

T cell lines specific for a synthetic Heymann nephritis peptide derived from the receptor-associated protein

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SUMMARY

Pathogenic antigens involved in the induction of Heymann nephritis (HN), an experimental rat model of human membranous nephritis, have been identified in megalin (gp330) and the receptor-associated protein (RAP) [1,2]. A pathogenic epitope has been identified in RAP (amino acid 1–86) that plays a significant role in the formation of immune deposits in glomeruli in HN. A synthetic peptide (P_{31–53}) derived from RAP_{1–86} contains a pathogenic epitope recognized by antibodies eluted from glomerular immune deposits and includes two putative RT-1B¹ MHC class II-binding motifs. We have investigated whether RAP P_{31–53} can be recognized by T cells. Five peptide-specific T cell lines were generated from regional lymph node (LN) T cells from Lewis rats immunized with P_{31–53}. The T cell lines were characterized by using a T cell proliferation assay for their specificity, FACS and MHC restriction assay for the phenotype, reverse transcription-polymerase chain reaction for TCR V β repertoire and cytokine expression, and cloning and sequencing for the analysis of the CDR3 sequence of TCR. The helper function of the T cell line was confirmed by autoantibody production *in vitro*. In this study, we clearly identify that the synthetic pathogenic peptide P_{31–53} contains a T cell epitope recognized by CD4⁺ Th2 cells in Lewis rats. This recognition was restricted by MHC class II RT1.B¹. These CD4⁺ Th2 cells were able to promote B cells to produce specific antibodies and used a restricted set of TCR V β genes with preferential usage of V β 18. A charged amino acid motif at the CDR3 region of predominant TCR V β subfamilies may contribute to the specific ability of these cells to recognize the immunogenic T cell epitope within RAP peptide P_{31–53}.

Keywords RAP Heymann nephritis epitope T cell line T cell receptor

INTRODUCTION

Active Heymann nephritis (HN) is an experimental rat model of autoimmune-mediated glomerulonephritis that shares evident similarities at pathophysiologic and immunohistologic levels with human membranous nephropathy [3,4]. Active HN is commonly induced by immunizing rats with a crude renal tubular antigen (RTA/Fx1A) emulsified in Freund's complete adjuvant (FCA) [5,6]. In HN renal injury is thought to be due to the binding of autoantibodies to autoantigens expressed on glomerular epithelial cells, leading to the formation of subepithelial immune deposits (ID) along the glomerular basement membrane with the activation of complement, resulting in severe glomerular dysfunction and proteinuria [7].

Both megalin (gp330) [1,2] and the receptor-associated

protein (RAP) [8] are considered to be major pathogenic autoantigens since active HN can be induced by immunization of rats with either. Recently significant progress has been made in mapping a pathogenic epitope within the 86 N-terminal amino acids of RAP (RAP_{1–86}) which plays a key role in the formation of subepithelial ID during the initiation of HN [9]. Furthermore, a synthetic peptide (P_{31–53}) derived from RAP_{1–86} has been shown to contain an ID-inducing epitope by SPOTs assay. Injection of antibodies raised against this synthetic P_{31–53} into rats can induce stable glomerular ID [10].

Previous studies have shown that the induction of an autoantibody response to RTA (Fx1A) is dependent upon CD4⁺ T helper (Th) cells, which provide help for autoantibody production [11]. The role of CD4⁺ T cells in the mediation of HN was further confirmed by the observation that anti-CD4 MoAb therapy can totally prevent the proteinuria [12]. Although CD4⁺ T cells have an established role in the production of pathogenic autoantibody in HN [11,12], little is known about the immunogenic T cell epitopes or their responding T cell receptors

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(TCR). One obvious candidate is RAP, which contains a well-characterized pathogenic epitope P₃₁₋₅₃ that binds antibodies eluted from the glomeruli of rats with active HN and initiates ID. This peptide contains two putative RT1.B¹ MHC class II peptide binding motifs (S x x x x E), thought to be important for generation of antibodies in Lewis rats [13]. Based on those findings, we reasoned that RAP P₃₁₋₅₃ may be presented by antigen-presenting cells (APC) in the context of MHC class II and recognized by T cells. If so, the identification of T cell epitopes and analysis of corresponding TCR should provide important information for the design of rational therapeutic interventions.

