Kidney International, Vol. 49 (1996), pp. 1222–1230

Heterogeneous T cell receptor Vβ gene repertoire in murine interstitial nephritis

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Heterogeneous T cell receptor Vβ gene repertoire in murine interstitial nephritis. Anti-tubular basement membrane disease (αTBM) produces T cell-mediated interstitial nephritis in SJL/J mice following immunization with heterologous renal tubular antigen. Initial mononuclear infiltrates appear *in vivo* after six to eight weeks, with subsequent progression to renal fibrosis and endstage kidney disease. Cultured lymph node derived nephritogenic T cells from these mice react to a small epitopic region of the 3M-1 target antigen and share a common amino acid motif in their Vβ CDR3 regions. We now have used RT-PCR to further characterize the renal expression of T cell receptor (TcR) Vβ gene repertoires during the course of this disease. Individual kidneys with focal mononuclear infiltrates characteristic of early αTBM disease express up to three different TcR Vβ genes; however, the same Vβ genes are not found in all kidneys at the same early stage of injury. DNA sequencing of the Vβ RT-PCR products reveals a heterogeneous population of VDJ recombinations and deduced CDR3 amino acid sequences. Our studies do not support TcR Vβ region gene restriction in histologically-detectable αTBM disease, but are more consistent with a dynamic, organ-specific autoimmune disease, directed at multiple autoantigenic epitopes.

Analyses of T cell receptor (TcR) variable (V) region gene repertoires have provided important information regarding the basic pathophysiology of autoimmunity, and have resulted in specific immunotherapeutic interventions for the treatment of autoimmune diseases [1]. Initial studies performed in experimental allergic encephalomyelitis (EAE) showed that the TcR Vβ gene repertoire of autoreactive, encephalitogenic T lymphocytes was highly restricted, and that specific anti-TcR Vβ-region antibodies could successfully ameliorate progressive disease [2, 3]. Confirmation of this work by others led to formulation of the "V-region hypothesis," which suggests that a single epitope of a single antigen results in clonal expansion of a small number of autoreactive lymphocytes (all utilizing the same V-region), which then cause disease [4]. More recent work in both animals and humans has demonstrated that TcR V gene repertoires in autoimmune diseases may not be as restricted as originally anticipated [4–10]. There has been little published data, however, regarding the TcR V-region gene repertoire in autoimmune renal disease [11, 12], and those studies that have been performed have focused on analysis of *in vitro* cultured T cell clones, not diseased kidneys [11, 12].

We have been studying a model of T cell-mediated autoimmune interstitial nephritis in mice, called anti-tubular basement membrane disease (αTBM disease), which is induced by immunization with heterologous renal tubular antigen in complete Freund's adjuvant (RTA/CFA) [13, 14]. Initial T cell infiltration occurs after six to eight weeks, and progresses to interstitial fibrosis, end-stage renal disease and death within 16 to 20 weeks [13, 14]. The target antigen, 3M-1, is a 48 kDa glycoprotein found on the basolateral surface of proximal tubular epithelial cells [15, 16]. Prior studies have revealed that a 3M-1-reactive, MHC II-restricted, $CD4^+$ helper T cell initially emerges after immunization, but that this cell is unable to directly transfer disease [17]. Instead, the $CD4^+$ helper cell induces a 3M-1-reactive, MHC I-restricted, $CD8^+$ effector T cell that mediates delayed-type hypersensitivity (DTH), renal proximal tubular cytotoxicity, and is capable of directly transferring disease upon adoptive transfer to naive syngeneic animals [17–19].

Our recent interests have been focused on analysis of the TcR V gene repertoire of nephritogenic T cells in this disease model, in an attempt to identify potential targets for specific immunotherapeutic interventions. We have previously isolated and characterized a panel of nephritogenic $CD4^+$ helper T cell clones from peripheral lymph nodes of animals immunized to get αTBM disease [12]. These T cell clones all recognize a single 14 residue immunodominant epitope contained within the target antigen, 3M-1 [12]. Analysis of TcR Vβ gene usage by these clones revealed the presence of multiple Vβ genes, with some preferential use of Vβ14, and evidence of common amino acid sequences across the putative CDR3 regions [12]. In order to extend these *in vitro* findings to a well-characterized *in vivo* model system, and to evaluate the TcR Vβ gene repertoire within the diseased organs themselves, we have undertaken a semiquantitative reverse transcription polymerase chain reaction (RT-PCR) analysis of the TcR Vβ gene repertoire throughout the course of anti-TBM disease.

Methods

Antigens

Rabbit renal tubular antigen (RTA) was isolated by previously published methods, and emulsified at 4 mg/ml with complete

Received for publication October 4, 1995
and in revised form November 28, 1995
Accepted for publication November 30, 1995

Freund's adjuvant (2 mg/ml of purified protein derivative from Connaught Laboratories Ltd, Toronto, Ontario, Canada mixed with incomplete Freund's adjuvant purchased from Gibco BRL, Grand Island, NY, USA) to produce RTA/CFA.

Immunization

SJL mice (H-2^s) four to six weeks of age (Jackson Laboratory, Bar Harbor, ME, USA) were immunized with RTA/CFA in multiple sites to get αTBM disease. Some animals were immunized with CFA alone as controls.

Antibodies

Fluorescein (FITC)-conjugated and phycoerytherin (PE)-conjugated antibodies to murine CD3 [20], TcR Vβ2 [21], TcR Vβ4 [21, 22], TcR Vβ6 [21, 23], TcR Vβ7 [21, 24], and TcR Vβ14 [25] were purchased from Pharmingen (San Diego, CA, USA). FITC-conjugated anti-TcR Vβ8.1, 8.2 [26] was a kind gift of F. Heinzel (Dept. of Medicine, Cleveland VA Med Center, Cleveland, OH, USA).

