Molecular Analysis of the Helper T Cell Response in Murine Interstitial Nephritis

T Cells Recognizing an Immunodominant Epitope Use Multiple T Cell Receptor Vβ Genes with Similarities across CDR3

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Abstract

Anti-tubular basement membrane disease (αTBM disease) produces T cell–mediated interstitial nephritis in SJL mice after immunization with renal tubular antigen. Initial mononuclear infiltrates appear in vivo after several weeks, with the subsequent progression to renal fibrosis and end stage renal disease over many months. We have analyzed the fine specificity of the autoreactive helper T cell repertoire in αTBM disease through the isolation and characterization of a panel of CD4+ Th1 clones harvested after 1–2 wk from animals immunized to produce disease. All clones capable of mediating αTBM disease are directed towards a 14-residue immunodominant epitope (STMSAEVPEAASEA) contained within the target antigen, 3M-1. Evaluation of the T cell receptor (TCR) Vβ repertoire used by these autoreactive T cells reveals the use of several Vβ genes, but with some preference for Vβ14. Sequencing across the putative CDR3 region of the TCR β chains suggests that common amino acids at the Vβ(N)Dβ junction and the Dβ(N)Jβ junction may contribute to the specific ability of these cells to recognize the immunodominant epitope. (*J. Clin. Invest.* 1994. 94:2084–2092.) Key words: autoimmunity • autoimmune disease • kidney diseases • gene rearrangement • β-chain • T cell antigen receptor

Introduction

Recent advances in immunology have facilitated the examination of autoimmune T cell repertoires (1–3), and have provided important insights into the molecular basis of autoreactive T cell recognition of target antigenic immunodominant epitopes (3–7). Analyses of T cell receptor (TCR)[1] variable (V) region genes used by autoreactive T cells in animal models of autoimmunity (3, 8–12) as well as in human autoimmune diseases (3, 13–16), have revealed numerous instances of autoreactive T cell repertoires containing the preferential use of individual Vβ region genes. Such work has resulted in successful, specific anti-Vβ gene immunotherapy (2, 3, 6, 17, 18). Despite these findings, there remains some controversy about the extent of restricted V region gene repertoires in autoimmune diseases (16), especially in systems lacking good information about epitope recognition.

We have been studying the cellular and molecular mechanisms involved in the expression of autoimmune interstitial nephritis (19, 20). In one such model, anti-tubular basement membrane (αTBM) disease in the SJL mouse, autoimmune interstitial nephritis is induced by immunizing susceptible animals with a rabbit renal tubular antigen preparation in complete Freund's adjuvant (RTA/CFA) (21). Animals develop nonpathogenic anti-tubular basement membrane antibodies in 7–10 d (22), with a subsequent renal mononuclear cell infiltrate at 6–8 wk (21, 22), that progresses to interstitial fibrosis, end stage renal disease, and death (21, 23). The development of αTBM disease depends upon the emergence of a CD4+ helper T cell reactive to the target antigen 3M-1 (7), which is expressed on the surface of proximal renal tubular epithelial cells (24). These helper T cells induce the development of 3M-1-reactive CD8+ effector T cells (25, 26), that mediate cytotoxicity and delayed-type hyper-sensitivity (DTH), and can adoptively transfer disease to naive syngeneic recipients (26, 27).

To further characterize the helper T cell repertoire in αTBM disease, we have set out to evaluate the peptide antigen specificity, and the selection of T cell receptor Vβ genes in CD4+ nephritogenic helper T cells.

Methods

Antigens. Rabbit renal tubular antigen (RTA) was isolated by previously published methods (28), and emulsified at 8 mg/ml with complete Freund's adjuvant (4 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) to produce RTA/CFA; ~ 0.2 ml was injected over multiple sites. Soluble renal tubular antigen (SRTA) was made from lyophilized RTA by collagenase digestion (29). Peptides P1 (LLRRRHGDRRSTMSAEVP), P2 (ASAEEQKEMEDKVTSPEKAEEA), and P3 (STMSAEVPEAASAE) were derived from the deduced amino acid sequence of the cloned cDNA for 3M-1 (7, 30) and were synthesized by standard peptide synthetic techniques (31), and HPLC purified (University of Pennsylvania peptide synthesis facility, Philadelphia, PA, and Research Genetics, Huntsville, AL).

Immunization. SJL mice (H-2^s) 4–6 wk of age (Jackson Laboratory,

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1. *Abbreviations used in this paper:* αTBM, anti-tubular basement membrane; DTH, delayed-type hypersensitivity; RTA, renal tubular antigen; SRTA, soluble RTA; TCR, T cell receptor.

Bar Harbor, ME) were immunized with RTA/CFA in multiple sites to get αTBM disease (21).

Isolation and maintenance of T cell lines and clones. T cell lines were established by standard methods (26, 32). Immune cells harvested from draining lymph nodes of SJL mice 10–14 d after immunization were placed in a 24-well culture plate in 2 ml of complete T cell media containing 20 μg/ml of appropriate antigen and cultured at 37°C in 5% CO_2 for 4 d. Complete T cell media consists of RPMI 1640 (JRH Biosciences, Lenexa, KS), supplemented with 10% complement-depleted fetal calf serum, 5% NCTC (Whittaker Bioproducts, Walkersville, MD), 2×10^{-5} M 2-mercaptoethanol, glutamine, and antibiotics. After 4 d, and then weekly thereafter, the cells were harvested and placed in fresh media containing antigen, 20% MLA 144 supernatant as a source of IL-2 and other growth factors (33), and $3–5 \times 10^6$ irradiated (2,500 rads) syngeneic splenic feeder cells (26). T cell lines were cloned twice by limiting dilution in 96-well plates (26), and then expanded and passaged weekly as described above. Some clones were stimulated weekly with SRTA (M33.25.6, M36.9.1, M36.6.1, and M30.2), while others were stimulated weekly with either the P1 (M34.7.3, M34.4.3, M31.1, M31B.3.3) or P2 (M35.2.7, M32.18.2.2) peptide, alone.