MATERIALS AND METHODS

Animals

Inbred male Lewis rats were obtained from the Animal Resources Centre in Perth, Western Australia. Rats weighing 180–200 g at the age of 8–10 weeks were used in all experiments.

Antigens

Peptide P₃₁₋₅₃ of RAP (sequence KAKRLHLSVRLAELHSD-LKIQE) was synthesized after the published data [10]. The irrelevant peptide sequence of a cellular structural protein was used (CESLYRQLERNSSLNELKLT) as a control. The two peptides were synthesized by Peptide Express (Colorado State University, Fort Collins, CO) and purified to >90% by reverse phase HPLC.

Immunization

Each group of four Lewis rats was immunized in both hind footpads with a 100- μ l emulsion of 300 μ g or 600 μ g of P₃₁₋₅₃ in FCA containing 1 mg *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI). Boosters were given subcutaneously with P₃₁₋₅₃ in Freund's incomplete adjuvant (FIA) at 3 weeks and 7 weeks. Five control rats were immunized with FCA prepared without P₃₁₋₅₃.

Production of T cell lines

T cell lines were independently developed from five rats. Popliteal lymph nodes were obtained 14 days after immunization and teased to a single-cell suspension. Lymph node (LN) cells (4×10^6) were stimulated with P₃₁₋₅₃ at a concentration of 20 μ g/ml for 7 days in complete RPMI medium supplemented with 2.5% syngeneic rat serum. Following stimulation, the T cell blasts were isolated on Lymph-prep (Cedar Lane, Canada). The T cells (1×10^6) were restimulated weekly with P₃₁₋₅₃ (20 μ g/ml) and 3×10^6 irradiated syngeneic splenocytes as APC in complete RPMI supplemented with 10% fetal calf serum (FCS) and 10% concanavalin A (Con A) supernatant. After the first round of stimulation, the cells were analysed for their specificity to P₃₁₋₅₃ in a proliferation assay. Following nine rounds of stimulation, the five T cell lines were characterized in detail.

T cell proliferation assay

After the first round of P₃₁₋₅₃ stimulation, 1×10^5 cells were seeded in microtitre plates with 3×10^5 irradiated (25 Gy) syngeneic splenocytes as APC in the presence of peptide at 20 μ g/ml. The culture was incubated in triplicate for 72 h. Each well was pulsed with 1 μ Ci of ³H-thymidine for the final 18 h. ³H-TdR uptake was determined by standard technique. Proliferation was expressed as mean ct/min. The results were expressed as mean ct/min and a stimulation index (SI).

FACS analysis

Cell lines were incubated with either OX-35 (anti-CD4) or OX8 (anti-CD8) FITC-conjugated anti-rat MoAbs (PharMingen, San Diego, CA) or an isotype control antibody. Samples were analysed using a Becton Dickinson (Heidelberg, Germany) FACScan analyser.

Determination of MHC restriction pattern

Anti-MHC MoAbs were used with the proliferation assay to determine the MHC restriction of T cell responses to P₃₁₋₅₃: OX6 (anti-RT1.B, class II), OX17 (anti-RT1.D, class II), OX18 (anti-RT1.A, class I) and an isotype control antibody.

P₃₁₋₅₃ specific immunoglobulin production in vitro

Demonstration of immunoglobulin production *in vitro* was essentially according to the method of Saoudi *et al.* [14]. Co-cultures were performed by seeding 1×10^5 cells from the P₃₁₋₅₃-specific T cell lines after nine rounds of peptide stimulation to 5×10^5 naive syngeneic spleen cells in a final volume of 0.2 ml/well in microculture plates in the presence of P₃₁₋₅₃ at 20 μ g/ml for 13 days. A volume of 150 μ l of culture supernatant was collected from each well at days 6, 9 and 13. Cultures were assayed for anti-P₃₁₋₅₃ autoantibodies by ELISA.

RNA extraction and cDNA synthesis

Total RNA was extracted from LN cells and T cell lines by acid guanidinium thiocyanate-phenol-chloroform extraction method [15]. cDNA was synthesized by using random hexamer primers (Promega, Madison, WI) and M-MuLV reverse transcriptase (GIBCO BRL, Grand Island, NY).

