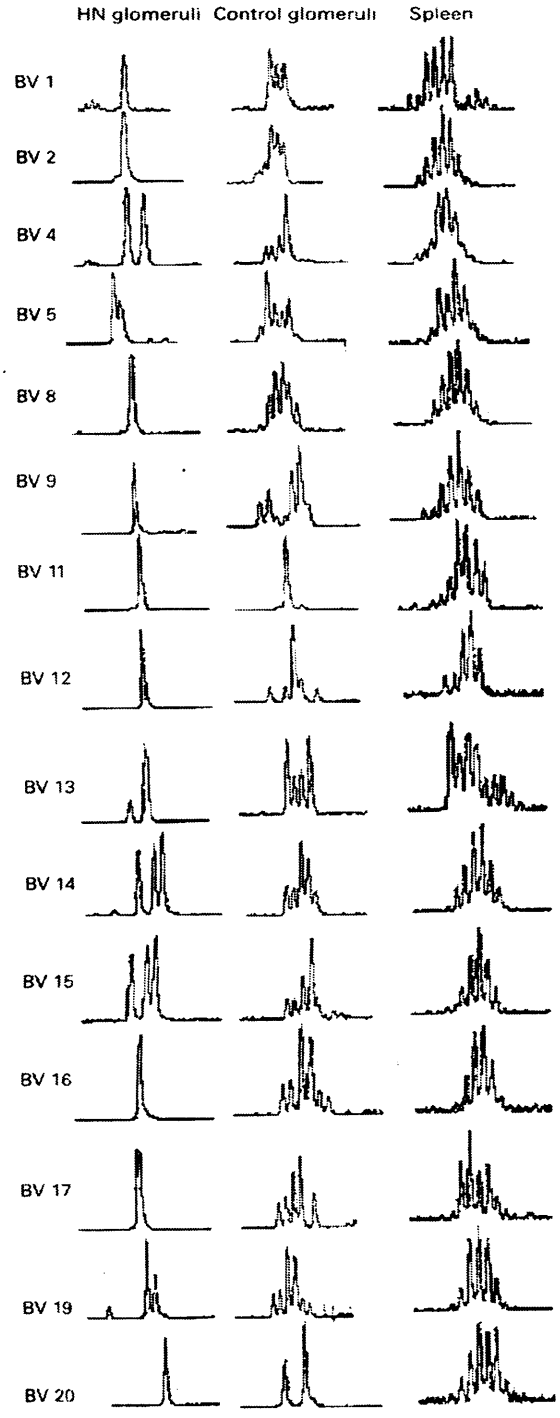


Figure 4.3 CDR3 spectratypes from a single animal with HN compared with those of a single control animal and spleen. The HN glomeruli showed marked restriction of multiple Vβ families. Control glomeruli also showed some restriction in their spectratypes but to a lesser degree than found in HN

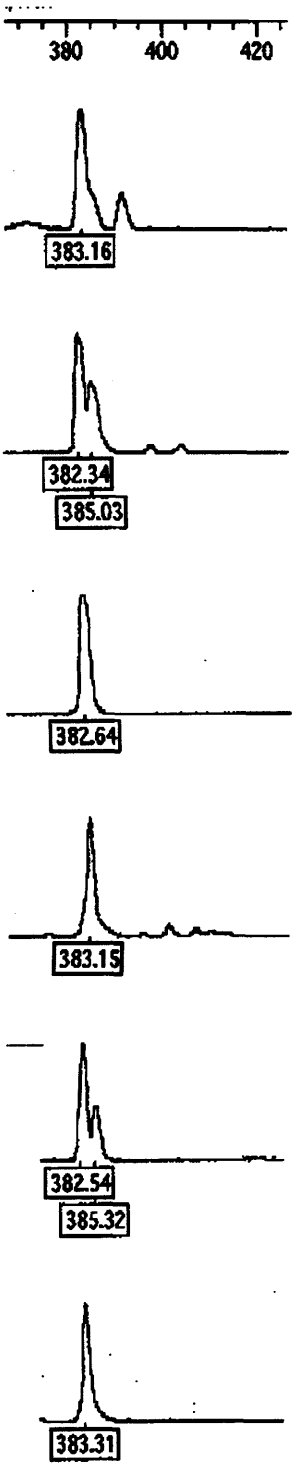


Figure 4.4 Vβ 5 spectratypes from six different HN rats show a common restricted size peak at 382/3 base pairs with little or no other signal present.

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	CASSLDRGNTEVFFGKGTRLTVV

Splenic sequences

1/5	CASSLPTGDEQYFGPGTKLTVL
1/5	CASSQQGRETQYFGPGTRLLVL
1/5	CASSQPGQTEVFFGKGTRLTVV
1/5	CASSLGSNTEVFFGKGTRLTVV
1/5	CASSLTPIGYDYTFGPGTRLLVI

Figure 4.5 Vβ 5 sequences from seven individual HN animals compared with splenic sequences. The public response is in bold. The nucleotide sequences relating to the public response were all identical.

V beta	J beta	Frequency	Sequence	
5	**2.6**	**8/9**	**97.05.01**	**CASSLSGGD---YEQYFGPGTKLTVL**
7	**2.6**	**7/7**	**100.07.06**	**CASSLGGLS---YEQYFGPGTKLTVL**
5	**2.6**	**9/9**	**106.05.02**	**CASSLDLFS---YEQYFGPGTKLTVL**
7	**2.6**	**6/6**	**107.07.01**	**CASNAGQVS---YEQYFGPGTKLTVL**
7	**2.6**	**8/8**	**114.07.08**	**CASSFWGSS---YEQYFGPGTKLTVL**
7	**2.6**	**3/8**	**112.07.04**	**CASSTGGAS---YEQYFGPGTKLTVL**
13	**2.6**	**4/6**	**116.13.13**	**CASSLAGGA---YEQYFGPGTKLTVL**
5	**2.6**	**2/11**	**113.05.17**	**CASSLGTDS---YEQYFGPGTKLTVL**
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---SPLYFAAGTRLTVT
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---NVLYFGEGSRLLVV
5	1.3	8/13	91.05.04	CASSLAGDG---NVLYFGEGSRLLVV
5	1.1	2/8	88.05.15	CASSLDRGN---TEVFFGKGTRLTVV

Figure 4.6 Sequences from T cell clones isolated from HN glomeruli. Clones bearing Jβ 2.6 are in bold.

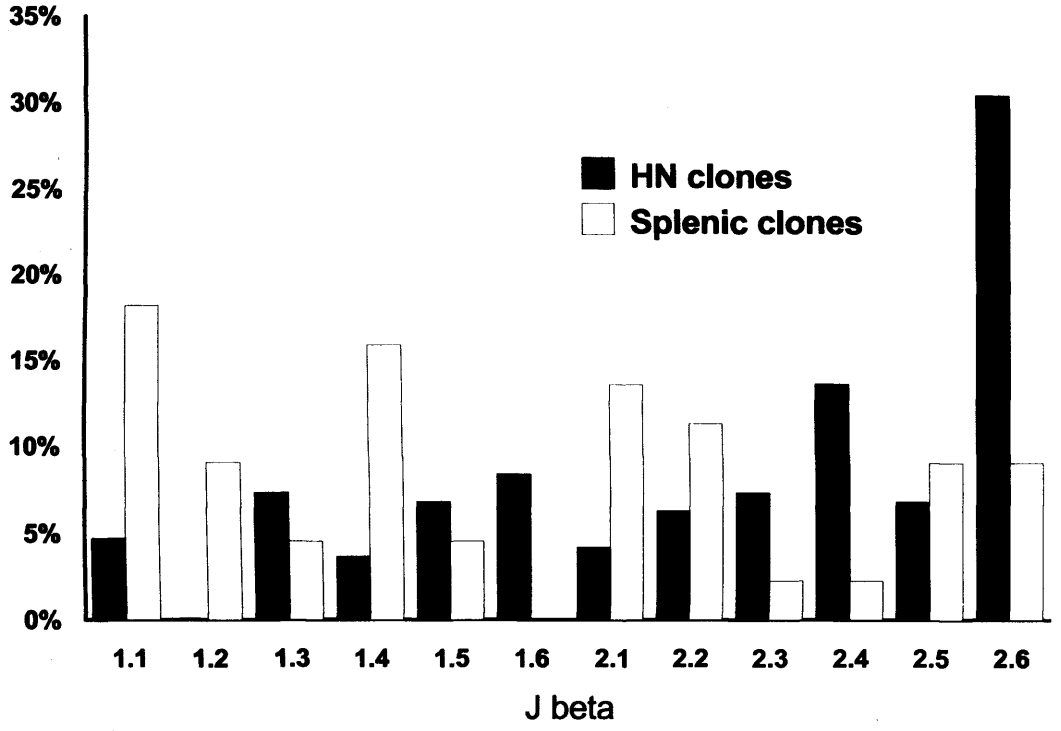


Figure 4.7 Jβ repertoire of HN clones (■) showing a striking over-expression of Jβ 2.6 when compared to splenic sequences (□).

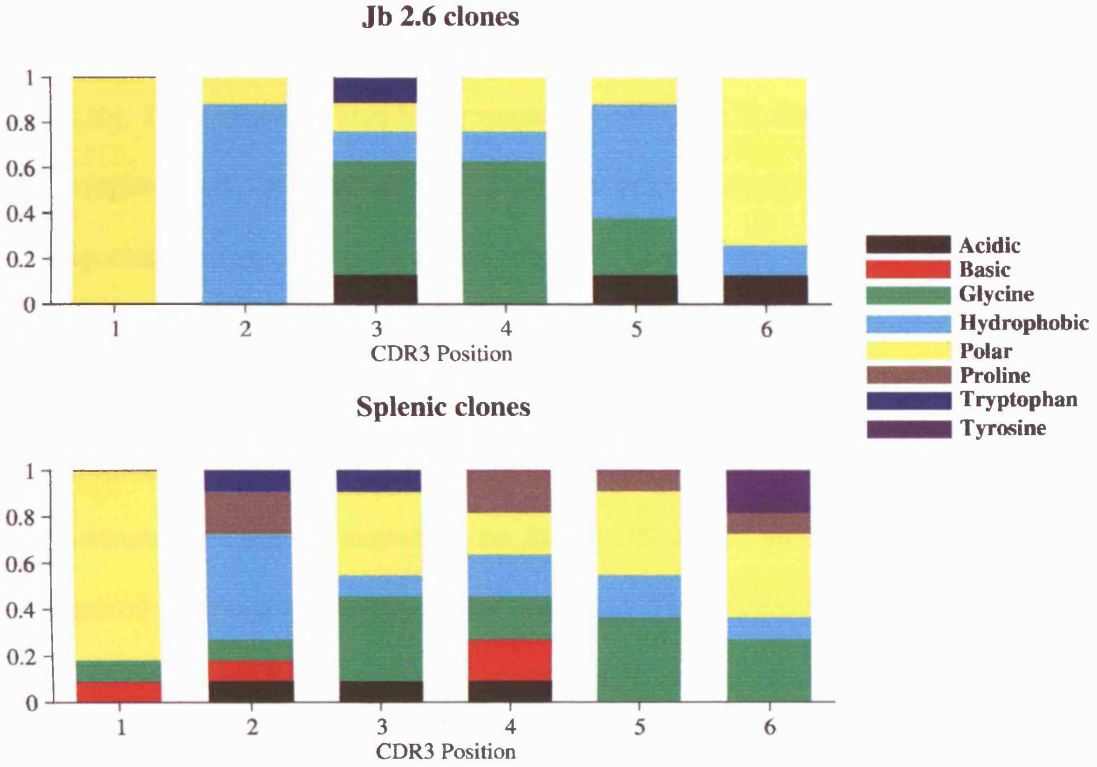


Figure 4.8 Profiles of six CDR3 positions in HN clones compared with the same positions in splenic sequences of the same length. Amino acids are grouped according to their properties. Positions 1, 2, 4 and 6 of the CDR3 profile of the HN clones show clear restriction when compared with splenic sequences of the same length ($P < 0.05$).

4.4 Discussion

Glomerular T cells are shown here to be present during the development of HN as reported previously (26). PCR shows a broad representation of Vβ TCR families with no specific family overrepresented. Subsequent CDR3 spectratyping demonstrated the presence of multiple oligoclones many of which shared identical CDR3 sizes across different animals. Sequencing of a subset of these clones found multiple clones with the same size CDR3 in several Vβ families expressing Jβ 2.6. HN clones strikingly overexpressed Jβ 2.6 when compared to spleen. A small public response was demonstrated in 3 animals.

Further examination of the sequences of the Jβ 2.6 HN clones showed a restricted CDR3 profile compared to splenic sequences of the same size. This suggests 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 (124). We have demonstrated that T cells in HN rats recognise specific peptidic antigens in the RAP (104).

Our data demonstrate that multiple Vβ 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 Vβ gene. Previous studies of HN have measured involvement of a particular Vβ gene by its overexpression in semi-quantitative PCR (28) (104). 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 Vβ 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 Vβ genes involved in clonal expansion. The presence of multiple epitopes makes the situation 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 overexpression of a particular Vβ gene which has subsequently been shown to contain pathogenic T cell clones (127). However this has been difficult to demonstrate in human diseases. More often than not, several Vβ 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 (128), psoriasis (129), Crohn's disease (130), pulmonary sarcoidosis (131), giant cell arteritis (132) and others. A study in giant cell arteritis even found differing Vβ expression in discrete inflammatory lesions in a single patient. If the Vβ gene is not always a critical point of contact with the peptide MHC complex then the same immune reaction could be generated with different Vβ genes on the same genetic background or even within the same individual. Thus, in a model, identical animals have the potential to produce diverse Vβ repertoires from a single antigen.

The nature of the peptide autoantigen being presented may dictate whether single or multiple Vβ genes are expressed in clonally expanded T cells. Recent work on crystal structures of MHC peptide TCR complexes (133) shows Vα to be an anchor to the MHC helices, Jβ to occupy the Vα/Vβ interface and Vβ to bind both to the MHC and to the peptide via its CDR3 region. Studies of the human TCR/Tax peptide/HLA A2 complex, however, showed that the

CDR3β pushes the TCR away from the MHC resulting in fewer TCR Vβ chain contacts with the MHC helices. This suggests that the important parts of the β chain are the Jβ, at the Vα/Vβ 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 Vβ genes but the same Jβ and similar CDR3 regions. In our model, we have demonstrated a subset of glomerular T cells which share similar CDR3 properties, use Jβ 2.6 and express different Vβ genes. We speculate that they are all responding to the same antigen.

4.5 Conclusion

These data show that glomerular T cells using Jβ 2.6 and a restricted set of CDR3 sequences in the context of at least two (but probably more) Vβ 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.

Chapter 5. DNA Vaccination Against Specific Pathogenic T Cell Receptors Reduces Proteinuria In Active Heymann Nephritis by Inducing Specific Autoantibodies

5.1 Statement

This project proceeded directly from my work on glomerular T cells in HN in the preceding chapter. At the time of its inception, my supervisor judged it to be unlikely that I would be able to remain in Australia long enough to complete the experiment. His judgement was later shown to be correct. Overall responsibility was therefore given to my immediate laboratory superior, Dr Huiling Wu. The majority of the work included in this chapter was performed by Dr Wu. My contribution includes preparation of DNA vaccine, generation of the disease model including injection of Fx1A into rats, injection of DNA vaccine, collection of urine using metabolic cages, sacrifice of animals, perfusion of kidneys, isolation of glomeruli, preparation of glomerular slides for subsequent histological staining. I did not perform the analyses contained within this chapter. These were all performed by Dr Wu. This work clearly validates and extends the observations made in chapter 4 of this thesis. The identification of glomerular T cells in HN does not of itself prove pathogenicity of those cells. This chapter utilises the data generated on infiltrating T cells and demonstrates that interruption of these cells alters the subsequent course of the disease, providing clear evidence of the pathogenicity of these cells and supporting the validity of the data presented in chapter 4. It is therefore presented here as part of my thesis.