Cytokine transcript production by T cell clones. T cell clones were harvested at the end of a 7 d cycle, and were isolated from any remaining dead splenocytes with Lympholyte-M™ (Cedar Lane Laboratories, Ltd., Hornsby, Ontario, Canada) as per manufacturer recommendations. $2\text{-}4 \times 10^6$ T cells were then cultured in 2 ml of T cell media in 24 well plates, with Concanavalin A (Con A) at 5 μg/ml, for 6 h. The supernatants were stored at −20°C for use in HT-2 cell assays, and the cells were washed with T cell media and pelleted. Total RNA was made from the T cells (as well as from snap frozen SJL mouse spleen) by the guanidinium isothiocyanate isolation procedure (34). First strand cDNA was made with mMLV (GIBCO BRL) and oligo-dT (34, 35). 500 ng of the resultant RNA/cDNA was used in a 100 λ polymerase chain reaction, containing 10λ of 10× buffer (Perkin-Elmer Cetus, Exton, PA) 200 μM dGTP, dCTP, dATP, dTTP, 0.25 λ Taq polymerase (Perkin-Elmer Cetus) and 40 pmol of each oligonucleotide primer. Primer sequences were derived from the cDNAs found in Genbank for IL-2, IL-4, and γIFN. All oligonucleotide primers were synthesized on an Applied Biosystems 391 DNA Synthesizer (Foster City, CA) and purified with an oligonucleotide purification cartridge as per manufacturer's recommendations. The sequences are as follows: IL-2 sense, 5′CCACTTCAAGCTCTACAGCCGG3′; IL-2 antisense, 5′CCTTATGTGTTGTAAGCAGGAGG3′; IL-4 sense, 5′GCTTTCTCTAACAGTAGAAACC3′; IL-4 antisense, 5′CCGACAGCGAGACCCAAATCTGTC3′; γIFN sense, 5′GGTCAGTGAAGTAAAGGTACAAGC3′; γIFN antisense, 5′GGAAGAGATTGTCCCAATAAGAATA3′. Predicted product sizes are: γIFN, 308 bp; IL-2, 463 bp; and IL-4, 302 bp. cDNA amplification was performed in a Perkin-Elmer Cetus DNA thermal cycler (Perkin-Elmer Cetus) for 35 cycles at 92°C denature for 30 s, 52°C anneal for 60 s, and 72°C elongation for 120 s. 15% of the resultant products were run out on a 2% agarose gel and photographed.

HT-2 proliferation assay. HT-2 cells (kindly provided by Michael Madaio, University of Pennsylvania) were maintained with twice weekly passage in T cell media supplemented with 2 U/ml murine recombinant IL-2 (Boehringer Mannheim Biologicals, Indianapolis, IN). Before use, the cells were pelleted, washed in fresh T cell media, and rested for 3 h at 37°C in 5% CO_2. 20,000 rested HT-2 cells were incubated with 100λ of T cell clone supernatant and 1 μCi [^{3}H]-thymidine (Amersham Corp., Arlington Heights, IL) in a total of 200λ, in 96-well culture plates for 16–48 h. In some experiments, anti-IL-2 blocking antibody (36) (kindly provided by Dr. Glen Gaulton, Department of Pathology, University of Pennsylvania), or murine polyclonal IgM (Bionetics, Charleston, SC), was added at 4 μg/ml. Cells were harvested and counted, and the results expressed as the mean of three experiments±SEM.

Cytofluorography. Splenic lymphocytes or T cell clones (at the end of a 7-d passage) were purified through Lympholyte-M™ and washed with phosphate buffered saline (PBS) with 0.1% BSA. $1–2 \times 10^6$ cells were suspended in 50λ of PBS/0.1% BSA and incubated with a 1:50 dilution of FITC-conjugated antibody for 30 min on ice. Specific FITC-conjugated antibodies for CD4, CD8 (37), TcRαβ (38), TcR Vβ7 (39), TcR Vβ3 (40), TcR Vβ14 (41), were purchased from Pharmingen (San Diego, CA). PE-conjugated αCD4 antibody was kindly provided by Bruce Freedman, M. D. (Dept. of Pathology, University of Pennsylvania, Philadelphia, PA). FITC-conjugated goat anti-rabbit antibody was obtained from Boehringer Mannheim Biologicals. The cells were washed three times in PBS/0.1% BSA, and resuspended in 2% paraformaldehyde/PBS. Fluorescence was recorded on a FACScan® cytofluorograph (24, 42), and analyzed with Lysis II software (Becton Dickinson and Co., San Jose, CA). 10,000 live gated cells were analyzed in each run.

Lymphocyte blast transformation assay. T cell clones were harvested at the end of a 7-d passage cycle, purified through Lympholyte-M™, and rested in complete T cell media for 4 h at 37°C in 5% CO_2 (24). 5×10^6 T cells were mixed with 3×10^6 irradiated syngeneic splenocytes and antigen in a total of 1 ml of T cell media, in a 24-well flat bottom plate, and cultured for 72 h at 37°C in 5% CO_2. 100-μl aliquots were removed and placed in flat bottom 96-well plates, mixed with 1 μCi [^{3}H]thymidine (Amersham Corp.) and incubated for an additional 24 h. The cells were harvested and counted and the results expressed as the mean of three experiments±SEM.

Effector cell induction assay. The assay was performed as previously described (25, 26, 43). Briefly, $30–250 \times 10^6$ nonirradiated syngeneic splenocytes were incubated with 20% MLA supernatant, 20 μg/ml of antigen, and 20% T cell clone supernatant (taken from the end of a 7-d culture) in a T25 or T75 culture flask (Falcon; Becton Dickinson Labware, Lincoln Park, NJ) for 5 d at 37°C in 5% CO_2. At the end of the culture period, the induced effector T cells were washed extensively in standard media followed by PBS, and were used for local adoptive transfer of delayed-type hypersensitivity (DTH) or subcapsular transfer.