Fluorescent activated cell sorter analysis (FACS) of peripheral lymphocytes

Pooled single cell suspensions of whole spleens were made after pushing each organ through a 60 mesh metal screen, and washing with DMEM/10% fetal bovine serum (Sigma, St. Louis, MO, USA). Enrichment for lymphocytes was performed by running the cell suspensions through Lympholyte-M (Cedar Lane Laboratories, Ltd., Hornsby, Ontario, Canada), as per the manufacturer's recommendations. One to 2×10^6 cells were incubated with a 1:100 dilution of labeled antibody in 50λ of PBS/0.1% BSA for 30 minutes on ice. The cells were washed three times with phosphate buffer saline (PBS) 0.1% BSA, suspended in 1 ml of freshly made 1% paraformaldehyde, and stored covered in foil at 4°C. FACS analysis was performed on a Becton/Dickinson flow cytometer and analyzed with C30 software.

Organ harvest

At various time points after immunization, the animals were sacrificed, and the organs perfused with 30 milliliters of phosphate buffer saline (PBS) via intracardiac puncture, to eliminate peripheral blood contamination. Each kidney was divided in half: one portion was snap frozen and used for polymerase chain reaction (PCR) analysis of Vβ genes, while the other portion was placed in formalin for histologic analysis. Slides were prepared and stained with hematoxylin and eosin by standard methods. Each slide was evaluated by a blinded investigator for the extent of disease involvement using the following scoring method: 0, no disease; 0.5, focal areas of lymphocytic infiltrates; 1, lymphocytic infiltrates in less than 10% of the renal cortex; 2, lymphocytic infiltrates in less than 25% of the renal cortex; 3, lymphocytic infiltrates comprising 50 to 75% of the renal cortex; 4, extensive infiltrates of greater than 75% of the renal cortex with tubular atrophy and scarring [17]. The spleen from each animal was also harvested and frozen for use in PCR experiments.

Polymerase chain reaction (RT-PCR) for TcR Vβ genes

Total RNA was prepared from snap frozen kidney or spleen tissue using RNAstat (Tel-Test Inc., Friendswood, TX, USA) as per manufacturer's recommendations. Oligo dT-primed first strand synthesis was performed with MMLV reverse transcriptase [27]. PCR was performed in 100λ total volume containing 10λ of 10× buffer (Perkin-Elmer Cetus, Exton, PA, USA), 200 μM dGTP, dCTP, dATP, dTTP, 0.25 λ *Taq* polymerase (Perkin-Elmer Cetus), 1 μ of RNA/cDNA template, 40 pmol of 3′ Cβ antisense primer PH12 (5′CTCAAACAAGGAGACCTTG3′) and 40 pmol of 5′ sense Vβ region primer. The 5′ Vβ amplification primers for Vβ1-Vβ19 were derived by William Smoyer and Carolyn Kelly (University of Pennsylvania, Philadelphia, PA, and University of Calfornia San Diego, San Diego, CA, USA) using a matrix analysis (MacVector sequence analysis software for the Macintosh, IBI, New Haven, CT, USA) and the murine Vβ gene sequences (manuscript in preparation). Each primer is a 20 mer designed to have minimal cross-reactivity to all other Vβ region genes and encodes a product of unique predicted size. The Vβ20 primer was designed later from the published sequence using the same criteria, and has the following sequence: 5′GCAGTTACACAGAAGCCAAG3′. Predicted sizes for PCR products using the 3′ PH12 primer and each of the 5′ Vβ primers are as follows: Vβ1–387 bp, Vβ2–256 bp, Vβ3–250 bp, Vβ4–213 bp, Vβ6–373 bp, Vβ7–165 bp, Vβ10–355 bp, Vβ14–253 bp, Vβ15–394 bp, Vβ16–234 bp, Vβ17–319 bp, Vβ18–327 bp, Vβ19–312 bp, and Vβ20–376 bp. The actual sizes of each product vary by several base pairs secondary to N region additions and deletions. A master mix of all reagents except the 5′ primers was made and divided into 14 tubes each containing one specific Vβ region primer. A fifteenth control tube was run containing two constant region primers, 5′-Cβ (5′TACCCTTGTGTGCTTGGCCA3′) and WS-2 (5′GAACTGCACTTGGCAGC-GGA3′), encoding a predicted product of 207 bp. Amplifications were performed in a DNA thermal cycler (Perkin-Elmer Cetus) for 32 to 38 cycles at 92° denature for 60 seconds, 54° anneal for 60 seconds, and 72° elongation for 120 seconds.

Southern blots analysis of RT-PCR reactions

Fifteen percent of each RT-PCR product was run on a 1.2% agarose gel and transferred to a Magna NT nylon membrane (Fisher Scientific, Pittsburgh, PA, USA) with a Posiblot positive pressure blotting system (Stratagene, La Jolla, CA, USA) as per the manufacturer's recommendations. The blots were then denatured for 10 minutes with 0.4 M NaOH, washed in 2 × SSC and UV cross-linked (Stratalinker, Stratagene).