Reverse transcription-polymerase chain reaction for TCR V β repertoire

Oligonucleotide primers used for rat TCR V β 1–20 were those described by Gold *et al.* [16]. Oligonucleotide primers for TCR C β (5'-tggtgtctgcgatctctgc-3'), for TCR V β and TCR C-C β primers (5'-accatgtggagctgagctg-3' and 5'-cactaccagcgtacttgacaaagc-3') specific for the constant region of TCR were designed using PCRprim software. The polymerase chain reaction (PCR) profile used was 1 min at each of 95°C, 60°C and 72°C for 32 cycles.

The specificity of each PCR product was verified by separate hybridization with Tris (2,2'-bipyridine) ruthenium (II) chelate (TBR)-labelled sequence-specific oligonucleotide probes. Sequence of probe specific for TCR V β PCR products was 5'-aggtctctgtttgagcca-3' and probe for TCR C-C β PCR products was 5'-acaatcctcgcaaccactc-3'. The electrochemiluminescent signal of the hybridized probe was detected with a QPCR 5000 system (Perkin Elmer, Norwalk, CT), as described [17,18]. The relative luminosity of each V β family member was expressed as a percentage of the total luminosity detected in all of the V β regions for a given sample.

To optimize the reverse transcription (RT)-PCR technique, two quantities of PBL cDNA were amplified with TCR C-C β primers with cycles from 24 to 41. QPCR luminosity units increased exponentially with the number of cycles until a plateau was reached. Thirty-two PCR cycles were used for the construction of a TCR C β standard curve in triplicate over a range of serial dilutions of known quantity of pooled PBL RNA for TCR C β . PCR amplification was performed in triplicate for TCR V β gene usage in all experimental samples in dilutions chosen so that the

luminescence reading for the experimental sample fell upon the steep part of the standard curves.

Semiquantitative RT-PCR for cytokine genes

Oligonucleotide primers used for rat cytokine genes IL-2, IL-10, IL-4 and interferon-gamma (IFN- γ) were those described [19,20]. TCR C- β primers specific for the constant region of TCR served as the internal control. The PCR profile used was 1 min each at 95°C, 60°C and 72°C for 30 cycles for TCR C- β , 32 cycles for IL-10 and IFN- γ and 35 cycles for IL-2 and IL-4. RT-PCR fragments of cytokine genes were electrophoresed on a 2% agarose gel and then photographed on a negative film (Polaroid 665). Signal was quantified by densitometry (Molecular Dynamics, Sunnyvale, CA).

To measure PCR products of cytokine genes semiquantitatively, standard curves of the linear range of amplification for each cytokine gene primer pair were constructed over three logs of a known concentration of pooled RNA obtained from Con A-stimulated rat T cells collected at different stimulation times. PCR amplification was performed in triplicate for cytokine genes in all experimental samples in dilutions chosen so that the densitometry reading for the experimental sample fell on the steep part of the standard exponential curve.

Cloning and sequencing of TCR V β R-PCR products

TCR V β PCR products were purified and then cloned into the pGEM-T vector system (Promega). DNA plasmid preparations were made from individual colonies using the Wizard plus SV miniprep kit (Promega). DNA sequencing was performed in both directions by the dideoxy chain determination method.

Antibody determination

Autoantibody titres were determined by ELISA as previously described [12]. Normal Lewis rat serum or culture media were used as the negative control. All samples were assayed in triplicate.

Immunofluorescence analysis

Kidneys were taken at 12 weeks post-inoculation for use in direct immunofluorescence studies. The frozen sections (4 μ m) were incubated for 30 min with FITC-labelled goat anti-rat IgG (Zymed Labs, San Francisco, CA).

Frozen sections (4 μ m) of normal Lewis rat kidney were fixed in acetone for 5 min and incubated with serum from a rat immunized with P₃₁₋₅₃ at 12 weeks of sacrifice. For controls, sections were incubated with serum either from a rat immunized with FCA alone as the negative control or from a rat immunized with Fx1A as the positive control. The sections were then incubated with FITC-labelled goat anti-rat IgG (Zymed Labs).