Local adoptive transfer of DTH. This assay has been previously reported in detail (25, 26). Induced effector cells, as described above, were resuspended in PBS at 2.0×10^7 cells/ml, with or without antigen (10 μg/ml), and 25λ of each cell preparation was injected into the hind footpads of recipient naive SJL mice (three per group). Footpad swelling as an index of DTH was measured 48 h later using a spring-loaded engineer's micrometer (Schlesingers for Tools, Brooklyn, NY). The magnitude of swelling was expressed as the mean increment between the antigen-challenged footpad and that injected with PBS in inches $\times 10^{-3}$±SEM. All measurements were performed by an individual blinded to the experimental protocol.

Subcapsular transfer experiments. Naive syngeneic mice were anesthetized and 10^8 induced effector lymphocytes (derived from an effector cell induction as described above) in 75λ of PBS were injected under the kidney capsule with a 30-gauge needle. After 7 d, the kidneys were harvested, fixed in 10% buffered formalin, and stained with hematoxylin and eosin. The stained sections were interpreted by an investigator blinded to the experiment (25, 43).

TCR Vβ gene analysis by cDNA amplification. Total RNA was prepared from Lympholyte-M™-treated T cell clones or snap frozen whole spleen by the guanididium isothiocyanate method (34). Oligo dT-primed first-strand synthesis was performed with mMLV reverse transcriptase (34, 35). PCR was performed in 100λ total volume, as described above, using 750 ng of RNA/cDNA template, 40 pmol of 3′ antisense primer PH12 (Cβ; 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 California, San Diego, CA) using a matrix analysis (Macvector sequence analysis software for the Macintosh, IBI, New Haven, CT) and the published murine Vβ gene sequences (44–46) (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 (47) using the same criteria, and has the following sequence: 5′GCAGTTACA-

CAGAAGCCAAG3′. Predicted sizes for PCR products using the 3′ PH12 primer (Cβ) and each of the 5′ Vβ primers are as follows: Vβ1,387 bp; Vβ2,256 bp; Vβ3250 bp; Vβ4213 bp; Vβ6373 bp; Vβ7165 bp; Vβ10355 bp; Vβ14253 bp; Vβ15394 bp; Vβ16234 bp; Vβ17319 bp; Vβ18327 bp; Vβ19312 bp; and Vβ20376 bp. The actual sizes of each product may 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 WAS-2 (5′GAACTGCACTTGGCAGCGGA3′), encoding a predicted product of 207 bp. Amplifications were performed in a DNA thermal cycler (Perkin-Elmer Cetus) for 35 cycles at 92°C denature for 30 s, 54°C anneal for 60 s, and 72°C elongation for 120 s. 15% of each reaction was run on a 2% agarose gel and photographed (34, 48).

A cDNA product encoding the T cell receptor Vβ region from one of the T cell clones was also obtained using anchored PCR (35). Briefly, first strand synthesis was performed using mMLV and a specific 3′ TcR Cβ antisense primer Cβdan, 5′TCAGGAATTCTTTCTTTTGA3′ (49). The cDNA was then G tailed, and amplifications were performed using an internal 3′ primer, PH12, and a 5′ poly C primer (35).

Southern hybridization. PCR products were transferred to a Zetabind (Cuno, Inc., Meriden, CT) membrane by capillary action using standard techniques, denatured for 10 min with 0.4 M NaOH, and UV cross-linked (Stratalinker; Stratagene, La Jolla, CA). Prehybridization and hybridization were performed as previously described (34) using 1 × 10^6 counts per ml of a ^{32}P-γATP-labeled (34) internal Cβ primer, PH15 (5′GAGTCACATTTCTCAGATC3′). The blot was washed twice in 6 × SSC at 4°C for 20 min, and then washed twice in 3 M tetramethylammonium chloride (Aldrich Chemical Co., Inc, Milwaukee, WI), 50 mM Tris HCl, pH 8.0, 2 mM EDTA, and 0.1% SDS at 55°C for 30 min, then autoradiographed.

Cloning and sequencing of the Vβ PCR products. Vβ PCR products were cloned into the TA cloning system (Invitrogen, La Jolla, CA). DNA was prepared for sequencing using the dideoxy chain termination (34, 35). Most Vβ genes, including the product isolated by anchored PCR, were subcloned from two different PCR reactions, and a single colony from each of the ligations was sequenced, in order to minimize any potential Taq polymearse-induced errors. Sequence analysis was done using the Macvector™ sequence analysis program for the Macintosh (IBI). For the isolation of the cDNA for the TcRβ chain of clone M30.2, we screened an M30.2 cDNA library in Lambda Zap II with a 530-bp TcR Cβ probe (inserted in a PstI site of PBR322) kindly provided by Dr. Mark Greene (Department of Pathology, University of Pennsylvania). A full length cDNA clone was obtained, and sequenced as well.

Sequence comparison of T cell receptor deduced amino acid sequences. 52 random murine TcRβ junctional sequences were obtained from the October 15, 1993 Entrez:Sequences program (NCBI, Bethesda, MD) using the keywords "T-cell receptor" and "T-cell receptor beta." Only sequences encoding functional TcRs in which the Vβ/Dβ and Dβ/Jβ junctions were identified were analyzed. Statistical comparisons were done using χ^2 analysis.

Results

Selection of potential antigenic peptides. Two potential antigenic epitopes, P1 and P2 were chosen from the deduced amino acid sequence of the cloned cDNA for the target antigen of αTBM disease, 3M-1 (7), using a software algorithm integrating a variety of determinants of protein conformation to assist in the prediction of peptide immunogenicity (50) (Fig. 1). P1 is an 18-amino acid peptide, separated by two amino acids from the 22-residue peptide, P2. We later chose a third, overlapping candidate 14 residue peptide, P3 (Fig. 1), that starts at a potential Cathepsin B site (after Arg-Arg), because Cathepsin B has been shown to be relevant in processing of other antigens presented in the context of MHC class II molecules (51).