5.2 Introduction

Human membranous glomerulonephritis is a major cause of end-stage renal disease in the adult population. Active Heymann nephritis (HN) is a model of membranous nephropathy (20) (23) induced in the Lewis rat by immunization with a crude renal tubular antigen (RAT/Fx1A) (100). It is associated with immune deposits in glomeruli, and infiltration of both glomeruli and interstitium by mononuclear cells. The pathogenesis of HN is thought to be through the binding of anti-Fx1A autoantibodies to the autoantigen (Gp330 or Megalin)

expressed on glomerular epithelial cells, resulting in severe glomerular injury and proteinuria (134). However, studies on the role of T cells in the aetiology of HN have shown that blocking $CD4^+$ cells by using anti-CD4 monoclonal antibody prior to immunization totally prevents proteinuria, glomerular Ig deposition and mononuclear cell infiltrates in the kidney in this model (24). More recently studies have shown that the appearance of activated T cells, principally Th1 and cytotoxic effector T cells, and macrophages within the glomeruli at 8 weeks, is coincident with the development of proteinuria (26). This results implies a role for glomerular infiltrating T cells in the pathogenesis of HN. Furthermore, permanent depletion of these $CD8^+$ T cells abolishes proteinuria and reduces glomerular infiltrates (135). These findings suggest that T cells and more specifically cytotoxic T cells may mediate glomerular injury.

T cell receptor (TCR) CDR3 spectratyping is a powerful tool for identifying autoimmune disease inducing T cells whose mechanism of action is poorly understood . We have demonstrated (chapter 4, p.54) clonal overexpansion of T cells using T cell receptors (TCR) encoding Vβ 5, Vβ 7 and Vβ 13 in combination with Jβ 2.6 (107) within glomeruli in this HN model . This was done using TCR spectratyping analysis and subsequent CDR3 region sequencing of spectratype-derived T cell clones. These overexpressed clones potentially represent pathogenic T cells. Vaccination with DNA encoding the Vβ region of an auto-reactive TCR has been shown to provide an effective means to prevent autoimmune diseases such as EAE by generating suppressor T cells (136). This study tested the effect of DNA vaccination targeting three specific TCR Vβ chains in HN. We sought to confirm the pathogenic nature of the subset of T cells bearing this restricted T cell receptor repertoire and investigate the therapeutic use of DNA vaccination in HN. The results of these studies are outlined below.

5.3 Materials and Methods

Further details on these methods are documented in Chapter 2.

5.3.1. Experimental animals and induction of active Heymann Nephritis

The disease model was created as described in chapters 2 (p.26) and 4 (p.54).

5.3.2. DNA vaccination

RNA was isolated from the HN kidney as described (107) and reverse transcribed into cDNA. cDNA was amplified by PCR with primers specific for either

Vβ 5 (5'-AAGATGTCTGGGGTGGTCCAGTCT-3'),

Vβ 7 (5'-AGC**ATG**TACTGGTATCGACAAGACCCAGATC-3')

or Vβ 13 (5'-AGA**ATG**ACTGGAGTCACCCAGTCCC-3)

and a reverse Jβ 2.6 primer (5'-CG**TCA**TAAAACCGTGAGCTTGGTGC-3).

cDNA was also amplified with a Vβ 8.2 primer as described (108) and a Cβ primer to generate a control vaccine. All forward primers were designed to include an in-frame ATG. PCR products were cloned into the pTarget plasmid DNA (Promega, Madison. WI) according to the manufacturer's instructions. Further details are documented in Section 2.12 (p.32).

5.3.3. Autoantibody determination

Anti-Fx1A antibody titres (total Ig) were determined by ELISA as described (24). Triplicate sample ODs were read at 405nm, corrected for a control sample of known strongly positive serum OD. IgG subclass were assessed by the same method using alkaline phosphatase-conjugated mouse monoclonal antibody to rat IgG1 and IgG2a (BD PharMingen, San Diego, CA USA).

5.3.4. Urinary protein estimation

Twenty-four hour urine samples were collected in metabolic cages at 4, 6, 8 and 12 weeks

post-Fx1A/CFA inoculation. Urine protein concentrations were determined by colorimetric assay (Biorad, Oakland, CA, USA). This is documented in more detail in Section 2.14 (p.33).

5.3.5. Immunoperoxidase staining

Glomeruli were isolated as previously described (107). Isolated glomeruli were enzymatically permeabilized for immunoperoxidase staining by incubation at 37 °C with a solution of 0.1mg/ml Collagenase D and 10mg/mg trypsin inhibitor in PBS. The sides were then washed in PBS for 15 min and stained with monoclonal Abs, followed by HRP-labelled goat anti-mouse IgG (BD PharMingen, San Diego, CA USA). The monoclonal antibodies used were W3/25 for $CD4^+$, OX-8 for $CD8^+$ and ED-1 for macrophages (Serotec, Oxford, UK).

5.3.6. RT-PCR for TCR Vβ repertoire

RNA was extracted from isolated glomeruli as described (107) and reverse transcribed to cDNA (104). Oligonucleotide primers used for the rat TCR Vβ 1-20 were those described (103) and documented in Table 2.2 (p.36). The PCR profile consisted of denaturation at 95°C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1 min for 40 cycles in a DNA thermal cycler (Perkin Elmer). Aliquots (1 μl) of the PCR products were re-amplified for 6 cycles with a nested 5'-Fam-labelled Cβ primer.

5.3.7. CDR3 spectratyping

Each labelled PCR product was loaded on POP4 sequencing gels together with size standard (GENESCAN-500 ROX) on an Applied Biosystems 310 DNA sequencer. Data were processed using the Genescan Analysis 2.1 Software (Applied Biosystems) which records the fluorescence intensity in each peak.

5.3.8. Semiquantitative RT-PCR for cytokine genes

Semiquantitative RT-PCR for cytokine genes was performed as previously described (104). Briefly, oligonucleotide primers used for rat cytokine genes IFNγ and IL10 were those described (137) (138) (26). GAPDH primers served as the internal control. The PCR profile used was one minute each at 95 °C, 55 °C and 72 °C for 30 cycles for GAPDH, 35 or 40 cycles for IFN-γ and 40 cycles for IL-10 . RT-PCR fragments of cytokine genes were electrophoresed on a 2% agarose gel. Signal was quantitated using Molecular Analyst software (Biorad, Hercules, CA).

5.3.9. Flow cytometry analysis and cell sorting

Mononuclear cells extracted from blood were stained with goat anti-rat IgG antibody conjugated with FITC (Zymed, San Francisco, CA) and anti-rat CD3 monoclonal antibody (clone G4.18) conjugated with PE (PharMingen San Diego, CA) or with anti-rat IgG conjugated with PE and anti-rat CD8α (clone OX 8) or anti-rat CD4 (clone OX35) conjugated with FITC (PharMingen San Diego, CA). All samples were analysed on a FACScan analyser (Becton Dickinson, Mountain View, CA). IgG^+CD3^+ cells were sorted using a FACStar (Becton Dickinson, Mountain View, CA).

5.3.10. Statistical analysis

Urine protein estimations, anti-Fx1A antibody levels for each group were expressed as mean ± SD. Comparison between groups were made by ANOVA. Paired T tests were used to determine whether changes in the levels of cytokine gene expression were significantly different.

5.4 Results

5.4.1. DNA vaccination protects against HN

Rats were divided into four groups: group 1(DV): DNA vaccination with TCR Vβ 5, 7, & 13

in HN; group 2 (DV control): DNA vaccination with TCR Vβ 8 used as a control vaccine in HN; group 3 (HN): HN; group 4 (CFA): immunization with CFA only as a HN control. DNA vaccination encoding TCR Vβ 5, 7 and 13 significantly reduced proteinuria at 6, 8, 10 and 12 weeks post Fx1A immunization (Figure 5.1 p.79) compared with HN and DV controls. There was no significant difference in proteinuria between the HN and DV control groups. Control rats immunized with only CFA never developed proteinuria.

Examination of isolated glomeruli from HN rats at 12 weeks demonstrated significant infiltrates of macrophages (ED1), $CD8^+$ and $CD4^+$ cells in the glomeruli as compared with CFA control rats (Figure 5.2 p.80). Macrophage and $CD8^+$ infiltrates in glomeruli in the DV group were significantly less than in the HN and DV control groups (p <0.005).

5.4.2. DNA vaccination reduces IFN-γ mRNA expression in HN glomeruli

To test whether DNA vaccination alters T cell function in the glomeruli, we examined cytokine profiles in isolated glomeruli at 12 weeks post-Fx1A/CFA immunization by semiquantitative RT-PCR. The results demonstrated lower mRNA expression for IFN-γ ($p<0.01$) in the DV group when compared with HN and DV controls (Figure 5.3 p.81) with levels not different from CFA controls.

5.4.3. DNA vaccination reduces the clonality of the T cell response in HN glomeruli

CDR3 spectratyping is a well described method used as a measure of oligoclonality of T cells. A normal splenic sample of a single Vβ 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. Our previous study demonstrated oligoclonal expansion of T cells in HN glomeruli (107) using CDR3 spectratyping and subsequent sequencing of the CDR3 regions of spectratype-derived T cell clones. To test whether DNA vaccination would change the pattern of TCR CDR3 spectratypes in

glomeruli, we examined CDR3 spectratypes of TCR Vβ 5, 7 and 13 in glomeruli in DNA vaccinated rats. HN glomeruli showed marked restriction with a single peak in CDR3 spectratypes of Vβ 5, 7 and 13 as we previously reported (107). The glomeruli isolated from DNA vaccinated rats showed diverse CDR3 lengths with more peaks in their CDR3 spectratypes (Figure 5.4 p.81) which matched the CFA control glomeruli. This result indicates that DNA vaccination may reduce the expansion of pathogenic T cell clones in the glomeruli.

5.4.4. DNA vaccination has no significant effect on anti-Fx1A antibodies

There was no difference in the level of serum anti-Fx1A antibodies or in the levels of IgG1 in DV, HN and DV control groups, however, IgG2a was suppressed by DNA vaccination early in the course of HN (Figure 5.5 p.82).

5.4.5. DNA vaccination induces specific autoantibodies

We analysed the mechanism underlying the protection induced by DNA vaccination. To test whether DNA vaccination with TCR Vβ 5, 7, and 13 would delete T cells expressing Vβ 5, 7 and 13 by producing autoantibodies, we analysed the TCR Vβ repertoire by RT-PCR in DNA vaccinated rats. We found that DNA vaccinated rats still expressed TCR Vβ 5, 7 and 13 in both peripheral blood and isolated glomeruli. We then asked whether DNA vaccination produces autoantibodies that bind the TCR. We sought to identify these T cells to see if they were deleted or functionally impaired. We stained mononuclear cells from DNA vaccinated and unvaccinated rats with PE conjugated anti-CD3 antibody and FITC-conjugated anti-IgG . A small population of $CD3^+IgG^+$ cells were found in DNA vaccination rats but not in unvaccinated HN rats (Figure 5.6 p.83). We also found that those $CD3^+IgG^+$ cells were CD8 cells by staining mononuclear cells from DNA vaccinated and unvaccinated rats with FITC-conjugated anti-CD8 antibody and PE-conjugated anti-rat IgG (Figure 5.7 p.83).

Furthermore, on analysis of their TCR Vβ repertoire, sorted $CD3^+IgG^+$ cells almost exclusively expressed TCR Vβ 5 and Vβ 13, two of the three Vβ families encoded in the DNA vaccination (Figure 5.8 p.84). Analysis of cytokine mRNA expression in the $CD3^+/IgG^+$ cells showed that IL-10 and IFN-γ mRNA were not detected (Figure 5.9 p.84). These results suggest that TCR DNA vaccination produced specific autoantibodies which bound to the TCRs encoded by the DNA vaccination, resulting in activation blockade of these T cells.

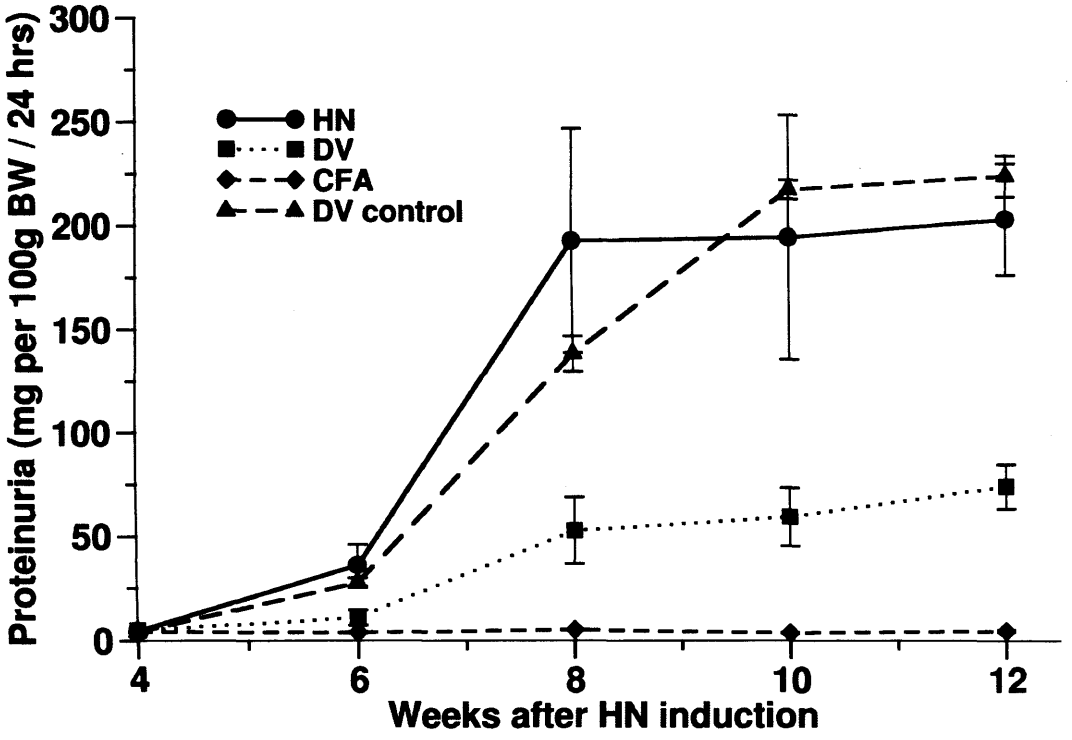


Figure 5.1 DV encoding TCR Vβ 5, 7 and 13 with Jβ 2.6 significantly reduced proteinuria at 6, 8, 10 and 12 weeks post immunisation compared with the control group ($p < 0.001$). HN: Heymann nephritis without DNA vaccination. DV: HN with DV with three TCR Vβs. CFA: CFA alone controls without Fx1A. DVctrl: HN with DV with Vβ 8 as control group for DV. All results were expressed as mean ± SD. N = 6 per group.

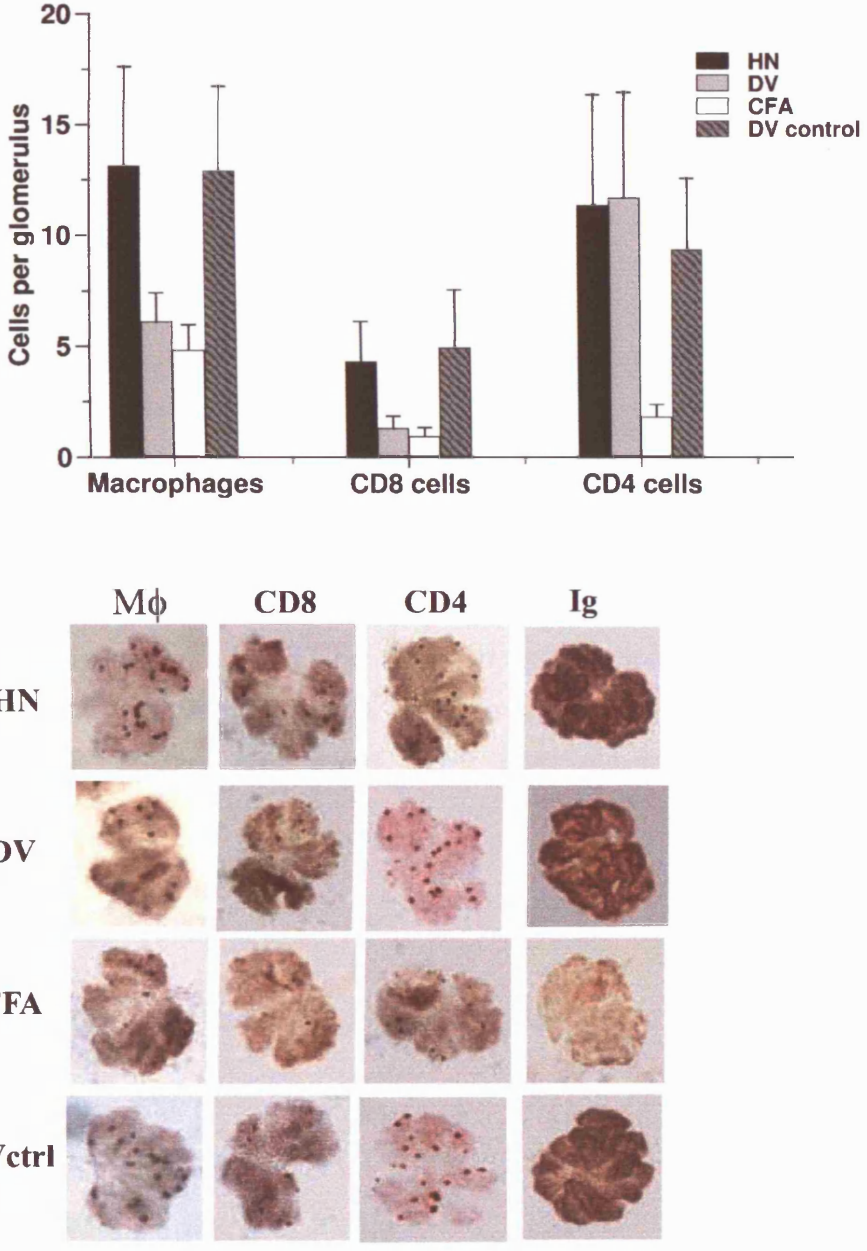


Figure 5.2 Immunoperoxidase staining of isolated whole glomeruli 12 weeks post immunisation with Fx1A/CFA. A. DV reduced macrophage and $CD8^+$ T cell infiltrates in glomeruli at 12 weeks ($p < 0.005$). All results were expressed as mean $\pm$ SEM of cells per glomerulus. N = 6 per group. B. A representative immunoperoxidase staining of isolated whole glomeruli from different treatment groups. No change in deposition of Ig was seen in glomeruli in HN compared with DV and DV control groups.