Four micrograms of a common internal Cβ oligonucleotide, Cβint, (5′GAGTCACATTTCTCAGATC3′), were end-labeled using 1 unit of polynucleotide kinase (Promega, Madison, WI, USA) 2λ of 10× buffer, and 3λ of ^{32}P-γATP (Amersham, Arlington Hts, IL, USA) in a 20λ volume for 45 minutes at 37°C. The labeled probe was separated from unincorporated nucleotides using a G25 spin column (Boerhinger Manheim, Indianapolis, IN, USA), as per the manufacturer's recommendations. Prehybridization was performed in 6 × SSC, 50 mM disodium phosphate pH 6.7, 5× Denhardt's solution, 0.1 mg/ml boiled salmon sperm DNA at 37°C for a minimum of three hours. Hybridizations were performed in 6 × SSC, 50 mM disodium phosphate pH 6.7, 5× Denhardt's solution, 0.1 mg/ml boiled salmon sperm DNA, 100 mg/ml dextran sulfate at 37°C with 6×10^5–1×10^6 counts per milliliter of labeled probe, overnight. The blots were washed twice in 6 × SSC at 4°C for 30 minutes, then twice at 55°C for 20 minutes in 3 M TMAC, 50 mM Tris HCl pH 8.0, 2 mM EDTA, and 0.1% SDS. The blots were analyzed on a

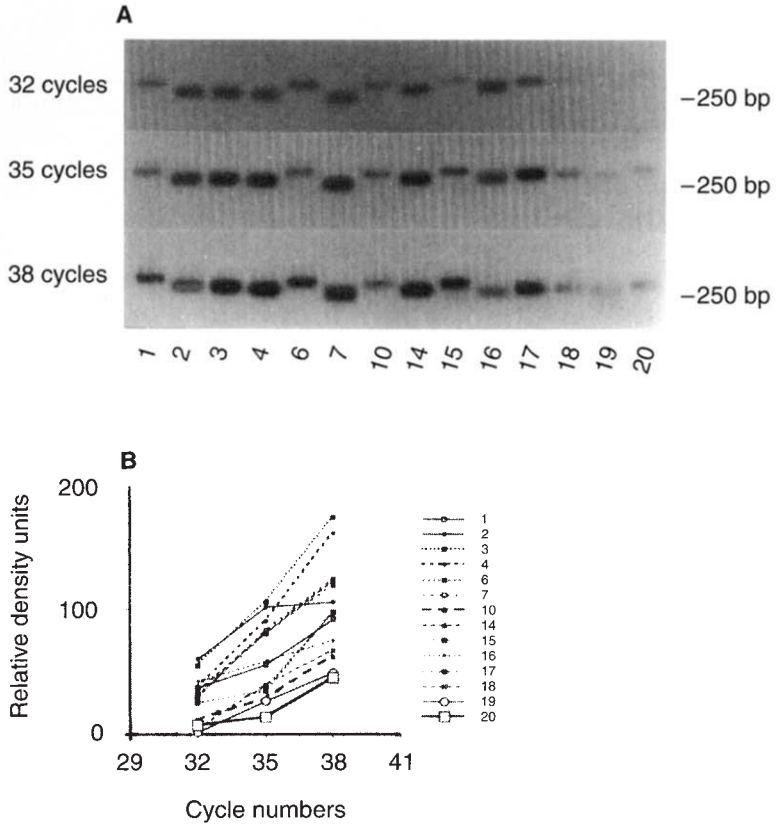

Fig. 1. *Effect of amplification cycle number on T cell receptor Vβ RT-PCR products.* **A.** Southern blots of Vβ RT-PCR products from splenic RNA, probed with internal oligonucleotide Cβint, for 32, 35 and 38 cycles. **B.** Quantification of the individual bands from A, using the Bio Rad GS-250 Molecular Imager.

BioRad GS-250 Molecular Imager (BioRad Laboratories, Hercules, CA, USA) using PhosphorAnalyst software (BioRad Laboratories) for the Macintosh. After subtraction of the background, the density of each band was determined, and was expressed as a percentage of the total of all of the Vβ regions for that organ. Statistical comparisons were performed using the Student's *t*-test. Some blots were additionally exposed to film and photographed.

Cloning and sequencing of Vβ RT-PCR products

Equal aliquots of each of the 14 indivdual Vβ RT-PCR products from selected diseased kidneys were mixed together, and shotgun cloned into the pCR 3000 TA cloning vector (In vitrogen, La Jolla, CA, USA) as previously described [28]. DNA plasmid preparations were performed on individual colonies using the Magic Miniprep kit (Promega), as per the manufacturer's recommendations. DNA sequencing was performed in both directions by the dideoxy chain determination method using fluorescein labeled primers as described by the manufacter (Pharmacia, Uppsala, Sweden), and run on a Pharmacia LKB A.L.F. Automated Sequencing apparatus. Sequence analysis was done using the MacVector sequence analysis program for the Macintosh (IBI, New Haven, CT, USA).

Statistical analysis

Statistical comparisons were done using the Student's *t*-test.

Results

Optimization of the RT-PCR technique

Initial studies were performed to determine optimal parameters for amplification of each Vβ PCR product. Figure 1 shows a representative experiment analyzing the effect of PCR cycle number on the peripheral Vβ gene distribution using 1 μg of splenic cDNA/RNA. Note that all of the PCR products are of predicted size, and bind to the internal primer (Fig. 1A). Vβ genes deleted in SJL mice [29] were not detected by RT-PCR (data not shown). The relative density of each detected band, as quantified using a molecular phosphorimager, is graphically depicted in Figure 1B. The signal intensity for each Vβ gene PCR product increases over the 32 to 38 cycle range, with the exception of Vβ2, which reaches a plateau after 35 cycles. Additionally, the relative percentage of each Vβ PCR product, expressed as a fraction of all of the Vβ products for that organ, is essentially identical at 32 and 35 amplification cycles (data not shown). We therefore performed all subsequent experiments with 1 μg of RT product per reaction, 92°C for one minute, 54°C for one minute, and 72°C for two minutes, for 35 amplification cycles.