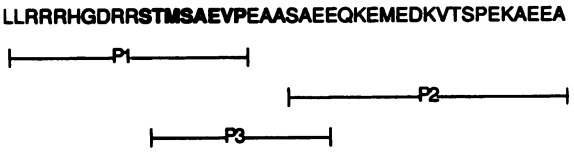


Figure 1. Amino acid sequences of the potential antigenic epitopes P1, P2, and P3. Abbreviations are the standard single letter amino acid codes. The eight residues in bold type represent the core amino acids of the immunodominant epitope recognized by the nephritogenic T cell clones.

General characterization of the T cell clones. To evaluate the relevance of peptides P1, P2, and P3 to the T helper cell response in αTBM disease, we prepared T cell clones from the draining lymph nodes of several SJL/J animals immunized to get αTBM disease. The T cell clones were stimulated weekly with either P1, P2, or SRTA. 10 clones were isolated from 8 different animals: 4 were stimulated with SRTA (M33.25.6, M36.9.1., M36.6.1, and M30.2), 4 were stimulated weekly with P1 (M34.7.3, M34.4.3, M31.1, and M31B.3.3), and 2 were stimulated weekly with P2 (M35.2.7, M32.18.2.2).

Previous work by our laboratory has shown that CD4$^+$ T cells overgrow all other T cell subtypes when isolated by our methods (26). The CD4$^+$, CD8−, TcR αβ$^+$ phenotype of each T cell clone was confirmed with FACS analysis using specific FITC-conjugated antibodies. A representative analysis of one clone is shown is Fig. 2. All of the isolated T cell clones expressed this identical phenotype.

Cytokine expression profile of T cell clones. The reverse transcription polymerase chain reaction (RT-PCR) was initially used to analyze the cytokine profile of each T cell clone. Pilot studies were done using total spleen RNA to determine optimal parameters for the detection of each transcript, and to show that amplification was in the linear range without evidence of plateau (data not shown). The cytokine profile of two representative T cell clones is shown in Fig. 3 *A*, and reveals appropriate sized products for γIFN and IL-2, but not IL-4, while all three transcripts are found in splenic RNA. All of the clones exhibited the same cytokine transcript profile. Confirmation of IL-2 production by selected Con A stimulated T cell clones is shown in Fig. 3 *B*. Supernatants induced proliferation of HT-2 cells that was almost completely blocked by anti-IL-2 antibody, but was unaffected by a polyclonal, control, isotype matched, mouse IgM. All clones tested behaved in a similar fashion (data not shown).

Antigen specificity as determined by local adoptive transfer of DTH. We initially chose to determine the antigen specificity of each clone using a functional assay, the ability to induce an antigen-specific, DTH-producing effector cell population. Each clone was tested with SRTA, P1, P2, and P3. As can be seen in Table I, the four clones stimulated weekly with SRTA (M26.9.1, M33.25.6, M30.2, M36.6.1) were able to induce an antigen-specific DTH response with SRTA, P1 and P3, but not with P2. Similarly, the four clones stimulated weekly with P1 (M34.7.3, M34.4.3, M31B.3.3, M31.1) had the same specificity for SRTA, P1, and P3, but not P2 (Table I). Notably, all eight clones reactive to SRTA were also reactive to P3, implying that P1/P3 is the sole antigenic epitope within the 3M-1 protein in

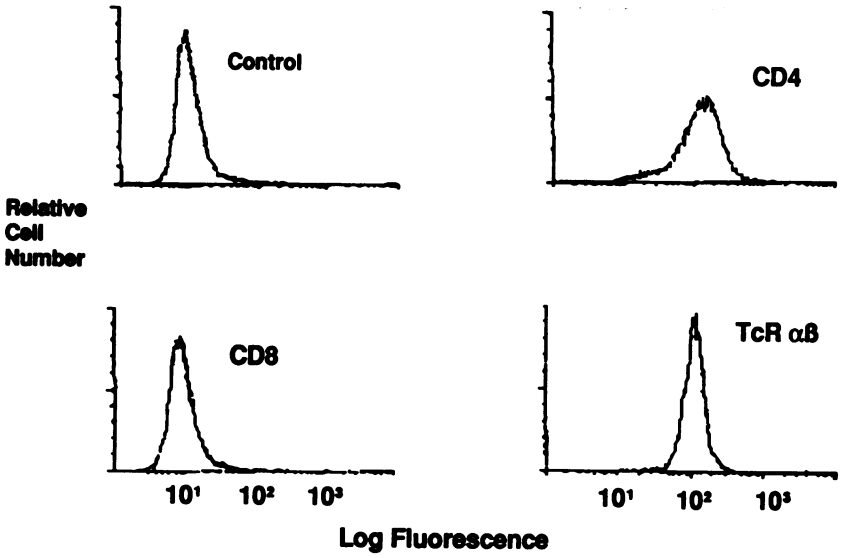
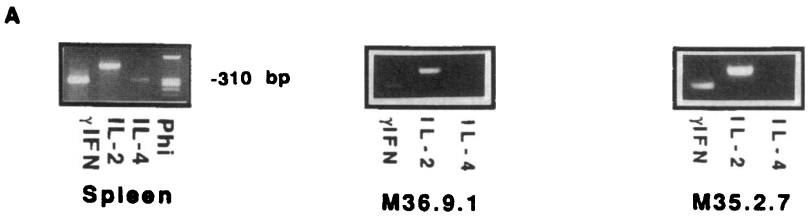


Figure 2. FACS analysis of clone M36.9.1 using FITC-conjugated antibodies to CD4 (*upper right panel*), CD8 (*lower left panel*), and TcR$\alpha\beta$ (*lower right panel*). A FITC-conjugated goat anti-rabbit antibody served as a negative control (*upper left panel*).

the SJL mouse. The two clones stimulated weekly with P2, however, were only able to induce a DTH response with P2, and not to the intact antigen, SRTA, or to the peptides P1 or P3 (Table I).