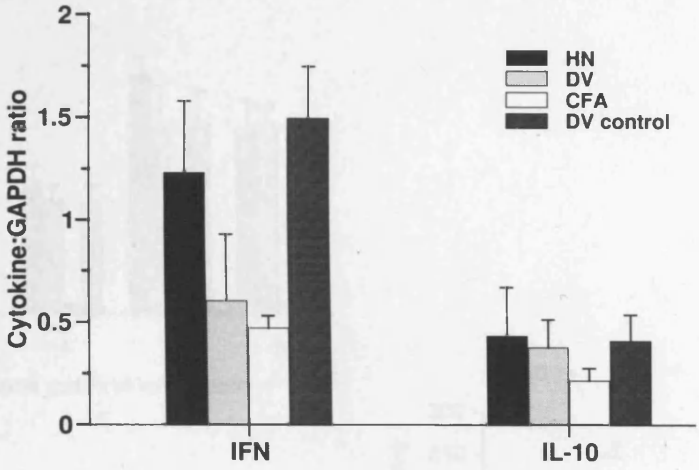


Figure 5.3 Semiquantitative RT-PCR analysis of cytokine expression in glomeruli from 12 week post immunisation with Fx1A/CFA. GAPDH gene served as an internal control. All results are expressed as mean ration of cytokine gene densitometry score to GAPDH densitometry score $\pm$ SD. n = 6 per group. IFN-γ expression was decreased in DV rats as compared with HN rats ($p < 0.01$).

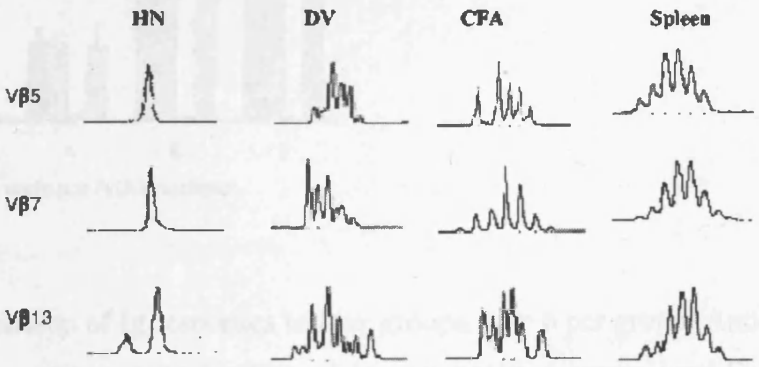


Figure 5.4 CDR3 spectratypes from a representative animal from DV group compared with those from HN, CFA groups and spleen. The HN glomeruli showed marked restriction of three TCR Vβs. DV glomeruli showed diverse CDR3 lengths with more peaks in the CDR3 spectratypes similar to CFA control glomeruli.

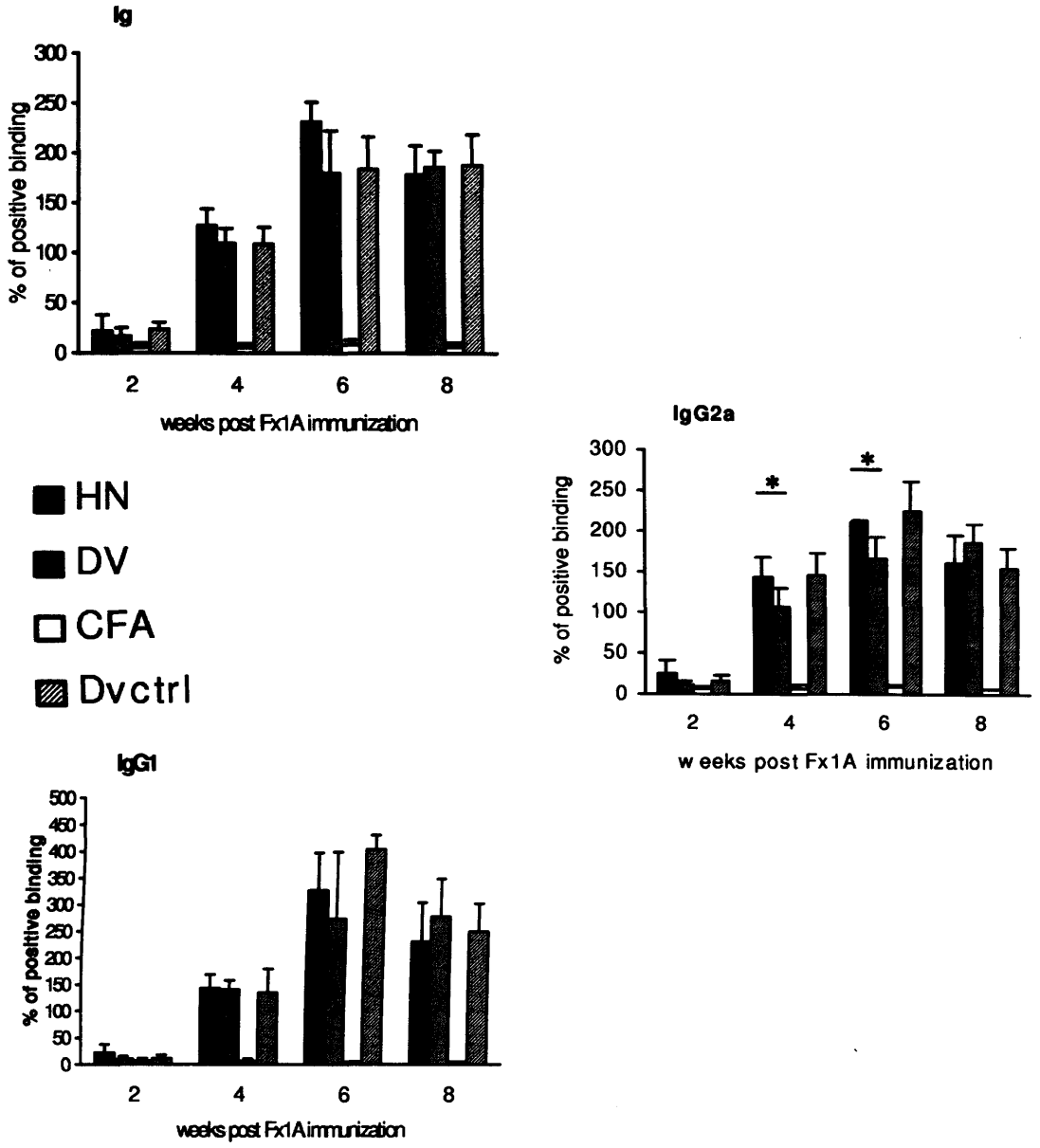


Figure 5.5 Comparison of Ig responses in four groups. N = 6 per group. Anti-Fx1A antibody levels expressed as percentage binding of known positive serum (anti-Fx1A titre 1:250) measured by ELISA and expressed as mean $\pm$ SD. There was no difference in the level of serum anti-Fx1A antibodies or in the levels of IgG1 in DV, HN and DV control groups. However, IgG2a was suppressed by DNA vaccination early in the course of HN ($p < 0.05$).

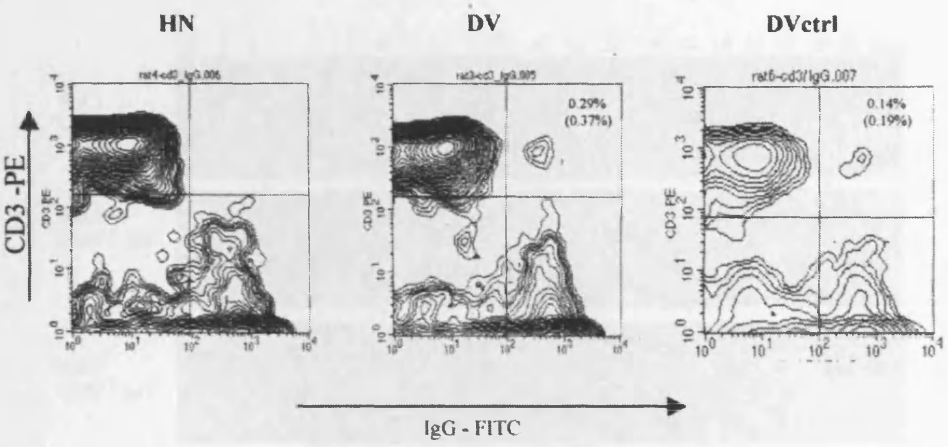


Figure 5.6 Identification of IgG^+CD3^+ cells in peripheral lymphocytes in DNA vaccinated rats by FACS. Freshly isolated lymphocytes from HN rats without DV (HN), with pathogenic TCR DV (DV) and with control TCR DV (DVctrl) were stained with anti rat Ig (FITC) and anti rat CD3 (PE). The number in brackets represents the percentage of IgG^+CD3^+ cells among total $CD3^+$ T cells. $n = 3$ per group. One representative experiment is shown.

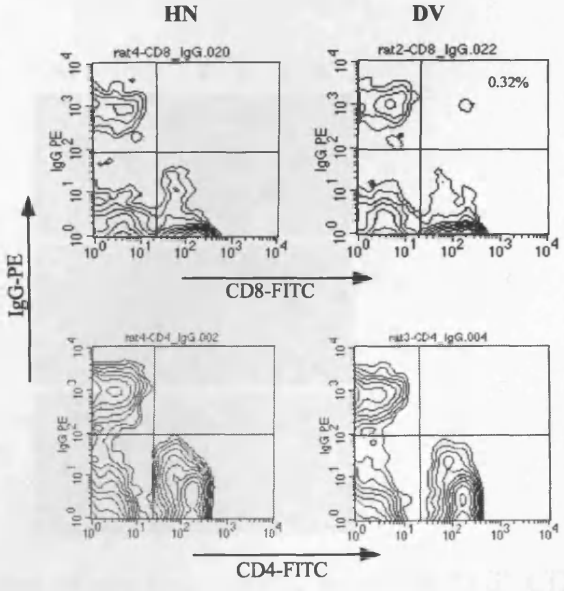


Figure 5.7 Identification of IgG^+ $CD8^+$ T cells in peripheral lymphocytes in DNA vaccinated rats by FACS. Freshly isolated lymphocytes from HN rats (HN) and DNA vaccinated rats (DV) were stained with anti rat IgG (PE) and anti rat CD8 (FITC) or anti rat CD4 (FITC). $n = 3$ per group. One representative experiment is shown.

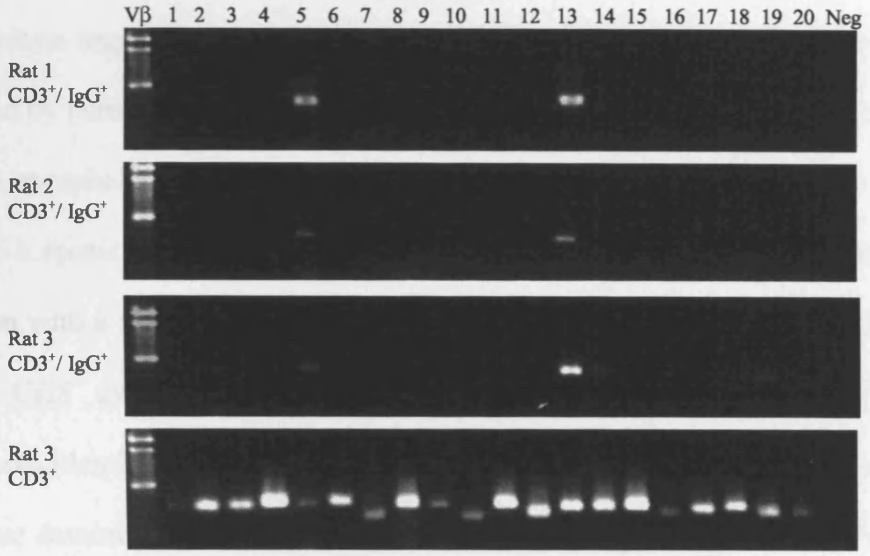


Figure 5.8 TCR repertoire analysis of sorted IgG^+CD3^+ T cells from peripheral lymphocytes of three rats vaccinated with DNA encoding TCR Vβ 5, 7 and 13. These $CD3^+/IgG^+$ T cells expressed Vβ 5 and 13 in contrast to the diverse TCR gene usage of the $CD3^+$ in the lower panel.

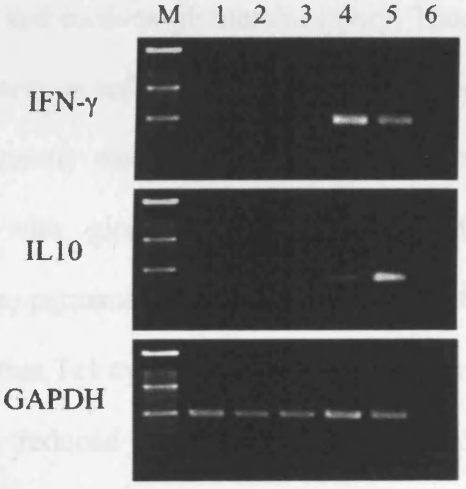


Figure 5.9 Expression of cytokine mRNA by sorted IgG^+ $CD3^+$ T cells from peripheral lymphocytes in rats vaccinated with Vβ 5, 7 and 13 by semiquantitative RT-PCR. IFN-γ and IL-10 were not detected in the sorted cells. The GAPDH gene served as an internal control. Lanes 1-3 represent PCR products from sorted IgG^+ $CD3^+$ T cells from vaccinated rats (n = 3). Lanes 4 and 5 represent PCR products from ConA stimulated T cells and Th2 cell lines we described previously, which served as positive controls for cytokine genes. Lane 6 represents a negative control for RT-PCR.

5.5 Discussion

In antigen driven organ-specific autoimmune diseases, it is possible define a restriction in the TCR used by pathogenic T cells. This restriction is seen in diseases such as experimental autoimmune encephalomyelitis (EAE). In EAE, encephalitogenic T cell lines and clones used mainly a restricted TCR Vβ 8.2 gene (139) (140). HN is induced in Lewis rats by immunization with a renal tubular antigen (RAT/Fx1A) or megalin (gp330). Recent studies suggest that $CD8^+$ cytotoxic T cells play a key role in causing glomerular injury in HN (135). We have identified HN-inducing TCRs by direct analysis of glomerular infiltrating T cells and have demonstrated that T cells within glomeruli in HN use T cell receptors (TCR) encoding Vβ 5, 7 and 13 in combination with Jβ 2.6 (107). In this study, immunotherapy using TCR-based DNA vaccines against these three Vβ families significantly reduced the severity of disease in HN, strongly suggesting that T cells bearing TCR Vβ 5, 7 and 13 are pathogenic and mediate glomerular injury. These findings support the evidence of clonality previously seen as reflecting expansion of pathogenic T cells.