Analysis of the peripheral TcR Vβ gene repertoire in nonimmunized mice

To define the baseline peripheral TcR Vβ gene repertoire, we performed RT-PCR for TcR Vβ genes with splenic RNA isolated from multiple control (nonimmunized) animals (Fig. 2). Figure

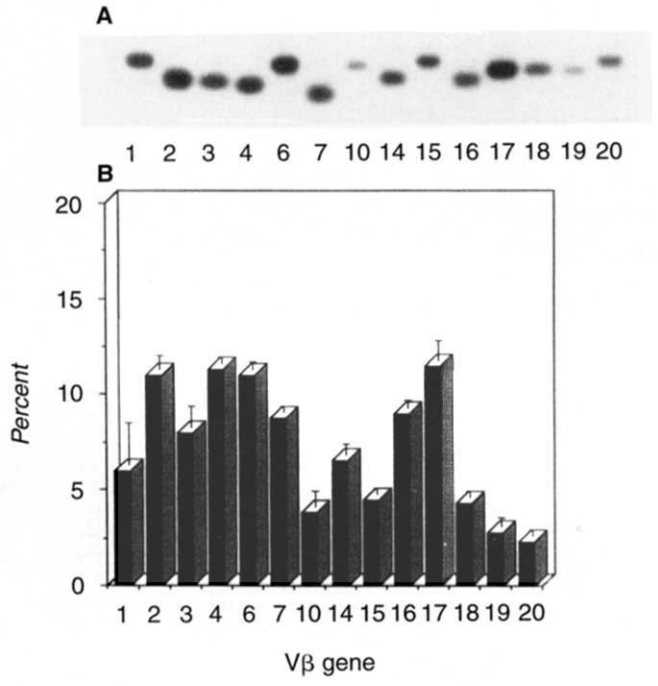

Fig. 2. *Peripheral T cell receptor Vβ gene repertoire in unimmunized mice.* **A.** Representative Southern blot of TcR Vβ RT-PCR products from splenic RNA of an unimmunized animal. **B.** Relative quantity of each individual Vβ RT-PCR product expressed as a percentage of the total of all Vβ genes combined ($N = 4$).

2A shows a representative autoradiograph of the peripheral TcR Vβ gene repertoire from a normal animal, while Figure 2B graphically depicts the cumulative results of multiple experiments ($N = 4$), and shows the relative expression of each Vβ gene family as a percentage of the total of all Vβ genes combined. SJL mice have a genomic deletion of a portion of the TcR Vβ genes and thus do not express Vβ5, 8, 9, 11, 12, or 13 [29]. Spleens from unimmunized control animals contain transcripts for all nondeleted Vβ genes, with Vβ2, 4, 6 and 17 comprising the highest individual percentages, while Vβ10, 15, 18, 19, 20 are present in significantly lower levels (Fig. 2B). This splenic TcR Vβ gene repertoire did not change over the 20-week time period that animals were maintained in our animal facility (data not shown). Additionally, we studied multiple different shipments of mice, both male and female, born over an 18 months period, with similar findings (data not shown).

Using commercially available antibodies specific for individual TcR Vβ gene families (Pharmingen), we next studied selected Vβ gene product expression on the surface of peripheral splenic lymphocytes. Figure 3 depicts a panel of two color FACS analyses evaluating the percentage of CD3+ splenic lymphocytes that additionally express Vβ2, 4, 6, 7, 8 and 14. Based on pooled lymphocytes from three individual spleens, 13.5% of CD3+ splenic cells express Vβ2, 13.9% express Vβ4, 10.7% express Vβ6, 10.5% express Vβ7, and 7.1% express Vβ14. $V\beta8^+$ was not detected, as expected from the known genomic deletion of this element in SJL/J mice [29]. These numbers are similar to the distribution determined by PCR analysis (Fig. 2B), where Vβ2, 4, 6, 7, and 14 comprise 11.0%, 11.3%, 11.0%, 8.8%, and 6.5% of the total Vβ genes, respectively.

Analysis of the peripheral Vβ gene repertoire in immunized animals

We next wished to evaluate whether immunization producing αTBM disease, or immunization with adjuvant alone, affects the peripheral Vβ gene repertoire. Splenic TcR Vβ gene expression 8 to 10 weeks after immunization with CFA alone, and after immunization with RTA/CFA to get αTBM disease is shown in Figure 4, and demonstrates no significant differences between the two groups ($P > 0.05$). Additionally, there are no significant differences ($P > 0.05$) between the peripheral Vβ gene repertoire of nonimmunized animals (Fig. 2B) and animals immunized with CFA alone or animals immunized with RTA/CFA to get disease (Fig. 4).

TcR Vβ gene distribution in diseased kidneys

To minimize background blood contamination, each kidney was perfused with 30 ml of PBS via cardiac puncture, prior to harvest. This procedure routinely resulted in a visible blanching of the kidney, with no detectable blood in routine histologic sections. Figure 5 shows a photomicrograph of a normal kidney from an unimmunized animal, with an accompanying Southern blot of the RT-PCR for TcR Vβ genes. As expected, there is minimal detectable TcR mRNA present in this normal organ, as compared to the splenic tissue. The strongest signals are Vβ2 and Vβ6, some of the same Vβ genes that are expressed in the highest levels in the periphery (Fig. 2), and presumably reflect residual blood contamination and the high sensitivity of the RT-PCR technique. TcR Vβ region gene analysis of histologically normal kidneys from animals immunized with adjuvant alone revealed similar findings (results not shown).

In contrast, kidneys with histologic grade 0.5 to 1 disease (focal mononuclear infiltrates in less than 10% of the renal cortex; Fig. 6A), predominantly express strong signals for up to three TcR Vβ PCR products (Fig. 6B), with the remaining signals being comparable to the background levels found in the histologically normal kidneys. Vβ2, 6, and 7 are found in many of the kidneys with αTBM disease, but other Vβ region PCR products (3, 14 and 16) are present as well. No single Vβ gene family is expressed preferentially in all kidneys. TcR Vβ PCR products were not detectable above background in kidneys of animals immunized with RTA/CFA, prior to the histological detection of disease (data not shown).