Antigen specificity as determined by lymphocyte proliferation assay. Selected T cell clones were additionally tested for their ability to proliferate specifically to the peptide antigens in a standard lymphocyte proliferation assay. As can be seen in Fig. 4, clone M36.9.1 and M33.25.6 proliferate to peptides P1 and P3, but not to P2, consistent with our DTH results.

Subcapsular transfer of induced effector cells. Previous work has shown that the M30.2 clone (SRTA, P1 and P3-reactive) can induce effector cells that cause renal interstitial injury within 5 d of renal subcapular transfer (7, 43). We next wished to test this finding on other P3-reactive clones and to evaluate whether P2-reactive clones could mediate renal inter-



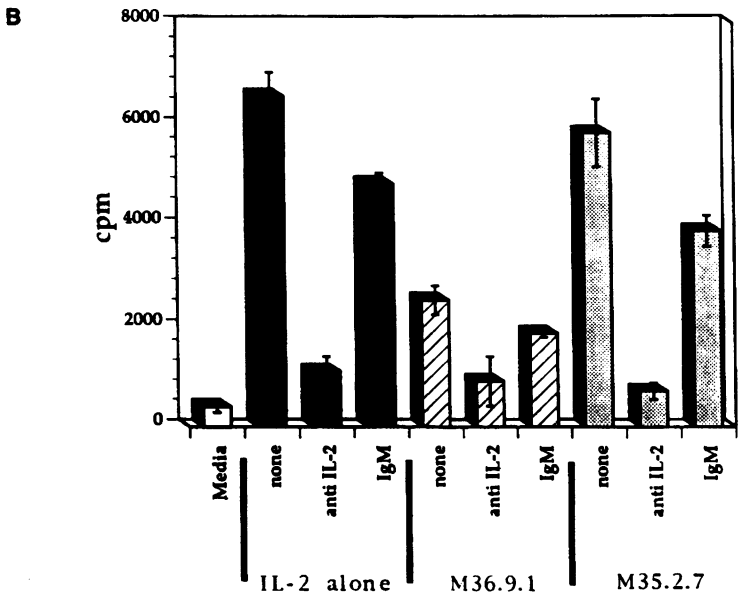


Figure 3. Cytokine profile of selected T cell clones. (*A*) Representative PCR results for γIFN (308 bp), IL-2 (463 bp), and IL-4 (304 bp) from total spleen RNA (*left*), clone M36.9.1 RNA (*middle*), and clone M35.2.7 RNA (*right*). Phi is a nucleotide marker. (*B*) HT-2 cell proliferation assay. (□) Media alone; (■) rIL-2 at 0.2 U/ml; (■) supernatants from Con A–stimulated T cell clone M36.9.1; (▩) supernatants from Con A–stimulated T cell clone M35.2.7. Experiments with rIL-2 and the T cell clone supernatants were performed with no additives (*none*), antiIL-2 antibody at 4 μg/ml (*anti–IL-2*), or a control polyclonal IgM at 4 μg/ml (*IgM*). $n = 3$ in each experiment; $P < 0.05$ between anti–IL-2 effect and the IgM control.

Table I. Antigen Specificity of T Cell Clones by DTH

Clone	Weekly stimulus	DTH			
		SRTA	P1	P2	P3
M30.2	SRTA	18.7±2.1	19.3±1.2	3.0±2.0	18.3±3.1
M33.25.6	SRTA	18.3±1.5	18.6±5.5	3.0±0.0	17.0±0.0
M36.6.1	SRTA	16.3±0.6	19.3±0.6	3.7±1.2	16.7±1.5
M36.9.1	SRTA	19.3±1.2	17.7±1.2	4.7±0.6	17.0±2.6
M34.4.3	P1	19.3±1.2	19.0±0.3	3.0±1.0	17.3±2.5
M34.7.3	P1	19.0±2.6	16.7±0.3	3.7±0.6	21±1.7
M31.1	P1	16.0±1.7	17.7±1.5	3.0±1.4	17.5±0.7
M31B.3.3	P1	18.0±1.0	20.0±1.0	3.3±1.2	18.0±1.7
M32.18.2	P2	3.3±1.2	2.3±2.1	18.0±3.1	3.7±2.5
M35.2.7	P2	3.3±1.2	3.7±0.9	15.3±1.0	3.0±1.7

stitial injury. Fig. 5 shows that clone M34.4.3 (P3 reactive) can mediate acute renal injury when P1 peptide is used in the effector cell induction culture (Fig. 5 *A*). A significant renal infiltrate with tubular degeneration and atrophy occurred within 5 d of subcapsular injection of M34.4.3/P1-induced effector cells (Fig. 5 *A*). The same clone was unable to cause renal injury when P2 was used in the induction culture (not shown), confirming the antigen specificity of this phenomenon. Similar interstitial renal injury was produced by all eight P3-reactive clones when the P3 peptide was used in the induction culture (data not shown). In contrast, a P2 reactive clone (M35.2.7) was unable to induce nephritogenic effector cells when either P1 (not shown) or P2 (Fig. 5 *B*) was used in the induction cultures. In these kidneys, we were able to find injected lymphocytes under the capsule of the kidneys, but the histology was indistinguishable from normal controls.

Analysis of TcR Vβ gene usage by nephitogenic helper T cell clones. We next wished to evaluate the TcR Vβ gene used by individual P3-reactive T cell clones. Specificity of the Vβ PCR primers was first demonstrated by Southern analysis of Vβ cDNA amplifications from total spleen RNA using an internal Cβ region primer, PH15 (Fig. 6). All Vβ PCR products were of predicted size and bound specifically to the internal primer. The genes encoding Vβ5, Vβ8, Vβ9, Vβ11, Vβ12, and Vβ13 are deleted from the genome of SJL mice (52), and were therefore not analyzed.