In HN, previous studies by Penny et al demonstrated that the onset of proteinuria is associated with glomerular infiltration of $CD8^+$ Tc1 cells and macrophages (26). Furthermore, permanent depletion of these $CD8^+$ T cells prevents the onset of proteinuria, suggesting that Tc1 cytotoxic cells may mediate glomerular injury (135). In this study, DNA vaccination reduced the glomerular infiltrates of $CD8^+$ cells and macrophages, but accumulation of glomerular $CD4^+$ cells was similar to HN controls. IFN-γ mRNA expression was significantly reduced in glomeruli of DNA vaccinated rats compared with HN rats, consistent with reduced infiltrates of $CD8^+$ T cells and suggesting that the major source of IFN-γ in HN is $CD8^+$ cells. Furthermore, CDR3 spectratype patterns in glomeruli with DNA vaccinated rats were similar to those seen in CFA controls, compared with an oligoclonal restricted TCR repertoire in HN glomeruli. Oligoclonally expanded

TCRs represent the presence of activated pathogenic T cells in target organs in many studies (108). Taken together, these results suggest that DNA vaccination suppressed the cytotoxic CD8 response, which is necessary for induction of proteinuria in this model (135).

We have demonstrated that TCR DNA vaccination produced specific antibodies to the TCRs which it encoded. These specific antibodies did not lead to the depletion of Vβ 5, 7 and 13 T cells. These results are consistent with the observations made in the EAE disease model (136) and a transplantation model (141) (142). In the EAE model, encephalitogenic T cell lines and clones mainly used a restricted TCR gene Vβ 8.2 (139) (140). Vaccination with DNA encoding the Vβ 8.2 region of an auto-reactive TCR protected mice from EAE (136). This protection was attributed to a shift from a Th1 to Th2 cytokine profile in the Vβ 8.2 target cells rather than due to their depletion. However, suppressive effects were obtained from other studies with no signs of a Th2 bias (108), suggesting that Th2 deviation is not essential for the protective effect of DNA vaccination. We have examined the cytokine profile in HN glomeruli. DNA vaccination reduced glomerular IFN-γ mRNA expression but did not elevate levels of Th2 cytokine mRNA. We also analysed the cytokine profile of $CD3^+IgG^+$ cells from DNA vaccinated rats. $CD3^+IgG^+$ cells which were $CD8^+$ T cells, had little IFN-γ and IL10 mRNA expression. It is interesting to note that $CD8^+$ T cells were significantly decreased in number in the glomeruli of the DNA vaccinated rats. We speculate that autoantibodies produced following DNA vaccination bind specific TCRs and block the activation of these T cells. Our results suggest that this specifically targeted pathogenic subset of the T cell population either did not infiltrate the glomeruli or failed to expand and were unable to express pathogenic cytokines. This would appear to demonstrate the specific suppressive effects of DNA vaccination. The interaction of the TCR with antigen in the context of MHC has been greatly advanced by the recent crystallographic evidence of TCR structure and mutational analysis of TCR activation. It is possible that antibodies binding to

the Vβ chain can directly limit the normal activation of these T cells preventing clonal expansion and cytokine production (143).

In conclusion, we have shown that immunotherapy with TCR-based DNA vaccination protects against HN. This is based on a previous identification of pathogenic Vβ TCRs by TCR CDR3 spectratyping analysis and subsequent CDR3 region sequencing of spectratype-derived T cell clones. The mechanism for this appears to be the induction of Vβ specific antibodies. DNA vaccination targeting the T cell receptors of pathogenic T cells is an attractive therapeutic alternative (107) to broad immunosuppression in the treatment of glomerulonephritis.

Chapter 6. Glomerular T cells are of restricted clonality and express multiple CDR3 motifs across different Vβ T cell receptor families in experimental autoimmune glomerulonephritis

6.1 Statement

This work is a collaboration between myself and the Renal Section of the Division of Medicine at Imperial College School of Medicine, Hammersmith Hospital, London, led by Professor Charles Pusey. The disease model was generated, the glomeruli separated and mRNA extracted in London. After first strand cDNA synthesis, the samples were shipped to me and analysis of glomerular T cells was then performed by me. Histology and other analyses of the model were all performed in London.

6.2 Introduction

Goodpasture's disease is an autoimmune disorder associated with severe crescentic glomerulonephritis and lung haemorrhage (144). The pathogenicity of the associated anti-glomerular basement membrane (GBM) antibodies is suggested by clinical studies and has been demonstrated using passive transfer experiments (65). The antigenic target of these autoantibodies has been identified as the α3 chain of type IV collagen (α3(IV)NC1), an important structural component of the GBM (145) (37) and B cell epitopes have been defined (146). T cells have been implicated in studies demonstrating that patients possess autoreactive T cells responding to α3(IV)NC1 (64) (71) and that the frequency of such T cells is high in active disease and falls in remission (147). As expected in a T cell dependent disease, MHC class II genes are important in susceptibility, with up to 80% of patients inheriting an HLA-DR2 haplotype (72). Further studies have suggested a hierarchy of association of DRB1 alleles, some inducing protection, others susceptibility (148). Treatment involves the removal of circulating antibodies by plasma exchange, combined with immunosuppression, usually with prednisolone and cyclophosphamide (149). Though effective if started sufficiently early, treatment induces side effects in a significant

proportion of patients. The development of more specific treatments depends upon increased understanding and characterisation of the pathogenic T lymphocytes which drive this disorder.

Experimental autoimmune glomerulonephritis (EAG) is a model of human Goodpasture's disease generated in susceptible strains of rat by a single injection of collagenase digested GBM in Freund's complete adjuvant (FCA) (29) (30) (31). It is characterised in the WKY rat by sustained anti-GBM antibody production, linear deposition of antibody on the GBM, severe focal necrotising glomerulonephritis with crescent formation and variable lung haemorrhage (150). T cell involvement in this model has been demonstrated by a reduction in severity of the disease with the use of Cyclosporin A (32), anti-CD4 (33) or anti-CD8 (34) monoclonal antibodies and T cell costimulatory blockade (151)(152). Furthermore the effectiveness of oral tolerance was associated with a decrease in Th1 responses *in vivo* and splenic T cell proliferative responses to GBM *in vitro* (153). Recent studies in a rat model of EAG induced by α3(IV)NC1 that disease can be transferred using autoreactive T cells (36) and more recently that a T cell epitope from the N-terminus of rat α3(IV)NC1 is capable of inducing severe crescentic nephritis (154). Studies of a mouse model of EAG have provided further evidence that T cells, in conjunction with specific MHC class II alleles, are necessary for the development of disease (41) (155).

The aim of this study was to characterise the T cells infiltrating the glomeruli of the WKY rat during the development of EAG, using PCR for the variable regions of the TCR β-chain, CDR3 spectratyping and DNA sequencing. a results demonstrate restricted clonality of glomerular T cells in EAG, suggesting that antigen-specific T cells are directly involved in pathogenesis of this disease.

6.3 Materials and Methods

6.3.1. Experimental animals

Male WKY rats, aged 8-12 weeks and weighing 120-150 g were purchased from Charles River UK Ltd. (Margate, United Kingdom). All animals were housed in standard conditions and had free access to normal laboratory diet and water. All experimental procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act.

6.3.2. Induction of EAG

Antigenic material was prepared from normal rat kidneys as previously described (30) (31) (150). EAG was induced in WKY rats by a single i.m. injection of collagenase-solubilised rat GBM at a dose of 5 mg/kg body weight in an equal volume of FCA (Sigma-Aldrich Company Limited) (41). Control rats received an i.m. injection of emulsion containing an equal volume of saline in FCA. Serial blood samples were taken by tail artery puncture under light anaesthesia with isofluorane, and 24 h urine specimens obtained by placing animals in metabolic cages.

6.3.3. Assessment of EAG

6.3.3.1. ELISA

Circulating anti-GBM antibodies measured in sera from animals with EAG by a solid-phase enzyme linked immunosorbent assay (ELISA), as previously described (30) (31) (150). Briefly, collagenase-digested rat GBM (10μg/ml) was coated on to microtitre plates (Life Technologies, Paisley, UK) by overnight incubation at 4 °C, and an optimum dilution of test or control sera was applied for 1 h at 37 °C. Bound anti-GBM antibody was detected by alkaline phosphatase conjugated sheep anti-rat IgG (Sigma-Aldrich Company Ltd), and developed using the substrate p-nitrophenyl phosphate (NPP, Sigma-Aldrich Company Ltd). The absorbencies for each well were read at 405 nm using an Anthos Multiskan ELISA plate

reader (Lab Tech International, Uckfield, UK), and the results initially calculated as mean optical density for each triplicate sample. In order to compare different experimental groups, results were finally expressed as a percentage of the binding obtained with a positive reference serum.

6.3.3.2. Rocket immunoelectrophoresis.

Urinary albumin concentrations were measured in 24 h urine collections from animals with EAG by rocket immunoelectrophoresis (Amersham Pharmacia Biotech, St Albans, UK) as previously described [13-15]. Briefly, urine samples from experimental animals were subjected to immunoelectrophoresis at 60 v in an electrophoresis tank containing Barbitone buffer (BDH Laboratory Supplies, Poole, UK), pH 9.5, for 6 h, using a 1% agarose gel (BDH Laboratory Supplies) containing rabbit anti-sera to rat albumin raised in a laboratory. Results were calculated using rat serum albumin standards (which were run at the same time) and expressed in mg per 24 h.

6.3.3.3. Direct immunofluorescence

Deposits of IgG and fibrin within the glomeruli were detected by direct immunofluorescence, as previously described (30)(31)(150). Tissue was embedded in OCT II embedding medium (Miles Inc, Elkhart, Indiana, USA) on cork discs, snap frozen in isopentane (BDH Laboratory Supplies) pre-cooled in liquid nitrogen, and stored at –70 °C. Cryostat sections were cut at 5μm and were incubated with fluorescein isothiocyanate (FITC) labelled rabbit anti-rat IgG (Serotec Ltd), or goat anti-rat fibrin (Nordic Immunology, Tilburg, The Netherlands). The degree of immunostaining, as judged by intensity of fluorescence, was graded from 0 to 3+ by a blinded observer.

6.3.3.4. Light microscopy

Kidney tissue was fixed in 10% neutral buffered formalin, processed, and embedded in

paraffin wax for light microscopy by standard techniques. Briefly, 3 μm sections were stained with haematoxylin and eosin, and periodic acid-Schiff. Fifty glomeruli per section were graded by a blinded observer as: normal, abnormal (small areas of glomerular hypercellularity and/or focal necrosis), or severe (>50% of the glomerulus affected by necrosis and/or crescent formation), and expressed as a percentage of glomeruli examine (30) (31) (150).

6.3.4. Immunohistology

Kidney sections were stained for T cells and macrophages using a standard avidin-biotin complex immunoperoxidase staining technique. Briefly, formalin-fixed, paraffin embedded kidney sections were stained with monoclonal antibodies W3/13 (T lymphocytes), W3/25 ($CD4^+$ lymphocytes), OX8 ($CD8^+$ lymphocytes), and ED1 (macrophages)(Serotec Ltd). Numbers of glomerular T cells and macrophages were detected using a biotinylated secondary antibody and avidin-biotin complex (Dako Ltd, Cambridge, UK). The cellular infiltrate was assessed by a blinded observer by counting the number of positively stained cells per 50 consecutive glomeruli in cross section (150).

6.3.5. Isolation of glomeruli

Glomeruli were isolated as previously described (101) and documented in detail in Section 2.3 (p.26). Microscopic examination confirmed purity of glomeruli of over 90% compared to non-glomerular fragments. Glomeruli were then processed for RNA extraction by standard methods.

6.3.6. Extraction of RNA and reverse transcription

Total RNA was extracted from isolated glomeruli using a modification of the method of Chomczynski and Saachi (102). Samples of glomeruli were dissociated in RNAzol B (Cinna/Biotec, Houston, TX.). RNA was then extracted following the standard protocol. The

final product was air dried, dissolved in DEPC treated water and stored at -80 °C. First strand complementary DNA was synthesised using the M-MLV Reverse Transcription kit (Gibco BRL). One microgram of RNA and random hexamer primers were used to prime the reaction.

Primers for rat TCR Vβ genes were published previously (156) and are documented in Table 2.2 (p.36). The protocol used for PCR is documented in chapter 2 (p. 28).

6.3.7. Detection of PCR products by QPCR System 5000

The specificity of each PCR product was verified by separate hybridisation 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 (104) (105) (157) and presented in further detail in section 2.9 (p. 29). 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 Vβ family member was expressed as a percentage of the total luminosity in all of the Vβ regions for a given sample.

6.3.8. CDR3 spectratyping of PCR products

2 μl of PCR product from each Vβ family was used as cDNA for a second round of PCR. Primers were as before with the addition of a Fam labelled Cβ reverse primer internal to the initial Cβ biotin primer. This proceeded for six to ten cycles. 1 μl of PCR product of this reaction was mixed with 12 μl of deionised formamide and denatured at 95°C for 2 minutes. This was run on an ABI 310 DNA sequencer. The results were analysed using Genescan and Genotyper software.

6.3.9. Cloning and sequencing of PCR products

PCR products of interest were purified using ethanol or isopropanol precipitation and cloned

into the p-Bluescript SK-T vector. Transformed colonies were screened using PCR with M13 primers. Positive PCR products were purified using ethanol precipitation. Sequencing reactions used the ABI Big Dye Terminator DNA sequencing kit. Purified reactions were then run on an ABI 310 DNA sequencer.

6.3.10. CDR3 region analysis

CDR3 sequencing data were stored in a database. Motifs detected on visual inspection were confirmed with database searches using regular expressions.

6.4 Results

6.4.1. Assessment of EAG

Animals immunised with GBM in FCA and killed at week 2 showed detectable circulating anti-GBM antibodies, intermittent linear deposits of IgG on the GBM, moderate proteinuria, slight focal glomerular abnormalities, and an increase in glomerular T cells (mainly $CD4^+$). Animals killed at week 4 after immunisation showed high levels of circulating anti-GBM antibodies, strong linear deposits of IgG along the GBM, marked proteinuria, severe focal and segmental necrotizing glomerulonephritis with extensive crescent formation, and large numbers of glomerular T cells (mainly $CD8^+$) and macrophages. Control animals given FCA alone showed no manifestations of EAG. Results are summarised in Table 6.1 (p.97) and illustrated in Figure 6.1 (p.98).

6.4.2. Vβ repertoires in glomerular T cells

cDNA from glomeruli was amplified with primers specific for rat TCR Vβ families. Vβ repertoires in individual animals showed overexpression of one or two families. Different Vβ families were overexpressed in each individual animal studied. The overall repertoire, combining the results from all seven animals studied (three killed at 2 weeks, four at 4 weeks), showed no consistent Vβ family overexpression across all the animals. A

representative Vβ repertoire from a single animal and the overall repertoire are shown in Figure 6.2 (p.99).

6.4.3. CDR3 spectratyping

Spectratypes from glomerular TCR PCR products showed that in spite of a lack of consistent restriction in Vβ repertoires, the infiltrating T cells were clearly oligoclonal. Many of the Vβ families showed spectratypes with either single peaks or otherwise clearly restricted distributions compared to those of control spleen. Spectratypes from a single representative animal are shown in Figure 6.3 (p.100).

6.4.4. CDR3 motifs

Common CDR3 spectratype peaks were studied further to look for similar T cell clones present in a single Vβ family across those different animals. Six of seven animals showed a restricted spectratype in Vβ 13 containing a size band measuring 370/1 base pairs. These spectratypes are shown in Figure 6.4 (p.100). DNA sequencing of these Vβ 13 products showed significant CDR3 similarities in 5 of these 6 animals. These similarities are shown in the Vβ 13 sequences shown in Motif 2 in Figure 6.5 (p.101). Interestingly, K7 was the only animal not to show a similar sequence in Vβ 13, in spite of having a similarly restricted spectratype. However the same CDR3 motif was subsequently found in Vβ 16. Other Vβ families showing common spectratype bands in different animals were sequenced but failed to show similar CDR3 regions confined to single Vβ families.

DNA sequencing was performed, as above, looking for similar sequences in common CDR3 spectratypes in single Vβ families. Mostly, with the notable exception of Vβ13, this failed to identify similar T cells in different animals. However, when sequence data were studied regardless of Vβ and CDR3 size, multiple CDR3 motifs were noted within the EAG animals. Two of these motifs were entirely absent in control animals. Motifs 1 and 4, from Figure 6.5 (p.101), were each represented by only a single sequence in over 50 CDR3 sequences from

control animals. Test animals showed over representation of motifs in small numbers of sequences from highly oligoclonal spectratypes, suggesting that they are well represented T cell clones within their respective Vβ families. Motifs were detected at both two and four week time points during disease development. Multiple Vβ families and CDR3 sizes were represented in each motif and no particular Jβ gene segment appeared to be overexpressed.