Figure 7 shows the TcR Vβ gene repertoire in kidneys with advanced αTBM disease. A diseased kidney with typical grade 3 to 4 histology is shown in Figure 7A, and reveals a marked interstitial infiltrate with peritubular edema and early fibrosis. The TcR Vβ genes found in 5 different kidneys with grade 3 to 4 histology are shown in Figure 7B, and reveals prominent expression of up to 10 different Vβ gene products in any individual organ, with no discernible reproducible pattern of expression of any single Vβ gene family. Notably, all of the organs were perfused with PBS prior to isolation of the RNA in order to minimize peripheral T cell contamination of the RT-PCR products. As can be seen, each of the TcR Vβ gene distributions in Figure 7 differ from that found in the spleen (Fig. 2A), with some TcR Vβ genes being totally absent, thus eliminating the possibility that the increased heterogeneity can be simply attributed to contaminating peripheral blood.

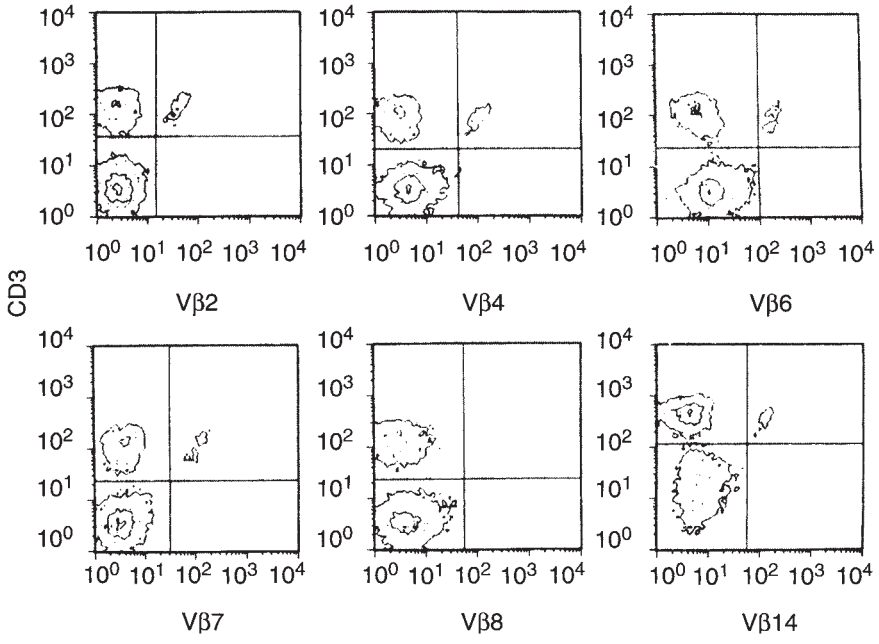

Fig. 3. *TcR Vβ gene product expression by peripheral lymphocytes.* FACS analysis of splenic lymphocytes from unimmunized animals using PE-conjugated anti-CD3 (Y axis) and individual FITC-conjugated anti-Vβ region antibodies (X axis). Splenocytes were pooled from three individual animals. The percentage of CD3+ T cells also expressing Vβ2, 4, 6, 7, 8, and 14 is 13.5%, 13.9%, 10.7%, 10.5%, 0%, and 7.1%, respectively.

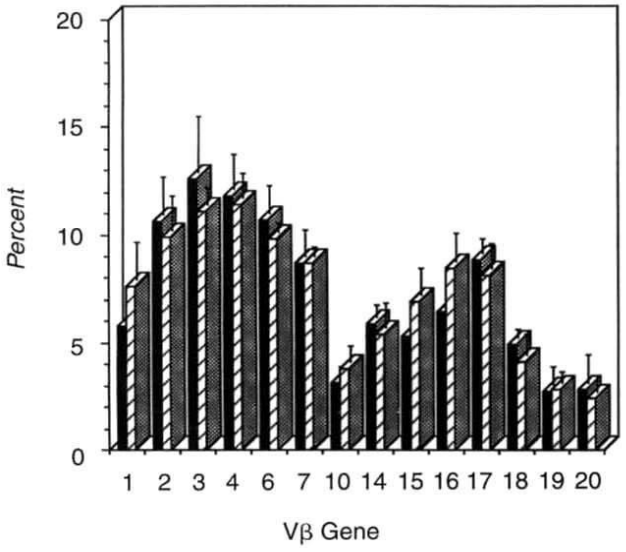

Fig. 4. *Peripheral T cell receptor Vβ gene repertoire from immunized animals.* Relative quantity of splenic Vβ RT-PCR products expressed as a percentage of the total of all Vβ genes combined. Black bars, animals immunized with CFA alone ($N = 4$); striped bars, animals immunized with RTA/CFA ($N = 7$). There are no statistically significant differences between groups, and no statistically significant differences between either group and the unimmunized animals (Fig. 2).

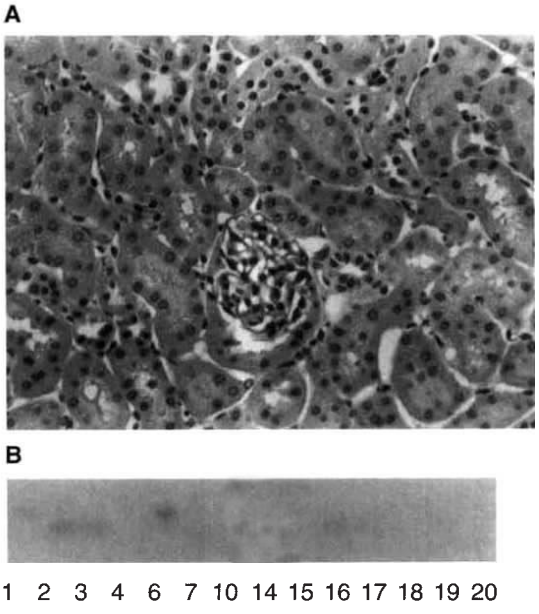

Fig. 5. *T cell receptor Vβ RT-PCR products in normal renal tissue.* **A.** Photomicrograph of normal kidney from an unimmunized mouse stained with hematoxylin and eosin (magnification 40×). **B.** Representative Southern blot of RT-PCR for Vβ genes using RNA from a normal kidney.