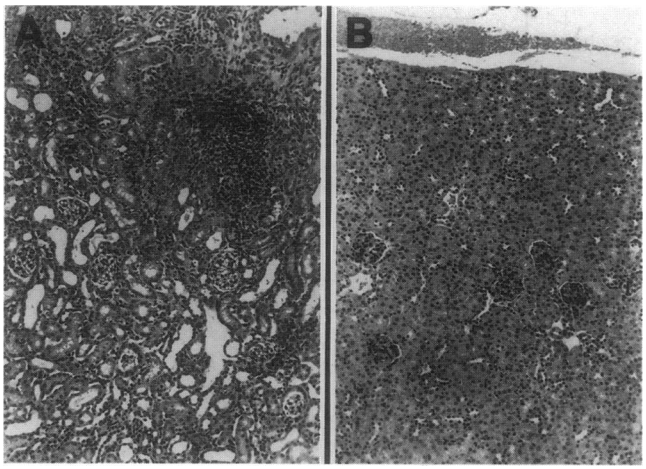


Figure 5. Subcapsular transfer of induced effector lymphocytes. (*A*) Effector cells induced with M34.4.3 (P1- and P3-reactive) and P1 peptide transfer florid interstitial nephritis with a mononuclear cell infiltrate, tubular degeneration and atrophy. (*B*) Effector cells induced with M35.2.7 (P2-reactive) and P2 peptide are not capable of transferring disease.

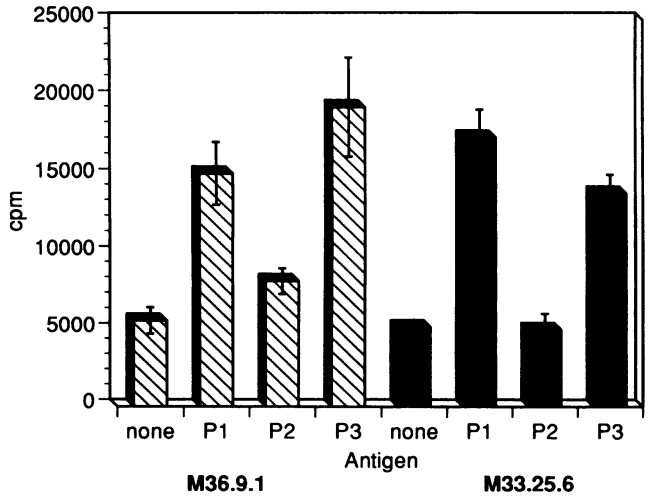


Figure 4. Lymphocyte blast transformation assay of selected T cell clones. (■) Clone M36.9.1; (■) clone M33.25.6. Peptide antigens P1, P2, or P3 were added at 50 μg/ml. $n = 3$ for each group.

Fig. 7 shows the results of the TcR Vβ analysis for two representative P3-reactive T cell clones. RT-PCR for TcR Vβ genes performed on RNA from clone M31.1 reveals a product for Vβ14 and a Cβ control (Fig. 7 *A, left*). Surface expression of Vβ14 was confirmed by FACS (Fig. 7 *A, right*). Similarly, clone M36.9.1 expresses Vβ7 (Fig. 7 *B*). The Vβ genes used by all 10 T cell clones are shown in Fig. 8. The Vβ genes expressed by six of the clones (those expressing Vβ3, 7, and 14) were confirmed by FACS. No antibodies for Vβ1, 10, 15, and 16 were available. Six Vβ genes were used by the eight different P3-reactive T cell clones, with three clones, M33.25.6, M31.1, and M31B.3., using Vβ14. The two P2-reactive clones used Vβ7 and Vβ1 (Fig. 8). To assess the baseline frequency of Vβ3, Vβ7, and Vβ14 within the peripheral T cell repertoire of the SJL mouse, we performed FACS analysis on freshly isolated splenic lymphocytes derived from unimmunized animals. Double labeling experiments using a PE-conjugated αCD4 antibody, and FITC-conjugated αVβ antibodies revealed that Vβ3, Vβ7, and Vβ14 make up 5%, 5%, and 6.5% of the $CD4^+$ T cell population, respectively (data not shown).

Analysis of the Vβ/Dβ/Jβ junctional sequences. The individual Vβ PCR products were then cloned into the PCR 3000 vector (Invitrogen), sequenced, and compared with the known

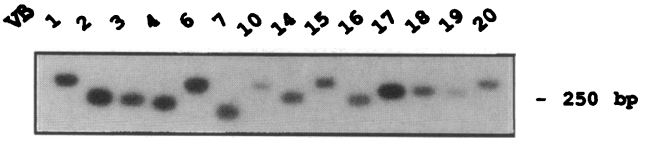


Figure 6. Southern blot of Vβ PCR products from splenic RNA probed with an internal Cβ oligonucleotide, PH15. Predicted product sizes are listed in Methods.

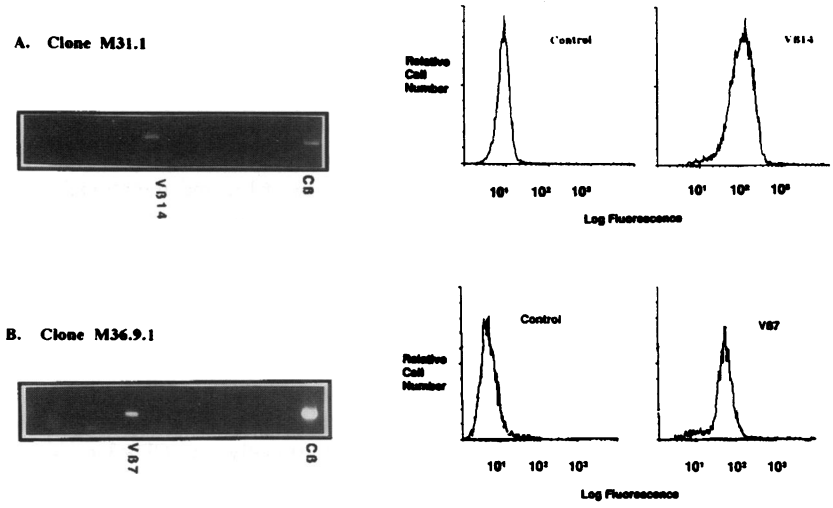


Figure 7. T cell receptor Vβ expression by individual T cell clones. (*A*) RT-PCR for Vβ genes using RNA from clone M31.1 revealing a PCR product for Vβ14 (253 bp) and for Cβ (207 bp), with confirmatory surface expression by FACS using a specific Vβ14 antibody. (*B*) RT-PCR for Vβ genes using RNA from clone M36.9.1 revealing a PCR product for Vβ7 (165 bp) and for Cβ (207 bp), with confirmatory surface expression by FACS using a specific Vβ7 antibody. An FITC conjugated goat anti-rabbit antibody served as a negative control for the FACS studies.