RESULTS

Primed LN cell proliferation in response to a synthetic RAP

P₃₁₋₅₃

Proliferation of the LN cells specifically in response to P₃₁₋₅₃ was detected after the first stimulation *in vitro*. As shown in Fig. 1, five rats manifested a strong T cell proliferative response to P₃₁₋₅₃ but not to an irrelevant control peptide. In the control group, the rats immunized with FCA alone had no T cell proliferation in response to both P₃₁₋₅₂ and an irrelevant peptide. The T cell proliferative response measured as a SI in the P₃₁₋₅₃-immunized rats to P₃₁₋₅₃ was 21.4 ± 16.4 and to the control

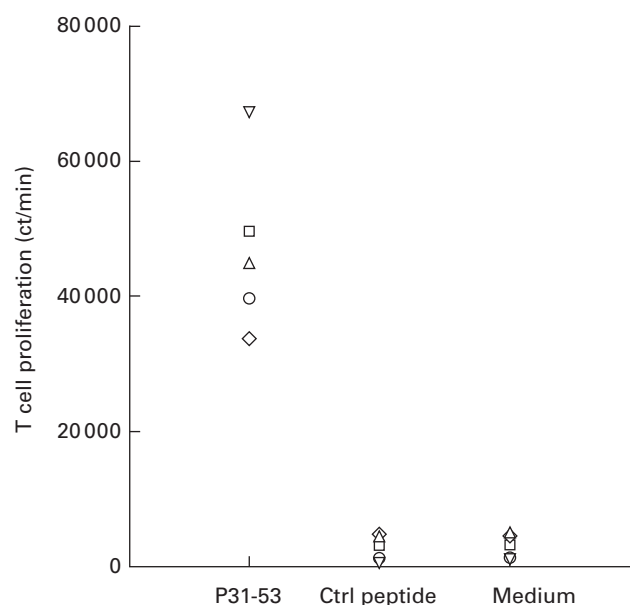


Fig. 1. Proliferative responses of lymph node cells primed *in vivo* and stimulated *in vitro* for 7 days with peptide P₃₁₋₅₃. The different symbols in each panel denote results obtained with individual rats. The data are expressed as mean ct/min in triplicate cultures.

peptide was < 1 or negligible. Thus, an antigenic T cell epitope was present within synthetic P₃₁₋₅₃.

T cell lines specific for P₃₁₋₅₃ were CD4⁺CD8⁻ and restricted by MHC class II RT1.B¹

Five T cell lines were generated successfully by *in vitro* restimulation of draining LN cells from five rats immunized with P₃₁₋₅₃. After nine rounds of stimulation with P₃₁₋₅₃, FACS analysis of the phenotype of peptide-specific T cell lines showed that 95–98% of T cells in each cell line were CD4⁺CD8⁻. Addition of anti-RT1.B MoAb reduced proliferation of all T cell lines by $> 70\%$, whereas anti-RT1.D or anti-RT1.A MoAbs had no significant effect on T cell proliferation (Fig. 2). These results

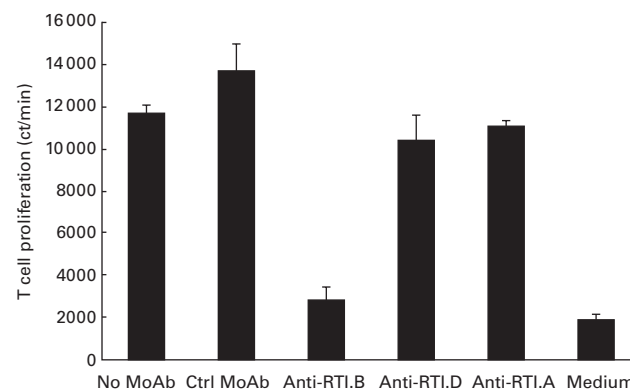


Fig. 2. Anti-MHC MoAb inhibition of peptide P₃₁₋₅₃-specific T cell line responses to P₃₁₋₅₃ after nine rounds of peptide stimulation *in vitro*. T cell lines were cultured with irradiated antigen-presenting cells and 20 μ g/ml P₃₁₋₅₃ in the presence of 10 μ g/ml MoAb specific for RT1.B (OX6), RT1.D (OX17), RT1.A (OX18) and isotypic control MoAb.