Sequence analysis of Vβ gene products in early αTBM disease

To analyze the β chain CDR3 sequences across the VDJ junctions of the TcRs from the diseased organs, we next subcloned and sequenced individual RT-PCR products from two kidneys with histologically focal (early) αTBM disease. For each kidney, two separate reverse transcription reactions, and four separate PCR reactions were used to generate the cDNA products used in the sequencing reactions. The deduced amino acid sequences are shown in Table 1. For each sequence, the open reading frame confirms the presence of a known Vβ and Jβ gene. In both of the kidneys analyzed, there is a predominance of Vβ7 and Vβ3, although other Vβ genes are present as well. The sequences from both organs also show some evidence for clonal expansion of individual T cell clones, in that several sequences were isolated from multiple different colonies, derived from different PCR reactions (Table 1). Several of the repeatedly isolated sequences from both organs contained Vβ3 and an n region encoded Arg

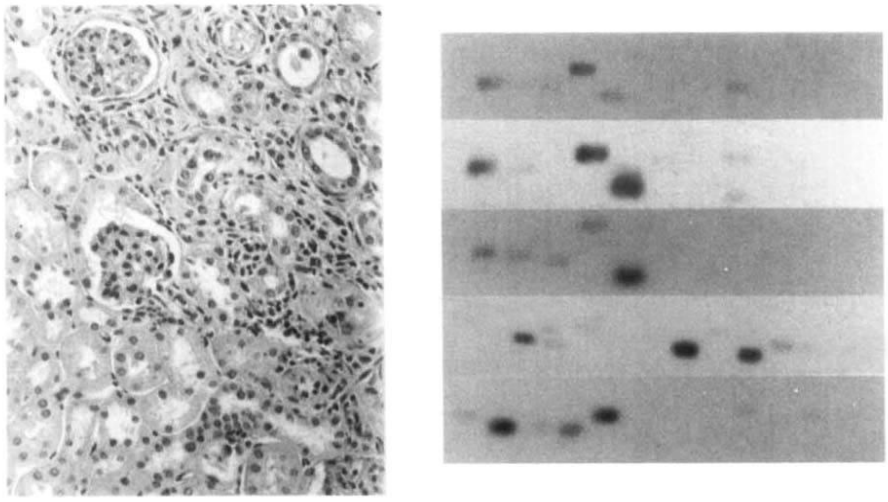

Fig. 6. *T cell receptor Vβ RT-PCR products in early αTBM disease.* **A.** Focal area of mononuclear cell infiltrate adjacent to histologically normal renal tissue in a kidney with grade 1 αTBM disease, stained with hematoxylin and eosin (magnification 40×). **B.** Southern blot of Vβ RT-PCR products from 5 individual kidneys affected with histologic grade 0.5 to 1 αTBM disease.

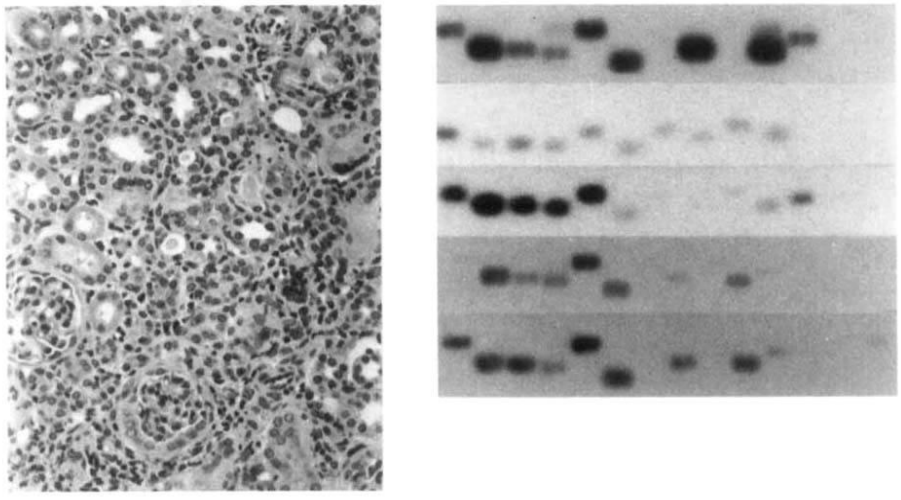

Fig. 7. *T cell receptor Vβ RT-PCR products in extensive αTBM disease.* **A.** Extensive mononuclear cell infiltrate with tubular destruction and peritubular edema in a kidney with grade 3 αTBM disease. **B.** Southern blot of Vβ RT-PCR products from 5 individual kidneys affected with histologic grade 3 to 4 αTBM disease.

residue. In fact, there is an overrepresentation of Arg residues encoded by n region additions/deletions in the sequences from both kidneys (Table 1). Interestingly, a similar n region encoded Arg (or structually similar Lys or His residue) was found in many of the CDR3β chain sequences from our CD4+ nephritogenic T cell clones [12]. On the other hand, there is remarkable heterogeneity in both the length of the CDR3 regions as well as in the individual residues found within the regions. These findings hold true even among those sequences cloned from the same organ, and sharing the same Vβ and Jβ genes.