Vβ (44–46), Dβ (53), and Jβ (54, 55) gene sequences. In all cases, the sequence of the Vβ PCR product corresponded to the known nucleotide sequence of that particular Vβ gene, further confirming the specificity of the cDNA amplification products. The nucleotide sequences, D gene, J gene, and deduced amino acid sequences of the 10 T cell clones are shown in Fig. 8. An appropriate open reading frame was present from the V region, through the VDJ junction and into the constant region of the T cell receptor in each case. The eight P3-reactive clones use five

Vβ/Dβ/Jβ Junctional Sequences

Clone	95	96	97	98	99	100	101	102	103	104	105	106	Peptide Specificity
	Vβ14						**Dβ1.1**				**Jβ1.2**		
M33.25.6	TGT	GCC	TGG	AGT		CGA	CAG	GGA	GCA	AAC	TCC	GAC	P3
	Cys	Ala	Trp	Ser		Arg	Gln	Gly	Ala	Asn	Ser	Asp	
	Vβ14						**Dβ1.1**				**Jβ1.2**		
M31.1	TGT	GCC	TGG	AGT		AAA	CAA	GGA	GCA	AAC	TCC	GAC	P3
	Cys	Ala	Trp	Ser		Lys	Gln	Gly	Ala	Asn	Ser	Asp	
	Vβ14						**Dβ1.1**				**Jβ1.2**		
M31B.3.3	TGT	GCC	TGG	AGT		CTA	CAG	GGG	GCA	AAC	TCC	GAC	P3
	Cys	Ala	Trp	Ser		Leu	Gln	Gly	Ala	Asn	Ser	Asp	
	Vβ3						**Dβ1.1**				**Jβ1.4**		
M30.2	TGT	GCC	AGC	AGT			CAG	GGG	GTC	AAC	GAA	AGA	P3
	Cys	Ala	Ser	Ser			Gln	Gly	Val	Asn	Glu	Arg	
	Vβ7						**Dβ1.1**				**Jβ1.2**		
M36.9.1	TGT	GCT	AGC	AGC			CGC	AGG	GGC		AAC	TCC	GAC	P3
	Cys	Ala	Ser	Ser			Arg	Arg	Gly		Asn	Ser	Asp	
	Vβ10						**Dβ1.1**				**Jβ2.5**		
M36.6.1	TGT	GCC	AGC				AGG	GAG	GGA	CTG	GAA	GAC	ACC	P3
	Cys	Ala	Ser				Arg	Glu	Gly	Leu	Glu	Asp	Thr	
	Vβ15						**Dβ2.1**				**Jβ2.4**		
M34.9.9	TGT	GGT	GCT	ACG				GGG	GGG	CAC	ACC		P3
	Cys	Gly	Ala	Thr				Gly	Gly	His	Thr		
	Vβ16						**Dβ2.1**				**Jβ2.3**		
M34.4.3	TGT	GCC	AGC	AGC	CCC	CGA	CTG	GGG	GGG	CGC	CAA	ACG	P3
	Cys	Ala	Ser	Ser	Pro	Arg	Leu	Gly	Gly	Arg	Gln	Thr	
	Vβ1						**Dβ1.1**				**Jβ2.5**		
M32.18.2	GCC	AGC	AGC	CTC	TAC	AGG	GGG	AGG		AGG	CAA	GAC	P2
	Ala	Ser	Ser	Leu	Tyr	Arg	Gly	Arg		Arg	Gln	Asp	
	Vβ7						**Dβ1.1**				**Jβ1.1**		
M35.2.7	GCT	AGC	AGT			CAC	AGG	GGG	ACA	GAA	GTC	TTC	P2
	Ala	Ser	Ser			His	Arg	Gly	Thr	Glu	Val	Phe	

Figure 8. Aligned nucleotide and deduced amino acid sequences of the ten T cell clones across the Vβ/Dβ/Jβ junction. The Vβ, Dβ, and Jβ genes used by each clone are in bold type above each sequence. Underlined nucleotides represent positions of N region additions/deletions. Amino acid residue numbers 95–106 are listed across the top of the figure.

different Jβ genes, with a preponderance of Jβ 1.2 (used by M33.25.6, M31.1, M31B.3.3, and M36.9.1). Six of the eight P3-reactive clones use Dβ1.1.

The deduced amino acid sequences of the TcRβ chains from the eight P3-reactive T cell clones are remarkably similar across the Vβ/Dβ/Jβ junction, with clones M33.25.6, M31.1, M31B.3, and M30.2 showing identical or conservatively substituted amino acids in 5 or 6 positions. All eight P3-reactive clones have a small nonpolar amino acid (Ala, Val, Leu, or Gly) at the Dβ/Jβ junction (residue 102 or 103), and five of the eight clones have a cationic, basic residue (Arg or Lys) at the Vβ/Dβ junction (residue 100). Residues in both of these positions are encoded primarily by N region deletions or additions (underlined in Fig. 8), and are thus potentially highly variable. Five of the eight P3-reactive clones additionally have an Asn residue 104. In the lower portion of Fig. 8 are the deduced amino acid sequences of the TcR β chains from the two, P2-reactive clones. The sequence of the Vβ/Dβ/Jβ junctions shows some homology to the P3-reactive clones, but position 103 contains a charged residue (Arg in M32.18.2 and Glu in M35.2.7) as opposed to a small nonpolar residue. Additionally, position 100 is Tyr or His in the P2 reactive clones, and not Arg as found in most of the P3-reactive clones.