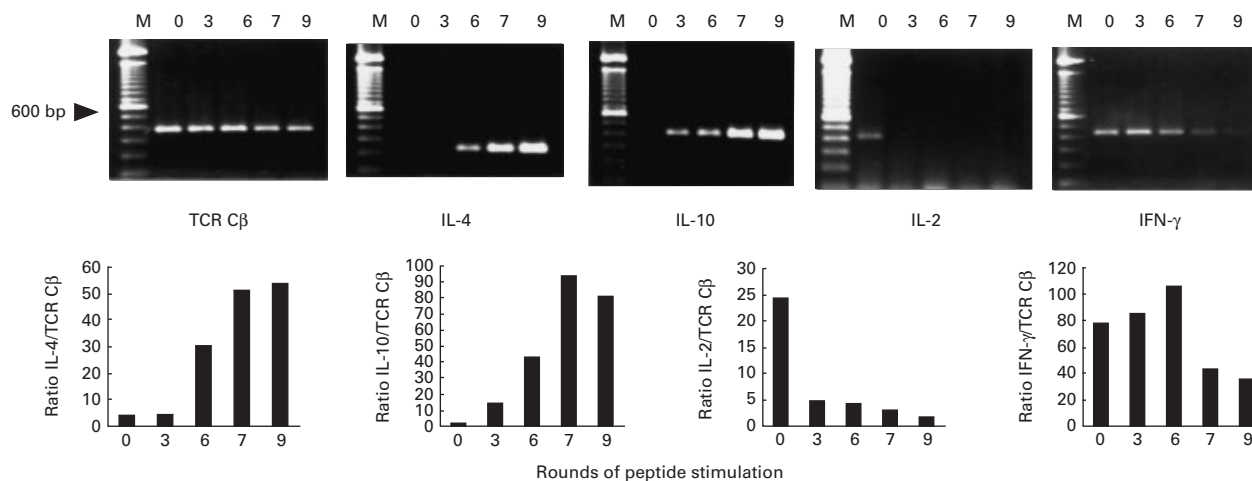


Fig. 3. The peptide-specific T cell line has a Th2 pattern of expression of cytokine mRNA with increasing rounds of peptide stimulation *in vitro* measured by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). The numbers at the top represent different rounds of peptide stimulation *in vitro*. The TCR β -chain constant region (TCR C- $\text{C}\beta$) was an internal control for the semiquantitative RT-PCR. Data show a representative repeated experiment from a cell line. All PCR products were separated on a 2% agarose gel (top panel) and of the expected size based on the molecular weight markers in lane 1. The results are expressed as ratio of cytokine gene densitometry score to TCR C- $\text{C}\beta$ densitometry score (bottom panel).

demonstrate that the antigen-specific T cells of the five lines were $\text{CD4}^+\text{CD8}^-$ cells and that T cell recognition of P_{31-53} was restricted by MHC class II RT1.B¹.

T cells specific for P_{31-53} had a restricted Th2 cytokine profile

After nine rounds of stimulation, T cell lines had a Th2 cytokine profile, in that IL-4 and IL-10 mRNA expression appeared to increase, whereas IL-2 and IFN- γ mRNA expression were almost undetectable (Fig. 3). To confirm these results further, we screened cytokine mRNA expression from T cell lines collected after 0, three, six and nine rounds of stimulation. Expression of mRNA encoding IL-4 and IL-10 genes increased substantially with increasing rounds of stimulation with P_{31-53} . In contrast, expression of mRNA encoding IL-2 and IFN- γ gene decreased with increasing rounds of stimulation with P_{31-53} .

Specific T cell lines promote B cell activation of antibody production

As shown in Fig. 4, low but measurable titres of autoantibodies (IgG) to peptide P_{31-53} were detectable in supernatant at 9 and 13 days after co-culture of T cell lines with naive spleen cells, but not at 6 days. In controls, no antibody could be detected in supernatants obtained from the cultures of T cell lines with or without irradiated spleen cells and spleen alone.