Discussion

Initial studies of T cell receptor Vβ gene expression in EAE [2, 3, 30], collagen arthritis [31, 32], and several other autoimmune disease models [reviewed in 33] suggested that autoreactive T cell repertoires are highly restricted. Based on such findings, many investigators hypothesized that autoimmunity was mediated by clonal expansion of a limited number of autoreactive T cells that recognize a single (or a few) determinant(s) derived from an autoantigenic protein [1, 4]. In fact, DNA sequencing of TcR β chains from encephalitogenic T cell clones later revealed a recurrent amino acid residue motif across the CDR3 regions [34], further suggesting that autoimmune responses could be uniquely directed at a small number of epitopes. Other studies in EAE [5, 7], however, as well as analyses of NOD mice with diabetes [10] and thyroiditis [9] have revealed a more heterogeneous T cell receptor Vβ gene repertoire. Additionally, studies of T cell receptor V region genes used in human autoimmune disease have yielded mixed findings; some cases of biased expression of TcRs have been noted [33, 35, 36], while others experiments suggest a more diverse repertoire [33, 37, 38].

Our studies are the first to comprehensively analyze the *in vivo* TcR Vβ gene repertoire in any model of autoimmune renal disease. The results described herein reveal that the T cell receptors detected in kidneys affected by αTBM disease are quite diverse, and suggest a polyclonal response. Although private repertoires of restricted TcR Vβ gene families (containing up to three different Vβ genes) are detectable in individual organs with

Table 1. TcR CDR3β sequences from kidneys with focal T cell infiltrates

A. Kidney 1			B. Kidney 2		
	CDR3	Vβ/Jβ		CDR3	Vβ/Jβ
CTCSA	P SYNSPLYFAAGTRL	2/1.6	CTCS	ADLGG AETLYFGSGTRL	2/2.3
CASS	LSGGL NYAEQFFGPGTRL	6/2.1			
CASS	QTGGAL AEQFFGPGTRL	4/2.1	CASS	QETGGAL AEQFFGPGTRL	4/2.1
			CASS	QEYN NYAEQFFGPGTRL	4/2.1 (2 colonies)[a]
CASS	RDRG DTQYFGPGTRL	3/2.5 (4 colonies)[a]	CASS	QDK DTQYFGPGTRL	4/2.5
CASS	LSR RD SDYTFGSGTRL	3/1.2			
CAS	RGQG YAEGFFGPGTRL	3/2.1	CASA	IRDRG SDTQYFGPGTRL	6/2.5
CASS	ARGF NSPLYFAAGTRL	3/1.6			
CASS	QGVN ERLFFGHGTKL	3/1.4 (2 colonies)[a]	CAS	RWYT GQLYFGEGSKL	3/2.2 (3 colonies)[a]
CASS	IQGGQ NTLYFGAGTRL	3/2.4 (2 colonies)[a]	CASS	RQGNNQAQH FGEGTRL	3/1.5
CASS	LAV NQDTQYFGPGTRL	3/2.5	CASSL	SR NYAEQFFGPGTRL	3/2.1 (2 colonies)[a]
CASS	RQEAF GNTNYFGEGSRL	3/1.3	CASS	AGA NSDYTFGSGTRL	3/1.2
			CASS	LKF SNERLFFGHGTKL	3/1.4
			CASS	LTGG NTLYFGAGTRL	3/2.4
CASS	PGLG YAEQFFGPGTRL	7/2.1	CASSL	TGS SPLYFAAGTRL	3/1.6
CA	QSI QNTLYFGAGTRL	7/2.4	CASS	DN SYEQYFGPGTRL	3/2.6
CASS	LYI NTLYFGAGTRL	7/2.4			
CAS	GYNQAQH FGEGTRL	7/1.5	CASS	PNRGRD AEQFFGPGTRL	7/2.1
CASS	RQGI TEVFFGKGTRL	7/1.1	CASS	LYRGI SNERLFFGHGTKL	7/1.4
CASS	ATF YAEQFFGPGTRL	7/2.1	CASS	GLGS NQDTQYFGPGTRL	7/2.5
CASS	LSGTK NQDTQYFGPGTRL	7/2.5	CASS	LSR RG TGQTYFGEGSKL	7/2.2
CASS	LLI SAETLYFGSGTRL	7/2.3	CASS	LEGASA ETLYFGSGTRL	7/2.3 (2 colonies)[a]

Standard single letter codes for amino acids are employed. Underlined residues are encoded by n region additions or deletions. Bold residues are positively charged arginine (R), histine (H), or lysine (K) residues encoded within the putative CDR3 regions.

[a] Multiple individual colonies contained identical sequences encoding these CDR3 regions

focal disease, and there is some evidence for clonal expansion of individual T cells, there is not an assured pattern of Vβ expression in all organs tested. The heterogeneity of CDR3β sequences, despite the presence of shared Vβ and/or Jβ genes, confirms the polyclonal nature of the response. Moreover, further diversity is manifested as the disease progresses, as indicated by the increased numbers of detectable Vβ genes by RT-PCR in kidneys with histologically more advanced lesions.

All of these findings must be interpreted in the context of known information about disease relevant autoantigens. Several published studies have noted that T cells expressing structurally distinct TcRs are capable of recognizing the same peptide/MHC complex [8, 10, 12, 38]. Previous work by our group has established that the autoreactive T cell and B cell repertoires in αTBM disease are highly focused towards a single immundominant region of the 3M-1 target antigen [12, 15, 39, 40]. We have shown that a panel of nephritogenic CD4+ T cell clones, expressing a variety of TcR Vβ genes (including Vβ14, Vβ3 and Vβ7), all recognize P3 (expressed in the context of I-A^s), a peptide derived from 3M-1 [12]. Interestingly, these T cell clones expressed TcRs containing positively charged CDR3β regions, with an over-representation of Arg, Lys, or His residues encoded by n region additions or deletions [12]. Many of the TcR sequences derived from our diseased kidneys express Vβ3 and Vβ7, and also contain similar positively charged residues encoded by n region additions or deletions. These structural similarities suggest that some of the TcR sequences obtained from diseased kidneys could be derived from T cells reactive to P3.