To confirm the relevance of the sequence findings, we analyzed a random sample of 52 Vβ/Dβ/Jβ sequences for the presence of Ala, Val, Leu or Gly residue at the Dβ/Jβ junction, or an Arg or Lys at the Vβ/Dβ junction. 14 of 52 sequences contained an Ala, Val, Leu, or Gly at the Dβ/Jβ junction, compared to 8/8 of our P3 reactive clones ($P < 0.001$), while 14/52 of the random sequences contained an Arg or Lys at the Vβ/Dβ junction, compared to 5/8 of our P3-reactive clones ($P < 0.05$). Additionally, 6/8 of the P3-reactive Vβ/Dβ/Jβ sequences are cationic (with the other two having an overall neutral charge), compared with the 5/52 incidence found in the randomly chosen T cell sequences ($P < 0.001$).

Discussion

We have successfully isolated a panel of CD4$^+$, TcR αβ-expressing T cell clones from mice immunized to get αTBM disease. The cytokine profiles and the functional characteristics (i.e., the ability to provide T cell help for the induction of DTH-

producing effector cells) of these clones are consistent with a Th1 phenotype (56). We have not tested these clones for their ability to induce a humoral immune response, however, and cannot fully exclude B cell help as a potential function of these cells. Additionally, we have not yet attempted to preferentially isolate Th2-like cells from these animals.

Antigen specificity was demonstrated functionally in all clones by an established biological assay (25, 43), and confirmed in selected clones through the use of a proliferation assay (24, 26). All clones stimulated weekly with SRTA or P1 specifically reacted to the P3 peptide, and not to P2, suggesting that these T cell clones recognize a core sequence of eight amino acids (STMSAEVP, bold letters in Fig. 1). Interestingly, this core sequence, as well as the entire P3 epitope, is anionic, and contains no positively charged residues (Fig. 1). Future work involving the elution of peptide from MHC II molecules (57), and a systematic evaluation of T cell reactivity to overlapping and mutated peptides, will be required to potentially identify the optimal peptide size, and the importance of individual residues in the recognition of this immunodominant epitope by the different T cell clones.

We also isolated two $CD4^+$ Th1 T cell clones which recognized the P2 peptide in vitro, but were unable to recognize the intact SRTA from which this peptide was derived. We find it curious that these T cell clones, isolated from animals immunized with RTA, can recognize the P2 peptide, but not the intact antigen. Although the explanation for this phenomenon is not readily apparent, the relative difficulty in isolating these clones (we were only able to isolate two) suggests that the target antigen of αTBM disease, 3M-1, is only rarely processed into P2 (or a peptide containing a significant portion of P2) by $I\text{-}A^s$ antigen presenting cells in the SJL mouse.

The relevance of P3 (but not to P2) to the development of αTBM disease was demonstrated through showing that the clones reactive to P3 were able to transfer disease to naive recipients, while a P2-reactive clone was not. In sum, these findings suggest that $CD4^+$, Th1 helper T cells derived from the peripheral lymphoid system of animals immunized to produce αTBM disease, and directed towards an immunodominant epitope contained within the P3 peptide, are nephritogenic. Interestingly, both the αTBM antibody response and the $CD8^+$ effector T cell response are also directed at P1 (7, 27) (reactivity towards P3 has not been tested), confirming the importance of this antigenic epitope in the pathogenesis of αTBM disease. Although our results demonstrate a single immunodominant peptide epitope relevant to the development of disease, it is possible that other, secondary, cryptic epitopes emerge within the kidney as the disease process progresses (58). Studies evaluating the peptide specificity of nephritogenic T cells derived from diseased organs will be required to address this issue.

Analysis of the TcR Vβ genes used by the P3 epitope-specific T cell clones revealed the use of several Vβ genes, with a recurrent use (3/8) of Vβ14. This 38% incidence of Vβ14 expression by P3-reactive clones, compared with 6.5% of the peripheral $CD4^+$ T cell population, provides evidence for the preferential use of the Vβ14 gene by the nephritogenic T cell clones. Collectively, our findings suggest that the anti–3M-1 TCR repertoire is oligoclonal. Whether these findings are representative of the TcR repertoire within diseased organs is an area of ongoing investigation. It should be noted however, that interpretation of TCR gene usage analyses in diseased whole organs is often hampered by the lack of definition of peptide specificity (16), and results may not correlate with findings derived from peptide specific T cell clones (16).

Perhaps more intriguing than the oligoclonality of the TCR response is the similarity of the deduced amino acid sequences across the Vβ/Dβ/Jβ junction. Based initially on comparative analyses between immunoglobulin and TcR sequences (59, 60), and later confirmed by statistical (61), and mutational (62–64) analyses, the Vβ/Dβ/Jβ junction of the TcR in $CD4^+$ cells has been proposed to constitute the putative CDR3 region, and thus make direct contact with its peptide/MHC class II ligand. The statistically significant overrepresentation (compared with a random set of functional TcRβ chains) of a small nonpolar amino acid at the Dβ/Jβ junction, and an Arg or Lys at the Vβ/Dβ junction, both determined by N region additions or deletions, suggests that these residues may be important in the specific recognition of P3. Additionally, the CDR3 region of all of the P3-reactive clones is neutral or cationic (Arg/Lys), with a distinct lack of negative charges (Glu in position 101 of M36.6.1 is the only one, and is counteracted by an adjacent Arg, Fig. 8), suggesting a direct interaction with the anionic P3 peptide. Analogous findings have been noted in another model of autoimmune renal injury (8). The precise determination of the role played by individual residues within the CDR3 in the specific recognition of the P3/MHC class II complex will require a mutational analysis of the TCR. Finally, the sequence of the TCR α chain needs to be defined before any definitive statements regarding the molecular basis of P3 recognition by autoreactive T cells can be made. Based on these experiments, however, it may be possible to direct specific immunotherapy at a common epitope within the TCRβ CDR3 region of nephritogenic, P3-reactive T cells.

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