T cell lines specific for P_{31-53} use a restricted set of TCR $\text{V}\beta$

By the ninth stimulation, TCR $\text{V}\beta$ gene usage by five peptide-specific T cell lines was largely restricted to $\text{V}\beta 18$. Percentages of $\text{V}\beta 18$ in total TCR repertoire of five cell lines were 54%, 47.4%, 41.4%, 36.4% and 21.1%, respectively. To demonstrate how the RAP peptide shapes TCR $\text{V}\beta$ repertoire over time *in vitro*, a representative cell line was analysed for TCR $\text{V}\beta$ gene expression

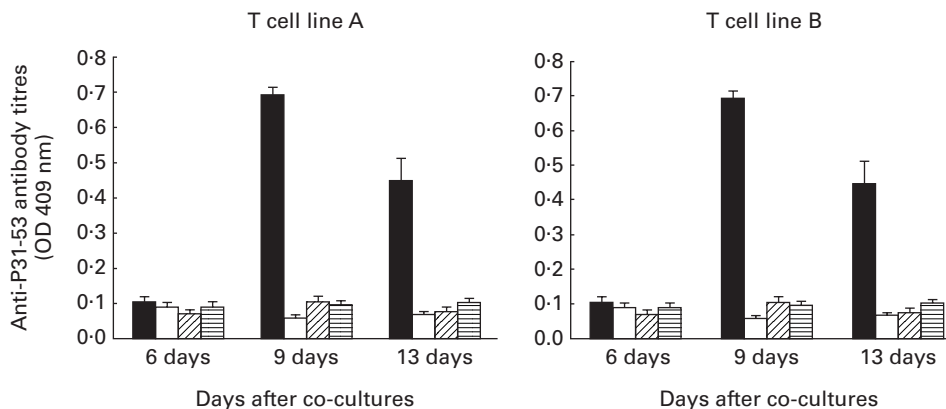


Fig. 4. Ability of two peptide-specific T cell lines to trigger *in vitro* antibody production by syngeneic spleen cells. Antibodies (immunoglobulin) to P_{31-53} were measured by ELISA. ■, ELISA optical density (OD) results from the supernatant from co-cultures of T cell line and naive syngeneic spleen cells; □, results from co-culture of T cell lines with irradiated syngeneic spleen cells; diagonal and horizontal hatched bars, respectively, show the results from supernatant of culture of T cell lines alone and the supernatant from culture of syngeneic spleen cells alone. Results are plotted as mean \pm s.e.m. of three individual cultures.

Table 1. T cell receptor (TCR) Vβ usage became progressively limited to Vβ18, Vβ4 and Vβ14

TCR Vβ	Primed*	Passage 3*	Passage 6*	Passage 9*
1	0.5	1.7	0.7	0.5
2	18.3	8.1	10.1	3.7
3	0.6	2.5	1.1	1.6
4	10.2	11.5	11.3	9.9
5	8.7	6.3	11.0	3.9
6	0.3	0.7	0.8	0.2
7	1.5	0.5	0.3	0.1
8.5	5.1	3.6	8.5	2.1
8.6	0.1	0.1	0.4	0.0
9	2.8	0.8	1.7	1.6
10	0.2	0.8	2.2	0.2
11	0.4	1.3	2.1	0.5
12	11.9	12.2	5.5	3.7
13	8.4	6.4	6.6	0.6
14	7.3	6.0	6.6	9.7
15	3.1	4.5	3.0	0.8
16	7.7	13.7	12.2	5.2
17	3.1	4.1	0.9	0.1
18	6.3	9.3	12.0	54.0
19	0.23	1.1	0.0	1.1
20	3.1	4.4	3.3	0.9

*Percentage of total repertoire.