	GBM/FCA		FCA alone
	2 wk	4 wk	2 wk
Circulating anti-GBM antibody (%binding)	60 ± 5	95± 4	21 ± 2
Deposits of IgG on GBM (intensity of fluorescence)	+	+++	-
Proteinuria (mg/day)	15 ± 8	120 ± 32	0.6 ± 0.4
Abnormal glomeruli (%)			
(a) Total	32 ± 10	100	
(b) Severe	7 ± 4	75 ± 6	0
Immunohistochemistry (cells per 50 glomeruli)			
(a) T cells	64 ± 12	135 ± 22	16 ± 7
(b) $CD4^+$ T cells	42 ± 15	5 ± 2	3 ± 2
(c) $CD8^+$ T cells	35 ± 17	120 ± 35	20 ± 6
(d) Macrophages	3 ± 1	150 ± 25	2 ± 1

Table 6.1 Summary of results from WKY rats immunised with GBM in FCA or FCA alone. Results are expressed as a mean ± standard deviation of each group at week 2 and week 4 after immunisation.

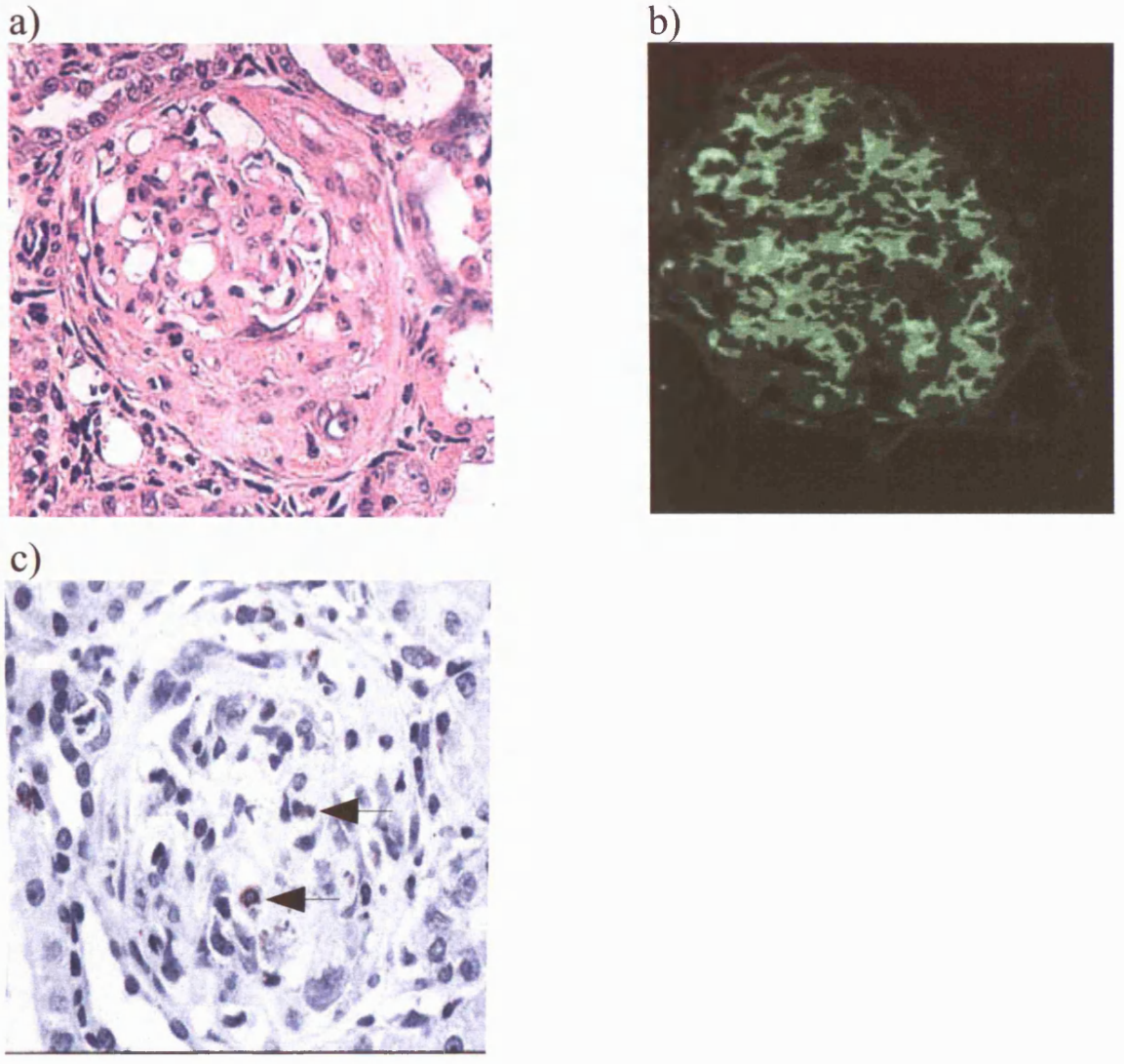


Figure 6.1 Light microscopy of kidney tissue at 4 weeks in WKY rats with EAG showing: a) marked segmental necrosis of the glomerular tuft with crescent formation (H&E x300); b) strong linear deposition of IgG along the GBM (immunofluorescence x300); c) T cells infiltrating the glomeruli and interstitium (immunoperoxidase x300).

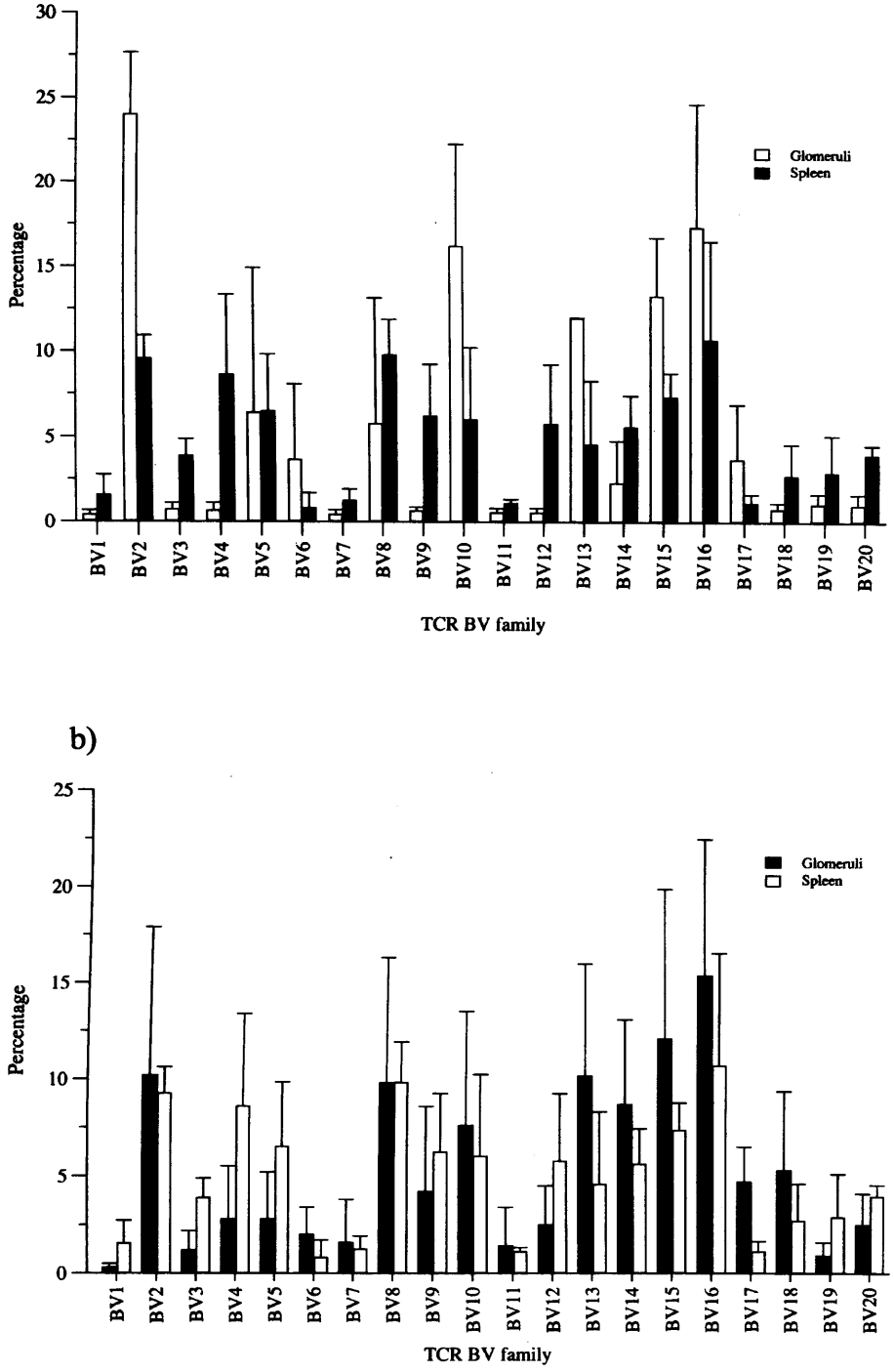


Figure 6.2 Comparison between glomerular and splenic TCR Vβ repertoires. a) glomerular T cell receptor Vβ repertoire from a single animal with EAG compared with the splenic repertoire showing overexpression of Vβ 2 and 13; b) Combined results from all animals showed no significant overall changes to the repertoires across all the animals. Results for all animals are expressed as mean ± SD for each group.

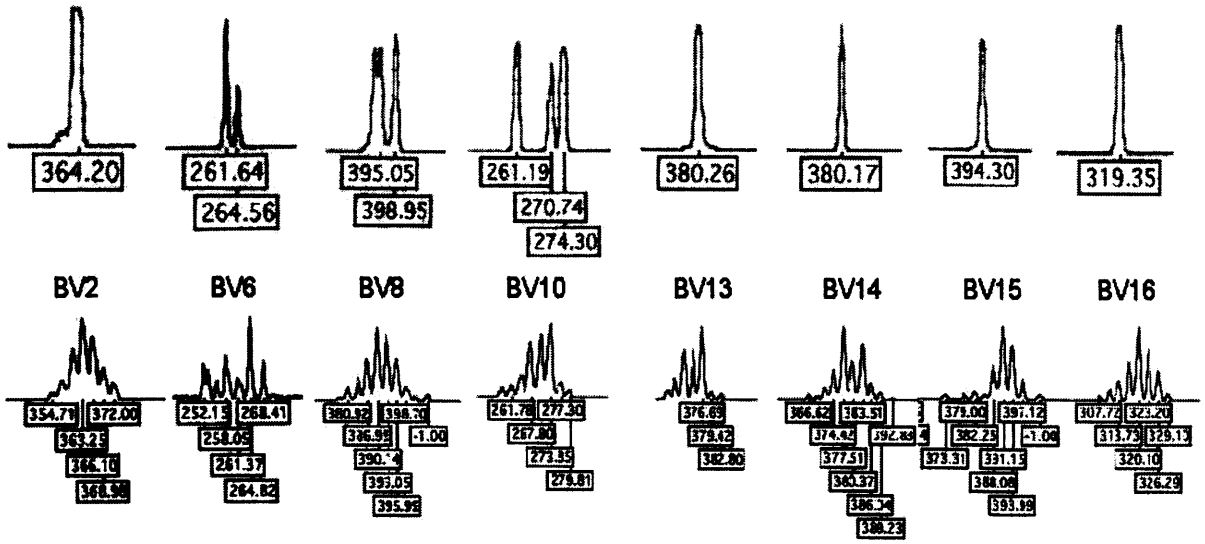


Figure 6.3 CDR3 spectratypes from glomeruli of a single rat with EAG (top) are compared with normal spectratypes from spleen (below) for the corresponding TCR family. Spectratype peaks are labelled with their approximate PCR product length in base pairs. A normal spectratype of a diverse T cell population consists of 7 or more peaks. There is clear restriction in multiple Vβ families in the glomeruli.

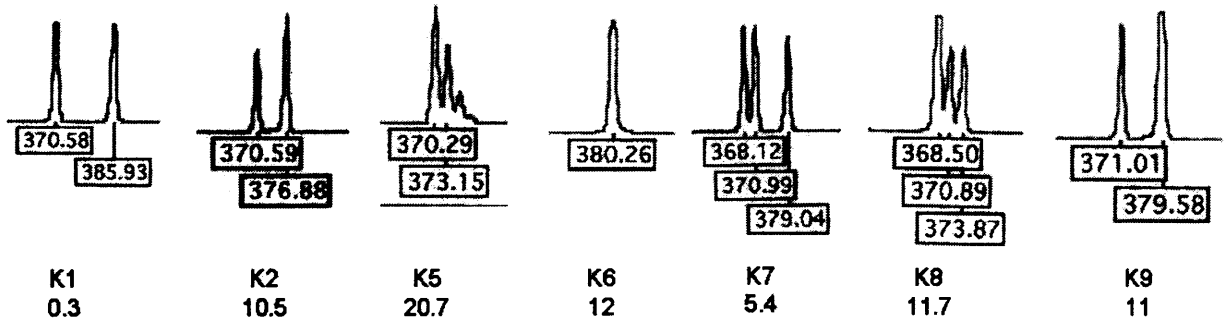


Figure 6.4 Vβ13 spectratypes from 7 animals showed restricted CDR3 spectratypes. Spectratype peaks are labelled with PCR product length in base pairs, animal reference and percentage expression in the TCR Vβ repertoire. A common band is present at 370/1 base pairs in 6 of the 7 animals.

Motif 1

Animal	V beta	CDR3 sequence	J beta	Clonality
K5	15	CGA------P-GQG----NQAQYF	1.5	1 of 3
K1	14	CAWL-----P-GQG----GAEQFF	2.1	3 of 8
K5	15	CGAA-----P-GQG----NTEVFF	1.1	2 of 4
K9	16	CASS-----Q-GQG----RERLFF	1.4	2 of 5
K8	5	CASSQ----P-GQG----GYDYTF	1.2	3 of 4
K5	13	CASS-----P-GTG----VETQYF	2.5	1 of 8
K5	15	CGA------P-GTG----GESATTDKIYF	2.3	1 of 1
K8	5	CASS-----PSGTG----GETQYF	2.5	1 of 4
K5	16	CASS-----PSGTG----NXDYTF	1.2	1 of 1
K2	16	CASS-----PDGTG----QLYF	2.2	4 of 6
Control	16	CASSLG---P-GQG----KNDYTF	1.2	1 of 54
CDR3 Position		12345 10 14 19		

Motif: Position 10: P or Q. 12: G. 13: Q or T. 14: G.

Motif 2

Animal	V beta	CDR3 Sequence	J beta	Clonality
K1	13	CASS--LDGNT---EVFF	1.1	10 of 10
K8	13	CASS--LEGNS---PLYF	1.6	1 of 1
K9	13	CASS--PDGNS---PLYF	1.6	4 of 4
K2	13	CASS--LDGQNT--LFF	2.4	14 of 14
K5	13	CASS--LEGQNT--LFF	2.4	1 of 6
K2	16	CASS--PDGTGQ--LYF	2.2	4 of 6
K7	16	CASS--LDGTEN--TLFF	2.4	1 of 6
CDR3 Position		12345 10 14 18		

Motif: Position 7: L or P. 8: Acidic. 9: G. 10, 11, 12: polar (QNTS)

Motif 3

Animal	V beta	CDR3 sequence	J beta	Clonality
K2	15	CGAK---DWG--QNTLFF	2.4	5 of 7
K2	14	CAWSR--DWGG-SDKIYF	2.3	2 of 2
K5	13	CASSLG-DWGG-LETQYF	2.5	2 of 2
K5	16	CASSPR-DWGG-SFYEQYF	2.6	1 of 1
K9	10	CASR---DWGD-QETQYF	2.5	3 of 3
K1	10	CASSS---WGD-SQNTLFF	2.4	5 of 5
K1	15	CGARV---WGE-RLFF	1.4	1 of 1
K7	10	CASSY---WG--STGQLYF	2.2	1 of 2
K1	10	CASSP---WGG-QETQYF	2.5	5 of 5
CDR3 Position		12345 10 14 19		

Motif: Position 8: D. 9:W. 10: G. 11: G or Acidic.