Alternatively, the heterogeneous TcR repertoire could reflect T cells reactive to a number of antigenic epitopes. Other data from studies in EAE implicate a second autoantigen, proteolipid protein (PLP), in addition to myelin basic protein, as an important target of the encephalitogenic autoimmune response [41, 42]. The description of autoreactive, pathogenic T cells recognizing multiple antigens, and several epitopes derived from each antigen, provides a reasonable explanation for the detection of a heterogeneous TcR Vβ repertoire during active disease [41, 42]. In an analogous fashion, while P3 is an immundominant determinant in the development of αTBM disease [12], it remains possible that the renal expression of a diverse TcR Vβ gene repertoire may reflect an immune response directed at other 3M-1-derived epitopes as well.

Progressively increasing heterogeneity of T cell receptor repertoires is also consistent with the recently described phenomenon of epitope spreading in autoimmunity [43, 44]. Epitope spreading is characterized by an initial autoreactive T cell response that is focused to a single (or a few) epitope(s), and that utilizes a restricted TcR V gene repertoire [43, 44]. The focused inflammatory reaction then leads to exposure of new antigenic epitopes, with up-regulation of costimulatory and chemoattractant molecules, eventually resulting in the influx of numerous T lymphocytes with specificities differing from the original disease-producing cells. This heterogeneous, newly infiltrating T cell population therefore expresses an expanded repertoire of TcR Vβ genes [43, 44]. Our data suggest that the initial lymph node derived autoimmune response in αTBM disease is focused towards P3 [12], and in individual animals, is initially biased towards selected Vβ regions. As the disease progresses, the heterogeneity increases, consistent with the recruitment of T cells directed at additional antigens. Importantly, both intramolecular and intermolecular epitope spreading in murine EAE are prominently detectable at two to three weeks after immunization. Since αTBM disease requires six to eight weeks to manifest itself histologically, it is conceivable that the autoreactive T cell response has spread to multiple autoantigenic epitopes by that time point.

The diversity of the T cell receptor repertoire may be partially

attributable to the fact that both CD4+ and CD8+ T cells are necessary for the phenotypic expression of αTBM disease, and that both T cell subtypes have been identified in the renal infiltrates [14]. Although it remains possible that the T cell receptor repertoire of the CD4+ or CD8+ subpopulations derived from renal infiltrates may be more restricted, our previously published findings of T cell receptor Vβ gene heterogeneity in CD4+, nephritogenic T cell clones argues that heterogeneity exists in at least the CD4+ subpopulation as well [28].

It is also possible that some of the increased heterogeneity of the TcR repertoire in severely diseased kidneys reflects recruitment of bystander lymphocytes attracted by nonspecific mediators of inflammation. Several studies in EAE have demonstrated the presence of nonspecific T cells in the brains or spinal cords of symptomatic animals [45–47], but the role played by nonspecific polyclonal infiltrates in autoimmune renal disease is unknown.

Importantly, we have defined the renal T cell receptor Vβ gene repertoire based on the detection of RT-PCR products, and we cannot be certain that this truly reflects TcR protein expression on the surface of T cells infiltrating diseased renal tissue. However, the similarity between TcR Vβ gene product expression by FACS and TcR Vβ transcript level by RT-PCR (Figs. 2 and 3), suggests that RT-PCR can provide a reasonable approximation of the TcR Vβ gene product repertoire *in vivo*.

These studies additionally define the baseline peripheral TcR Vβ gene repertoire in SJL/J mice within the constraints of the 20 known Vβ gene families, and allow for a comparison with the peripheral repertoire from diseased animals. The similarity between TcR Vβ gene product expression by FACS and TcR Vβ transcript level by RT-PCR, suggests that RT-PCR can provide a reasonable approximation of the TcR Vβ gene repertoire *in vivo*. Our findings are additionally consistent with the relative expression of Vβ genes detected by other investigators [29, 48]. Interestingly, the peripheral distribution of TcR Vβ genes varies minimally between unimmunized animals, does not change over the 20 weeks during which the animals were maintained in our animal facility, and is not affected by immunization with adjuvant alone. Additionally, immunization with RTA/CFA has little effect on the relative expression of peripheral TcR Vβ genes, even when the disease is present histologically in the kidney. It remains likely, however, that clonal expansion of individual T cells does occur peripherally in response to immunization with RTA/CFA, but that such expansion does not affect the overall T cell receptor repertoire of the animal.

In conclusion, these studies, in conjunction with those from our previously described, nephritogenic T cell clones [12], do not support a strict "V-region hypothesis" of T cell responses in murine αTBM disease, and suggest that restricted T cell repertoires are not a generalizable phenomenon of all autoimmune diseases. T cells detected in diseased organs affected with αTBM disease express heterogeneous T cell receptors, most consistent with a polyclonal response directed at multiple antigenic epitopes. The diversity of TcR Vβ genes expressed in diseased kidneys, even in the early stages of disease, suggests that immunotherapy directed at individual Vβ gene elements would not be efficacious in the prevention or treatment of this disease. Theoretically, even successful elimination of a single Vβ gene family, using a specific anti-Vβregion antibody, would leave a residual autoreactive T cell population capable of producing renal injury. Immunotherapeutic approaches targeting immunodominant epitopes [49], or targeting common structural characteristics of the autoreactive TcRs within complementarily determining regions [50], may be more appropriate alternatives for the treatment of this disease entity.

Acknowledgments

This work was supported, in part, by the Medical Research Search Service of the Department of Veterans Affairs, and by grants from the National Institutes of Health (DK-30280, DK-07006, DK-45191, DK-41110, DK-46282, DK-08446, and DK-42155). P.H. is a recipient of a Clinical Investigator Award (K08 DK-02125 to 01) from the National Institute of Health. W.S. is a recipient of a National Research Service Award (1 F32 DK 08607 to 01) from the National Institute of Health.

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