Motif 4

Animal	V beta	CDR3 Sequence	J beta	Clonality
K2	15	CGA----RDGGG---TEVFF	1.1	2 of 7
K5	15	CGA----RAGGG---AEQFF	2.1	2 of 3
K8	15	CGA----RSGG----DETQYF	2.5	6 of 6
K7	16	CASST--RQGG----YEQYF	2.6	1 of 4
K7	16	CASST--RTGG----AEQYF	2.6	1 of 4
K5	15	CGARI--RTGG----NQAQYF	1.5	2 of 3
K9	15	CGA----RVGG----PNTLFF	2.4	2 of 2
K9	13	CASS---RTGGG-SYAEQFF	2.1	5 of 5
K9	14	CAWS---RLGG-VSYAEQFF	2.1	3 of 3
Control	15	CGA----RTGG----AETQYF	2.5	1 of 54
CDR3 Position		12345 10 14 19		

Motif: Position 8: R. 10, 11: G. 17: E.

Table 6.5 CDR3 amino acid motifs from animals with EAG. Multiple Vβ families and CDR3 sizes are represented in each motif, but no particular Jβ is overexpressed.

6.5 Discussion

These data show clear evidence of T cell restriction in the glomeruli of animals with EAG. This suggests that T cells responding to antigens within the glomerulus may be involved in the pathogenesis of EAG. This may be relevant to human Goodpasture's disease, in view of the extensive immunological and pathological similarities between the two conditions. In EAG induced by GBM, a major autoantigen is α3(IV)NC1 (38) (39), although α4(IV)NC1 (158) may be involved. The nephritogenicity of α3(IV)NC1 has now been demonstrated by several groups (39) (38) (158). In patients with Goodpasture's disease, α3(IV)NC1 is clearly the main target of autoantibodies (145) (37) and the major B cell epitope has been defined (146). The pathogenicity of these anti-GBM antibodies has been clearly demonstrated in mouse and rat models of EAG (41) (159), as in human disease (65). The role of T cells in EAG has been clearly demonstrated in the model induced by rat GBM (150). There is an early phase of $CD4^+$ T cell infiltration, which is followed by $CD8^+$ T cells and macrophages. Similarly, in the human disease, both $CD4^+$ and $CD8^+$ T cells are demonstrated on renal biopsy, but with a predominance of $CD4^+$ cells (160). T cell infiltration has been shown to precede macrophage infiltration, but the time course of $CD4^+$ versus $CD8^+$ T cell infiltration has not been studied. In EAG, T cells have been shown to proliferate to α3(IV)NC1 *in vitro* and antigen specific $CD4^+$ T cell lines are capable of inducing crescentic nephritis (36). However, a role for $CD8^+$ T cells has also been demonstrated by therapeutic studies using anti-CD8 monoclonal antibodies (34). In patients with Goodpasture's disease, there is a high frequency of $CD4^+$ T cells reactive with α3(IV)NC1 (147). However, involvement of $CD8^+$ T cells is suggested by the generation of $CD8^+$ T cell clones reactive with GBM (71). Finally, the renal and pulmonary pathology in EAG and Goodpasture's disease is remarkably similar (144) (150).

Previous studies of oligoclonal T cells thought to be specific for a particular antigen or set of

antigens in autoimmunity have produced variable results. T cells in several animal models of autoimmune disease have been shown to use highly restricted Vβ repertoires. In EAE, initial results suggested the predominant use of Vβ 8.2 in spinal cord lesions (161). Pathogenicity of these T cells was confirmed on adoptive transfer. Subsequent studies, however, implicated multiple other Vβ families, including Vβ 3, 6, 10, 12 and 17 (19). Common CDR3 motifs were identified in experimental autoimmune carditis, but only in a single Vβ family (Vβ 4) (162). Other models have shown the use of multiple Vβ and single Vα genes (163) or vice versa (164). In each of these models, CDR3 motifs, when found to be present, have been confined to one or two Vβ families. In a study of glomerular T cells in Heymann nephritis, we have previously shown similar CDR3 regions in the context of three Vβ families and an overexpression of Jβ 2.6 (165).

This study has shown a restricted set of T cells infiltrating the glomeruli of rats with experimental autoimmune glomerulonephritis. Initial analysis using RT-PCR did not show any overall overexpression of specific Vβ families, although a small number of families were over-represented in each animal. However, subsequent CDR3 spectratype analysis gave clear evidence of oligoclonality within the infiltrating T cells. This was further confirmed by sequencing the spectratypes, which showed a limited number of sequences from each spectratype. Single peak spectratypes generally produced a single sequence of T cell receptor DNA. An initial search for similar T cell sequences within single Vβ families with shared CDR3 spectratypes produced a strong motif present in Vβ 13 in five of seven animals studied. This proved to be the exception rather than the rule, with no other similarities found in four other Vβ families studied. Further study of the sequences, however, showed that when CDR3 regions were compared, irrespective of Vβ family and CDR3 length, strong motifs were present in test animals that were mostly absent from controls. Only two sequences with the CDR3 motifs of interest were found in control

animals across all Vβ families and these were represented by only a single sequence in a highly diverse population. The other two motifs were absent from the control animal CDR3 sequences. Highly similar CDR3 regions were used by the infiltrating T cells though in combination with different Vβ and Jβ gene segments.

This is the first time that CDR3 motifs have been shown across multiple Vβ families in so many animals. This data in EAG shows multiple clear CDR3 motifs present in the majority of animals with EAG across several Vβ families, demonstrating a broader network of similar T cells than has been shown before. Studies of T cell receptor repertoires have previously relied heavily on the relative expression of Vβ families as a simple screening test for oligoclonality. This study shows that, even in the face of inconsistent changes in the T cell repertoire, extensive oligoclonality may exist in infiltrating T cells. An unchanged Vβ family expression at 10% becomes remarkable when spectratyping shows a single CDR3 band (e.g. K6 in Figure 4). The increased availability of CDR3 spectratyping makes more detailed studies of oligoclonal T cells a great deal easier than it was even five years ago. It is perhaps not surprising that studies such as this should show increasingly complex T cell responses.

In human disease, a similar spectrum of T cell responses has been documented. Recognition of myelin basic protein (MBP) amino acids 83-99 presented by HLA-DR2 in humans is mediated by Vβ 13 cells expressing limited CDR3 regions (44). T cells specific for an influenza matrix protein shared overexpression of a single Vβ combined with multiple Jβ and a strong CDR3 motif (166). In EBV specific T cells, three different Vβ families expressed different public CDR3 motifs, all in combination with Vα15. The CDR3 motifs were confined to a single Vβ family. The α chain expressed different CDR3 features depending on its paired β chain (167). Two previous studies in man have shown common CDR3 motifs, but involving only a few individuals (129) (168).

Diseases in which there is overexpression of single Vβ families are likely to be amenable to

TCR-based treatment strategies, including DNA vaccination or antisense therapy to part of the β chain. In a previous study of the glomerular T cells in Heymann nephritis we have demonstrated oligoclonal T cells utilising three Vβ families, Jβ 2.6 and similar CDR3 regions. DNA vaccination against these cells has subsequently been shown to ameliorate disease with a clear decrease in proteinuria and T cell and macrophage infiltration (169).The prospects for T cell based therapy become less clear, however, in more complex responses such as those described here. Neither the Vβ or Jβ are obvious targets for treatment, but there does remain the possibility of designing a specific treatment targeted to the CDR3 motifs. The model of EAG described in this study utilizes immunisation with collagenase digested rat GBM, so animals could be generating a more complex T cell response than might be seen in Goodpasture's disease where the immune response is restricted to α3(IV)NC1 collagen. It is therefore possible that generating EAG with a more defined antigen, as can be done with α3(IV)NC1 (38) (39) (158), may simplify the T cell responses seen and provide a better opportunity to design specific therapy.

Chapter 7. Oligoclonal T cells in human rapidly progressive glomerulonephritis

7.1 Statement

This work is a collaboration between myself and the Renal Laboratories at Leicester General Hospital headed by Professor John Feehally. Renal biopsy material was identified from the Department of Pathology renal biopsy archive held by Professor Peter Furness. Appropriate cases were identified by Dr Alice Smith and Elaine Foster. The archival biopsies were obtained, mRNA extracted and first strand cDNA synthesised prior to transfer of the samples to me for analysis. All further analysis was performed by me.

7.2 Introduction

Rapidly progressive glomerulonephritis is characterised by a crescentic glomerulonephritis with infiltration of mononuclear cells, fibrinoid necrosis of the glomerular capillary tuft and, in renal vasculitis and idiopathic RPGN, a notable absence of immune deposits in the affected glomeruli. Goodpasture's disease is also associated with linear deposition of antibody along the glomerular basement membrane. For a considerable time, the investigation of the role of antibodies had dominated research in this area but there is evidence for the involvement of T cells in the pathogenesis of these diseases both in animals and, increasingly, in humans.

Evidence in animal models has shown that T cells are crucial in the pathogenesis of RPGN. In a model of anti-GBM glomerulonephritis, Tipping et al showed that $CD4^+$ cells were crucial for development of crescentic nephritis. Absence of $CD8^+$ worsened disease (69). A model of crescentic nephritis in the mouse have also showed clear evidence of the importance of T cells in that disease could only be transferred in the presence of T cells. In their absence, antibody was deposited in the glomeruli but no crescentic changes or severe proteinuria occurred (41). More recently, Xiao et al showed direct pathogenic role for ANCA antibodies with specificity for myeloperoxidase in the development of crescentic disease. In

the absence of splenocytes, however, the antibodies were deposited in the glomeruli and produced only a mild proliferative GN with no crescents, indicating the need for cells, most likely T cells, for the development of severe disease (82). Further data in the involvement of T cells in RPGN is documented in the introduction on page 19.

I have analysed renal biopsies samples from patients who suffered from RPGN, extracted their RNA and analysed their TCR repertoires using PCR, CDR3 spectratyping and DNA sequencing of their CDR3 regions.

7.3 Methods

7.3.1. Renal biopsies and PBMC samples

Renal biopsies were identified from a bank of biopsy tissue at the Department of Nephrology, Leicester General Hospital, UK. Diagnoses were either Goodpasture's disease or idiopathic rapidly progressive glomerulonephritis. Peripheral blood mononuclear cells were obtained by venesection of healthy volunteers followed by density gradient centrifugation. Serial dilutions of subsequent PBMC cDNA were performed for comparison with renal biopsy samples in order to control for dilution in the study of oligoclonality of the infiltrating T cells.

7.3.2. RNA extraction and reverse transcriptase PCR

mRNA was extracted from biopsy material using oligo-dT conjugated magnetic beads (Dynal mRNA Direct kit). cDNA was then synthesised using M-MLV reverse transcriptase and random hexamer primers, and stored at -80°C until required. PCR was performed. 2 ml of cDNA was added to a reaction mixture consisting of 10x PCR buffer (Mg^{++} 1.5 mM, Roche), dNTPs 0.2 mM, Taq polymerase 0.03 U/ml, Cβ and Vβ primers to a final concentration 0.3 mM. Primers used in these reactions are presented in Table 1. Samples were run for 40 cycles. 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). Products were then analysed on a 2% agarose gel. Repertoires were analysed using UV densitometry of the agarose gel. Results are expressed as percentage of total Vβ signal in each sample.

7.3.3. CDR3 spectratyping

Two microlitres of PCR product from each Vβ family was used as cDNA for a second round of PCR. Primers were as before with the addition of a Fam labelled Cβ reverse primer internal to the initial Cβ biotin primer. This proceeded for six to ten cycles. 1 μl of PCR product of this reaction was mixed with 12 μl of deionised formamide and denatured at 95°C for 2 minutes. This was run on an ABI 310 DNA sequencer. The results were analysed using Genescan and Genotyper software.

7.3.4. Cloning and sequencing of PCR products

PCR products of interest were purified using ethanol or isopropanol precipitation and cloned into the p-Bluescript SK-T vector. Transformed colonies were screened using PCR with M13 primers. Positive PCR products were purified using ethanol precipitation. Sequencing reactions used the ABI Big Dye Terminator DNA sequencing kit. Purified reactions were then run on an ABI 310 DNA sequencer.

7.3.5. Real time PCR for CCβ and GAPDH

Primers and probes to detect Cβ and GAPDH using real time PCR were designed. The Cβ oligonucleotides were designed using Primer Express Software (Applied Biosystems, CA). The sequences are shown in Table 3.1 (p.44). The protocol used for real time PCR is documented in section 2.16 (p.34).

7.4 Results

7.4.1. Vβ TCR repertoires

Five biopsies surrendered adequate RNA for subsequent analysis. Figure 7.1 (p.112) shows

their Vβ repertoires. Biopsies A to E show differing levels of repertoire diversity. Biopsy E shows the most restriction with only 12 Vβ families detectable. Biopsy D demonstrates a more diverse TCR repertoire with almost all Vβ families represented at similar levels. Four of the five biopsies show a clear degree of restriction in their TCR cDNA.

7.4.2. Oligoclonal CDR3 spectratypes

All detected Vβ families PCR products were run on a high resolution sequencing gel to study their CDR3 spectratypes. All spectratypes from the biopsies showed clear oligoclonality with some striking restriction in multiple Vβ families. These restricted spectratypes are shown in Figure 7.2 (p.114) along with the estimated percentage expression from the TCR repertoire. Under these conditions, many highly restricted spectratypes represent a significant percentage of the total TCR repertoire. This is an indication of a highly oligoclonal population of T cells infiltrating these biopsies.

In order to confirm that the apparent spectratype oligoclonality was not simply due to dilution of the TCR cDNA, PBMC cDNA was diluted to a similar level of CCβ signal and subjected to the same experiment. Real time PCR using Cβ primers was performed using serial dilutions of PBMC in order to ascertain the level of dilution of a diverse T cell repertoire that would be required to produce an equivalent Cβ signal to the renal biopsies being studied. As shown in Table 7.1 (p.115), a 1000 fold dilution of PBMC gave a threshold cycle equivalent to or higher than the biopsies. This sample therefore represents an equivalent or more dilute TCR sample than the biopsies providing a control for oligoclonality. A TCR repertoire was performed followed by CDR3 spectratyping. The resulting spectratypes are shown in the Figure 7.3 (p.116). The PBMC spectratypes are clearly more diverse than those from the renal biopsies. Some single peaks are present but they represent a minor fraction of the total repertoire. Thus, the spectratypes of the renal biopsies are clearly restricted due to TCR oligoclonality rather than overdilution of cDNA or

poor recovery of RNA .

7.4.3. Agarose gel densitometry gives an estimate of the TCR repertoire compared with real time PCR.

Gel densitometry is a limited technique which provides an approximation of a T cell repertoire. The above spectratyping studies raise the question of whether the technique was distorting the Vβ repertoires such that otherwise poorly represented Vβ families were being significantly overestimated. In order to assess the extent of repertoire changes in this technique, I measured a TCR repertoire on 1000-fold diluted PBMC using both real time PCR and agarose gel densitometry. The results are shown in Table 7.2 (p.117). The main question to be addressed was the possible overamplification of poorly represented Vβ families with restricted spectratypes. The results show that agarose gel densitometry gives a 'flatter' repertoire than other measures, reducing high percentage expressions and increasing lower figures. However the overall shape of the repertoire remains intact. Under-represented Vβ families continue to be under-represented. There is no evidence to show that agarose gel densitometry grossly distorts the overall Vβ repertoire.

7.4.4. Sequencing results

Restricted and well represented Vβ families in the biopsy repertoires were cloned and sequenced. The results in Table 7.3 (p.118) show the ten most strongly represented sequences for each sample. The percentage of TCR signal is calculated from the percentage expression of the Vβ family multiplied by the proportion of TCR clones from that family that carried the identical sequence. The results show clonally expanded T cell sequences in each sample, which account for a highly significant proportion of the total T cell signal within the sample. These results suggest that relatively few T cell clones may account for a striking percentage of the total T cell receptor signal within kidneys with RPGN.

7.4.5. Shared CDR3 motifs in multiple biopsies

Sequencing of the biopsies also revealed that there are clear shared CDR3 motifs in the TCRs infiltrating the kidneys in RPGN. These are shown in Table 7.4 (p.119). Not only are these shared motifs present, but they are also in the five most common CDR3 sequences detected within each biopsy. This would suggest that they are likely to represent T cells that are either highly activated expressing large amounts of TCR mRNA or present in the tissue in disproportionate numbers. Either way, they are likely to be involved in disease pathogenesis.

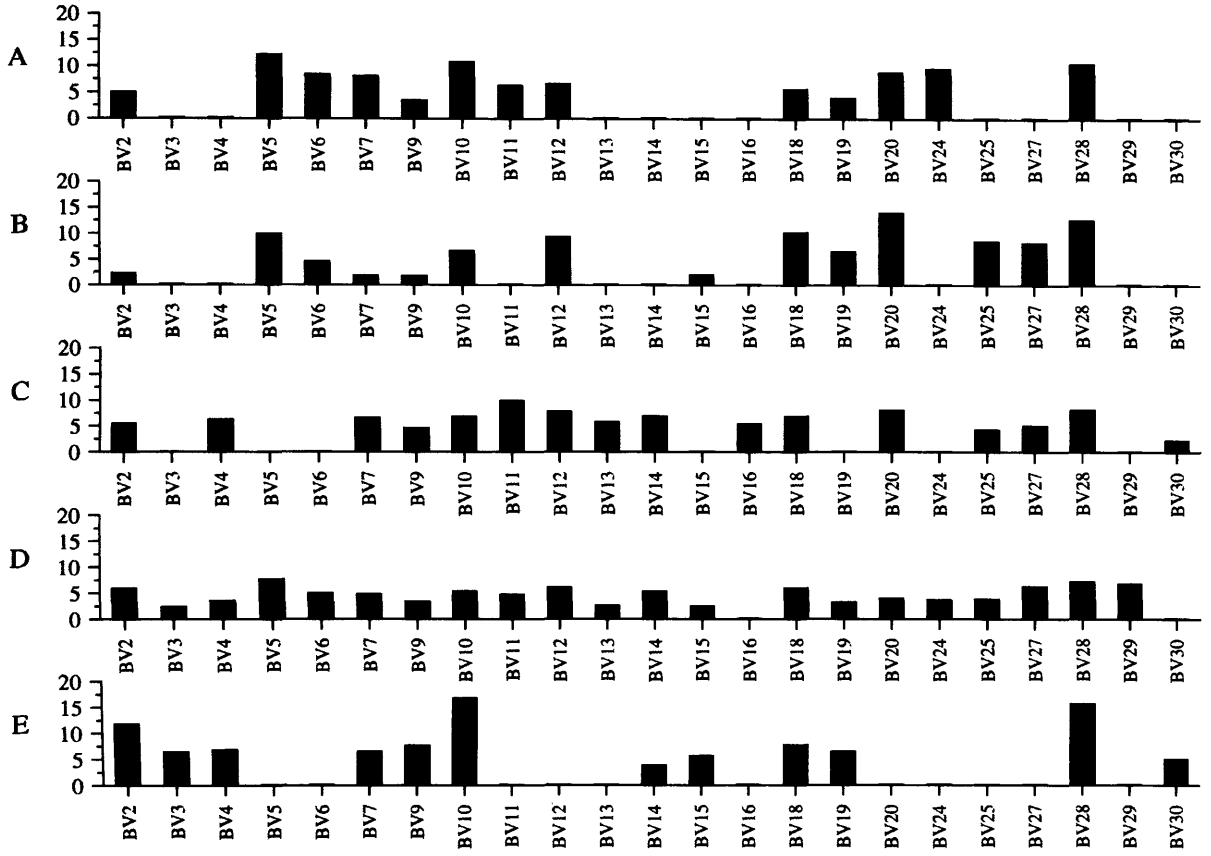
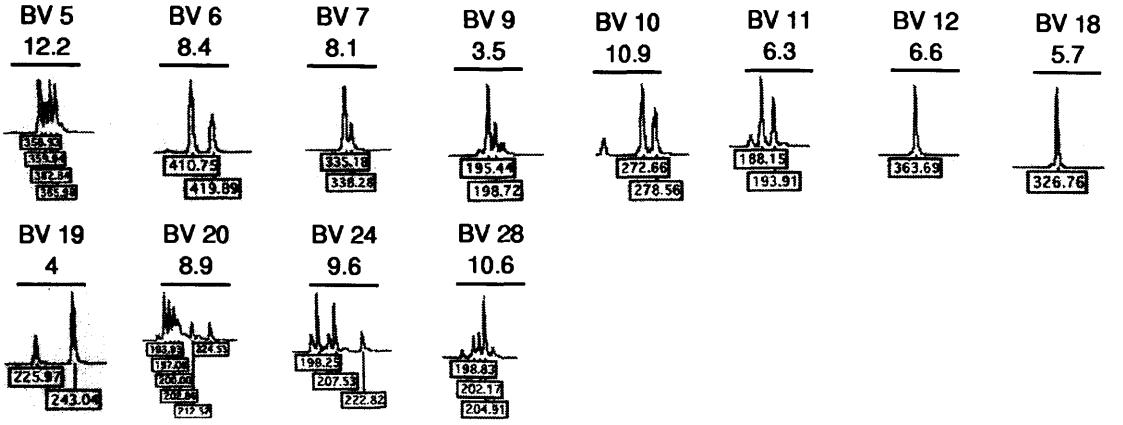
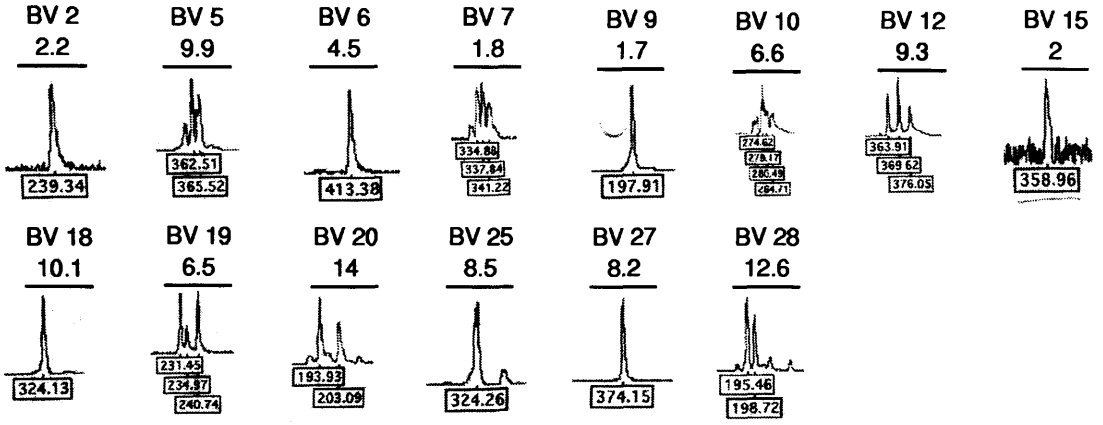


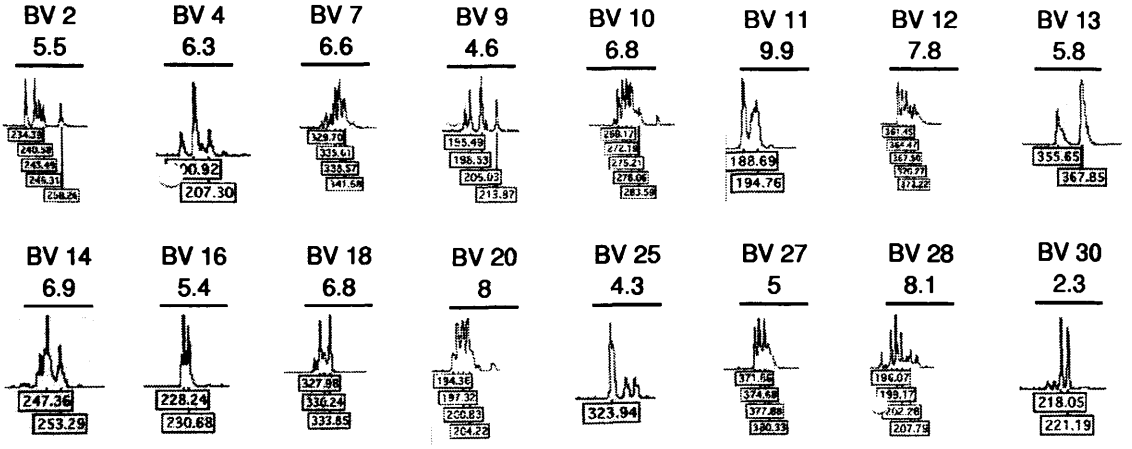
Figure 7.1. TCR repertoires for biopsies A to E. Four of five biopsies show some restriction in the expression of Vβ families. No particular Vβ overexpression is seen across biopsies.



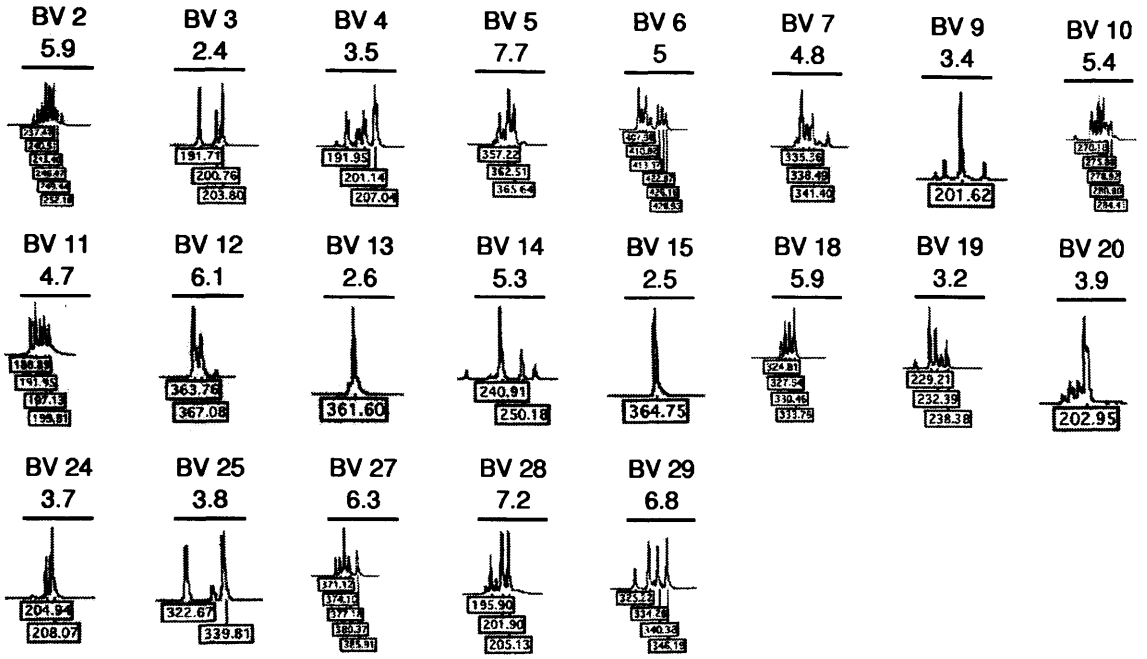
Biopsy A spectratypes



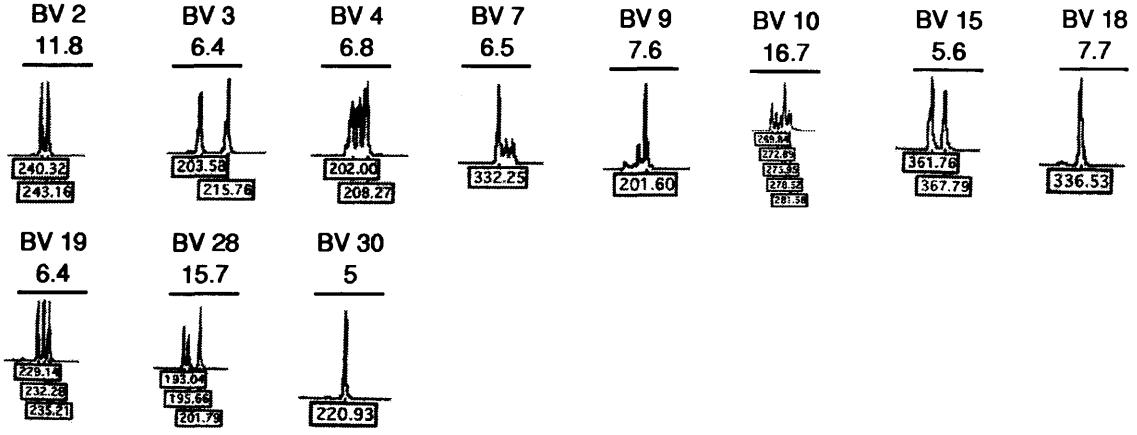
Biopsy B spectratypes



Biopsy C spectratypes



Biopsy D spectratypes



Biopsy E spectratypes

Figure 7.2. Spectratypes from biopsies A to E. Each spectratype is labelled with BV family name and estimated percentage expression by PCR. Multiple restricted CDR3 spectratypes are demonstrated in each biopsy. Many of these are strongly expressed within the biopsy as indicated by their percentage expression within the repertoire. Single peak spectratypes generally represent single clones of T cells.

	GAPDH Ct	CC beta Ct
Peripheral blood dilutions		
PBMC 10	24.6	24.5
PBMC 100	27.7	27.7
PBMC 1000	31.4	31.7
PBMC 10000	34.7	35.1
Renal biopsies		
A	27.2	31.4
B	25.3	31.5
C	25.3	29.6
D	25.4	29.1
E	26.0	32.2

Table 7.1. Real time PCR threshold cycle numbers for GAPDH and CCβ in serial dilutions of PBMC and archival renal biopsies. Biopsy samples show a CCβ signal equivalent or greater than a 1000 fold dilution of PBMC cDNA.

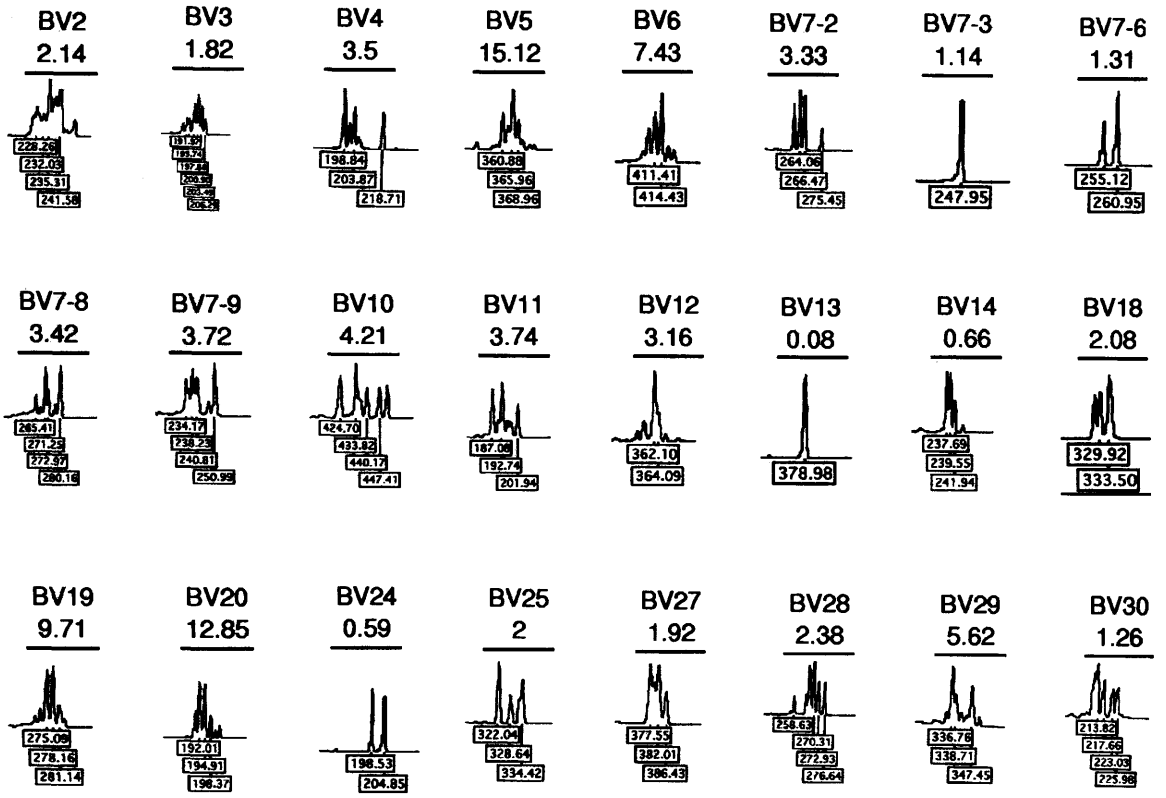


Figure 7.3. Spectratyping of a 1000 fold dilution of PBMCs shows a considerably more diverse T cell population than in renal biopsy samples with an equivalent CCβ signal. Single peak spectratypes are represented at a low level in the TCR repertoire as demonstrated by a low percentage expression.

	Percentage expression in TCR repertoire	
Vβ family	Gel	Real time
BV 2	3.87	1.74
BV 3	**0.79**	**0.77**
BV 4	2.7	3.19
BV 5	3.48	9.20
BV 6	1.48	6.09
BV 7-2	3.19	4.27
BV 7-3	4.22	1.93
BV 7-6	0.77	1.49
BV 7-7	**0.67**	**0.19**
BV 7-8	**2.21**	**0.62**
BV 7-9	7.62	11.01
BV 9	5.48	5.05
BV 10	3.35	1.80
BV 11	7.62	4.28
BV 12	6.23	1.67
BV 13	**3.89**	**0.33**
BV 14	5.33	1.21
BV 15	**1**	**0.19**
BV 16	**1.96**	**0.71**
BV 18	3.63	2.35
BV 19	3.44	9.66
BV 20	6.31	19.84
BV 24	0.86	1.64
BV 25	**0.75**	**0.05**
BV 27	6.18	3.42
BV 28	**2.59**	**0.66**
BV 29	8.08	5.58
BV 30	2.31	1.07

Table 7.2. A comparison between TCR repertoire analysis using agarose gel densitometry and real time PCR. PCR was run for 40 cycles in triplicate. Percentages were calculated by dividing each Vβ family signal by the total measured signal multiplied by 100. Figures in bold show families measured at less than 1% by real time PCR and their corresponding densitometry figures.

Sample	BV	CDR3 sequence	BJ	Clonality	Length	Percentage	Cumulative Percentage
A	12	CASSPPRGFTEAFF	1.1	5 of 5	14	6.6	6.6
	18	CASSRRDDPSGYTF	1.2	6 of 7	14	4.89	11.49
	6	CASARARDRTDTQYF	2.3	8 of 16	15	4.8	16.29
	7	CASSLGAGHETQYF	2.5	5 of 11	14	3.68	19.97
	19	CAAGTAKETQYF	2.5	3 of 5	12	2.4	22.37
	6	CASSYGGHEQYF	2.7	4 of 16	12	2.4	24.77
	6	CASRKGLTYEQYF	2.7	4 of 16	13	2.4	27.17
	7	CASSLSTGVDEQFF	2.1	2 of 11	14	1.47	28.64
	7	CASIPSAGGYNEQFF	2.1	2 of 11	15	1.47	30.11
	19	CASSIGPLAGGPAGELFF	2.2	2 of 5	18	1.6	31.71
B	25	CASSDGGAQPQHF	1.5	9 of 9	13	8.5	8.5
	27	CASSLSHRQGPGFF	2.1	6 of 6	14	8.2	16.7
	20	CSASGLAGEISFYNEQFF	2.1	4 of 8	18	7	23.7
	18	CASSPNRGTEAFF	1.1	7 of 11	13	6.43	30.13
	28	CASSLQARRETQYF	2.5	6 of 12	14	6.3	36.43
	28	CASSYKGYYGYTF	1.2	6 of 12	13	6.3	42.73
	6	CASGRRVNEKLFF	1.4	1 of 1	13	4.5	47.23
	18	CASSPFGEETQYF	2.5	4 of 11	13	3.67	50.9
	20	CSARSPGYEQYF	2.7	2 of 8	12	3.5	54.4
	20	CSAKYGLAGSYEQYF	2.7	2 of 8	15	3.5	57.9
C	18	CASSPTGYFETQYF	2.5	11 of 16	14	4.68	4.68
	25	CASSVRGKETQYF	2.5	2 of 2	13	4.3	8.98
	4	CASSQDWGEQGAYEQYF	2.7	2 of 3	17	4.2	13.18
	11	CASSLGESQPQHF	1.5	3 of 10	13	2.97	16.15
	14	CASSPGPRPHEQYF	2.7	5 of 12	14	2.88	19.02
	16	CASSQSQGAKNIQYF	2.4	6 of 12	15	2.7	21.72
	16	CASSSLAGAFYDEQFF	2.1	6 of 12	16	2.7	24.42
	13	CASSFIVRSSYNEQFF	2.1	2 of 5	16	2.32	26.74
	14	CASSPGLGREQYF	2.7	4 of 12	13	2.3	29.04
	4	CASSQSSGSSEKLFF	1.4	1 of 3	15	2.1	31.14
D	12	CASRPQGAYYGYTF	1.2	2 of 4	14	3.05	3.05
	12	CASSFGQGYRELFF	2.2	2 of 4	14	3.05	6.1
	13	CASSHGTGGDEQYF	2.7	6 of 6	14	2.6	8.7
	15	CATSSLLGQPYNEQFF	2.1	6 of 6	16	2.5	11.2
	14	CASSQTGVRLFF	1.4	2 of 6	12	1.77	12.97
	9	CASSGTVRMNTEAFF	1.1	2 of 4	15	1.7	14.67
	3	CASSLYGEQFF	2.1	4 of 6	11	1.6	16.27
	14	CASSQTGVKLFF	1.4	1 of 6	12	0.88	17.15
	14	CASSQVAGAPETQYF	2.5	1 of 6	15	0.88	18.03
	14	CASSQAGVRLFF	1.4	1 of 6	12	0.88	18.92
E	2	CASSERSLGAEAFF	1.1	3 of 4	14	8.85	8.9
	28	CASSLVLPLNEKLFF	1.4	3 of 6	15	7.85	16.7
	18	CASSPPRTLSGANVLTF	2.6	5 of 5	17	7.7	24.4
	7	CASSLVEVQPQHF	1.5	1 of 1	13	6.5	30.9
	9	CASSFSGTKGDEQFF	2.1	7 of 9	15	5.91	36.8
	28	CASSPGSPDTGELFF	2.2	2 of 6	15	5.23	42.0
	30	CAWSVQGTPEAFF	1.1	9 of 9	13	5	47.0
	3	CASSPGTAVNEKLFF	1.4	7 of 9	15	4.98	52.0
	15	CATSRDLVGTVGNEQFF	2.1	2 of 3	17	3.73	55.8
	2	CASTGNGQETQYF	2.5	1 of 4	13	2.95	58.7

Table 7.3. The ten most strongly expressed T cell clones in each biopsy make up a significant proportion of TCR mRNA signal in each biopsy as estimated by PCR and densitometry.

Biopsy	*BV*	*CDR3 sequence*	*BJ*	*Clonality*	*CDR3 Length*	*Percentage*
A	12	CASS **P-PR**G-FTEAFF	1.1	5 of 5	14	6.6
B	18	CASS **P-NR**G-TEAFF	1.1	7 of 11	13	6.43
C	14	CASS **PGPR**P-HEQYF	2.7	5 of 12	14	2.88
E	18	CASS **P-PR**T-LSGANVLTF	2.6	5 of 5	17	7.7

Table 7.4. Similar CDR3 sequences from 4 of 5 archival renal biopsies. All clones were within the 5 most represented sequences in each biopsy.

7.5 Discussion

I have shown that T cells infiltrating the kidneys of patients with rapidly progressive glomerulonephritis are restricted in their TCR Vβ expression and have restricted CDR3 spectratypes. The ten most frequently expressed T cell clones appear to make up a considerable proportion of the mRNA signal in each biopsy, accounting for between 18 and 55% of the total TCR signal. The restriction of spectratypes is not due to cDNA dilution since manual dilution of a diverse T cell repertoire produces much more diverse spectratypes. There may be some debate as to the use of agarose gel densitometry to assess the TCR repertoires since this is clearly not the method of choice. However a control experiment with diverse TCR cDNA shows that it does not skew the repertoire to any great extent. Further evidence from the spectratypes of diluted PBMC also suggests that restricted spectratypes with high levels of expression within the repertoire are likely to be genuine since there are none within the control experiment.

The use of real time PCR, spectratyping and sequencing to document oligoclonal T cell infiltration is not new. However, the demonstration of the cumulative level of mRNA expression of the top 10 T cell clones has not been done previously. This data strongly suggests that the network of infiltrating T cells in a tissue driving an autoimmune disease may be a great deal smaller than previously assumed. This leads to the possibility of a smaller T cell target to interrupt the disease. Interruption of 5 to 10 T cell clones in an individual is clearly a viable option for selective T cell immunotherapy. Data from animal models including my own, documented in chapter 5, has shown that interruption of a very limited set of T cells defined by their TCR can have a profound impact on disease pathogenesis.

The limitations of this study are mainly methodological. Very limited cDNA was available for study from these biopsies frozen for some years. Real time PCR was not available to us

at the commencement of the study thus some compromises had to be made with regards to quantification of mRNA. Standard PCR usually requires several PCR experiments to eliminate the possibility of over amplification of cDNA. This was not feasible since this process necessarily utilises much larger quantities of cDNA than were available to me. Some degree of overamplification within these results is therefore highly likely. The extent of this does not appear to negate the remarkable results. Control experiments are clear in showing that a diverse TCR sample diluted to similar or greater levels would not produce the results I have demonstrated. Clearly this needs to be confirmed in subsequent real time PCR experiments on more recently acquired renal biopsy tissue with adequate controls. This project is currently ongoing, utilising the method described in chapter 3.

This study suggests that a highly restricted set of T cells may be responsible for the majority of a T cell infiltrate in the kidneys of patients with rapidly progressive glomerulonephritis and that selective T cell immunotherapy may be possible on an individual patient basis. The interruption of ten T cell clones is a potential treatment option. Further studies are needed to confirm this result.

Chapter 8. Summary and Conclusions

8.1 Summary

This thesis sets out to identify potentially pathogenic T cells in kidney tissue with a view to the design of novel T cell specific immunotherapy. The glomerular T cells found in two animal models are analysed on the basis of the TCR Vβ expression. The results of one of these models is then used to design a DNA vaccination treatment which is shown to ameliorate the disease. A study of human renal biopsy tissue shows infiltrating oligoclonal T cells are also clearly identifiable by their Vβ chains in human RPGN. A method to analyse human TCR Vβ chain expression is described using real time PCR. This will enable subsequent human studies to be more accurately quantified and forms the basis for further work in this area.

The study of glomerular T cells in HN (chapter 5, p.71) was based upon the premise that infiltrating glomerular T cells had been described coincident with the development of proteinuria in this model. Glomeruli were isolated and mRNA extracted to identify these potentially pathogenic T cells. The analysis demonstrated the presence of T cells with a high degree of oligoclonality in multiple Vβ families. There was only limited evidence of a public response across multiple animals in spite of the fact that the model is generated in an inbred strain of rats. Further analysis of the T cells using DNA sequencing of CDR3 sequences showed a group of T cells bearing CDR3 regions of equal length. These regions showed significant restriction in the selection of amino acids at several positions, suggesting that they may be part of a population of T cells responding to a nephritogenic antigen. T cells bearing TCR Vβ 5, 7 and 13 and Jβ 2.6 with a CDR3 length of 6 amino acids were identified in this context.

Having suggested that these infiltrating T cells in HN may be pathogenic, we designed a study to attempt to interrupt them. DNA vaccines based upon the PCR products of Vβ 5, 7

and 13 families in conjunction with Jβ 2.6 were designed, ligated and purified. Test animals were vaccinated prior to induction of HN. The results showed a significant reduction in proteinuria in vaccinated animals. This was associated with a reduction in infiltration by $CD8^+$ T cells and macrophages and a reduction in IFN-γ expression in the glomeruli. Infiltrating T cells in vaccinated rats showed an increase in the diversity of their CDR3 regions in Vβ 5, 7 and 13. This suggests that either the infiltration or expansion of specific oligoclonal T cells was prevented by vaccination. DV treatment had no apparent effect on the infiltration of $CD4^+$ T cells or the deposition of anti-Fx1A antibodies in the glomeruli. The mechanism of action of DV was investigated, showing the development of specific anti-TCR antibodies in vaccinated animals with a reduction in the expression of IFN-γ. This study has demonstrated that the initial study into glomerular T cells is highly likely to represent a real population of pathogenic T cells infiltrating the glomeruli and that DNA vaccination is a potentially useful therapeutic tool.

A study into a second animal model, EAG, also showed extensive T cell oligoclonality in multiple Vβ families. In this case, there was not an obvious population of T cells to be targeted with specific therapy applicable to multiple animals. Each individual animal carried a different infiltrating T cell repertoire in its glomeruli, each overexpressing different Vβ families. CDR3 spectratyping showed highly restricted spectratypes in multiple TCR families. DNA sequencing demonstrated several CDR3 motifs expressed in conjunction with multiple Vβ families. Clearly this particular result makes it difficult to envisage a treatment that could be used in a group of animals or patients. It does not, however, exclude the possibility of individualised therapy based upon Vβ TCR expression.

The same approach was then used to study infiltrating T cells in archival renal biopsy tissue from patients with RPGN. This was performed on whole renal tissue rather than glomeruli. Standard PCR was used without calibrating experiments to avoid overamplification. This

was due to the small amounts of cDNA available for experimentation. The results showed a variable level of restriction in the Vβ repertoire expression with some biopsies having a very limited repertoire and others fairly broad. Subsequent CDR3 spectratyping showed that even where the Vβ repertoire did not appear to be restricted, there was striking evidence of oligoclonality. This is a clear demonstration of the fact that TCR Vβ repertoires when used alone are a very insensitive tool for the detection of T cell clones within tissue. Combining the repertoire and spectratyping data, I have suggested that oligoclonal T cell mRNA may make up a significant proportion of the Vβ mRNA signal within a tissue. The ten most frequent mRNA species, presumably reflecting ten T cell clones, appeared to account for between 20 and 59% of total TCR Vβ mRNA signal. This suggests that designing a T cell therapy for an individual may require the interruption of relatively few T cell clones to induce a clinical effect.

The main weakness of this human study is the inadequate quantification involved in standard PCR of small quantities of mRNA. In order to address this for future studies, a real time PCR experiment was performed using a re-designed set of Vβ primers to compare a fluorogenic probe with SYBR green, a non-specific dye which binds all ds-DNA. The primers were shown to work well in conjunction with the fluorogenic probe and SYBR green. The SG, however, failed to work well on using samples which were either of too low a signal or mixed with non-TCR cDNA. The fluorogenic probe is therefore the appropriate method to use for subsequent experiments on small amounts of renal biopsy tissue.

8.2 Future studies.

The results presented in this thesis suggest several lines for further research. The finding that DNA vaccination ameliorates the development of Heymann nephritis is interesting. It remains a study in prevention of disease which does not address truly therapeutic issues. Further studies need to address therapy with treatment given after the onset of disease. This

would more fairly represent the scenario of treatment within the human population. The identification of T cells in renal tissue or glomeruli does not in itself demonstrate pathogenicity. Neither does the interruption of a group of T cells demonstrate pathogenicity of the entire interrupted population. Other studies envisaged would address the difference in TCR repertoires and detectable T cell clones in both activated T cells and populations of regulatory T cells in peripheral blood. An analysis of regulatory T cells within renal tissue is necessary to prevent the targeting of these cells in any potential future therapeutic manoeuvres.

Further studies are required to confirm the results of the human renal biopsy study. The real time PCR for TCR Vβ families needs to be performed on relatively fresh biopsy samples with subsequent CDR3 analyses to demonstrate the relative levels of T cell clones. Analyses need to be carried out on a wide range of glomerulonephritides including ANCA associated vasculitis, Goodpasture's disease, IgA nephropathy, membranous glomerulonephritis, and the renal manifestations of systemic lupus erythematosus. Once T cell clones have been identified in an individual patient, clonotypic PCR can be designed to detect the clone in PBMC or urine. In the case of progressive or relapsing diseases, the time course of these T cell clones can then be studied in relation to their clinical course. Results from animal models suggest that even in an inbred population, different animals utilise different Vβ families and CDR3 sequences to drive the same immunological process. The same is therefore highly likely to be true in an outbred population such as humans. It then seems unlikely that T cell therapies based on Vβ families will be anything other than an individualised therapeutic option. Multiple questions require answering prior to that becoming reality. The studies suggested above would answer the question of whether specific T cell clones can be identified and tracked during the disease. Other studies would need to address whether the same T cell clones are used in serial relapses of the same disease

in an individual patient and whether interruption of these clones could be used to treat the disease.

8.3 Conclusions

The findings in this thesis demonstrate that restricted populations of oligoclonal T cells are detectable in animal models of renal disease and in human renal biopsies. In Heymann nephritis, DNA vaccination based on TCR Vβ expression is shown to be effective in preventing disease. This suggests that the glomerular T cells identified are likely to be pathogenic. A limited number of T cell clones appear to be responsible for driving the immune process in human rapidly progressive glomerulonephritis. Individualised Vβ specific T cell immunotherapy may be a viable treatment option for patients with T cell mediated glomerulonephritis.

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