Table 2. CDR3 sequences of T cell receptor (TCR) Vβ reverse transcription-polymerase chain reaction products from a T cell line after nine rounds of P₃₁₋₅₃ stimulation

Vβ	NDN	Jβ	Clonotype Frequency
	←-----CDR3-----→		
Vβ18 CSS G	<u>DGGR</u>	AEQF FG	Jβ2.1 12/12
Vβ4 CAS S	<u>QDLGGAR</u>	EQT FG	Jβ2.6 10/10
Vβ14 CAT S	<u>EGL</u>	TEQT FG	Jβ2.6 5/10
CAT S	TC	QNTLF FG	Jβ2.4 4/10
CAT S	<u>HRGT</u>	NTEVF FG	Jβ1.1 1/10

Standard single-letter codes for amino acids are employed. Underlined residues are encoded by D region. Bold residues are positively charged arginine (R) and negatively charged aspartic acid (D), or glutamic acid (E) residues encoded within the putative CDR3 regions. CDR3 include the 5' S residue of the conserved C-A-S(S) motif of most TCR Vβ gene and the 3' amino acid that precedes the conserved (X)-F-G-X-G motif presenting all TCR Jβ gene [25].

after 0, three and six cycles of peptide-specific stimulation. Table 1 show that TCR Vβ gene usage became progressively limited to Vβ18 (54%), Vβ4 (10%) and Vβ14 (10%). By the ninth stimulation, these three Vβ subfamilies accounted for >70% of all the cells in the line.

Analysis of CDR3 sequence of Vβ PCR products

The deduced amino acid sequences are shown in Table 2. Analysis of CDR3 sequence encoding Vβ18 RT-PCR products from a representative T cell line after nine rounds of peptide stimulation demonstrated that 12 out of 12 clones shared identical CDR3 sequences based on identity at the nucleic acid level. In contrast, Vβ18 RT-PCR products derived directly from LN cells were remarkably heterogeneous, as shown in Table 3. Of 34 clones, 28 showed different CDR3 sequences at the nucleic acid level. Usage of CDR3 region of TCR Vβ18 segments in the cell line became highly restricted with increasing rounds of P₃₁₋₅₃ stimulation *in vitro*. Six out of 34 clones (17.6%) derived directly from LN cells primed with P₃₁₋₅₃ had the same CDR3 sequence as that seen in the T cell line studied after nine rounds of peptide

Table 3. CDR3 sequences of T cell receptor (TCR) Vβ reverse transcription-polymerase chain reaction products of lymph node (LN) cells from a rat immunized with the P₃₁₋₅₃

Vβ18	NDN	Jβ	Clonotype Frequency
	←-----CDR3-----→		
CSS G	VLQG	NEVF FG	Jβ1.1 1/34
CSS G	S	NEVF FG	Jβ1.1 1/34
CSS G	DI	NQAPL FG	Jβ1.5 1/34
CSS G	RGQPS	NQAPL FG	Jβ1.5 1/34
CSS G	GGHGL	QAPL FG	Jβ1.5 1/34
CSS G	EGRTGS	NERLF FG	Jβ1.4 1/34
CSS G	EGRTG	NERLF FG	Jβ1.4 1/34
CSS G	TAGD	NERLF FG	Jβ1.4 1/34
CSS G	DGV	ETQY FG	Jβ2.5 1/34
CSS G	RQEQ	ETQY FG	Jβ2.5 1/34
CSS G	Q	ETQY FG	Jβ2.5 1/34
CSS G	E	Y FG	Jβ2.5 1/34
CSS G	DRARG	YEQY FG	Jβ2.6 1/34
CSS G	DMKG	EQY FG	Jβ2.6 1/34
CSS G	GGNV	EQY FG	Jβ2.6 1/34
CSS T	GK	YEQY FG	Jβ2.6 1/34
CSS G	GLGGT	YEQY FG	Jβ2.6 1/34
CSS G	QD	EQY FG	Jβ2.6 1/34
CSS G	DPGD	GQLY FG	Jβ2.2 1/34
CSS G	QGT	NTGQLY FG	Jβ2.2 1/34
CSS G	DGGR	AEQF FG	Jβ2.1 6/34
CSS E	DRAY	SYAEQF FG	Jβ2.1 1/34
CSS P	GGAH	SYAEQF FG	Jβ2.1 1/34
CSS G	AGLGE	AEQF FG	Jβ2.1 1/34
CSS G	GRG	AEQF FG	Jβ2.1 1/34
CSS G	REGF	YAEQF FG	Jβ2.1 1/34
CSS G	QLGPN	AEQF FG	Jβ2.1 2/34
CSS G	VMGT	SQNTLF FG	Jβ2.4 1/34

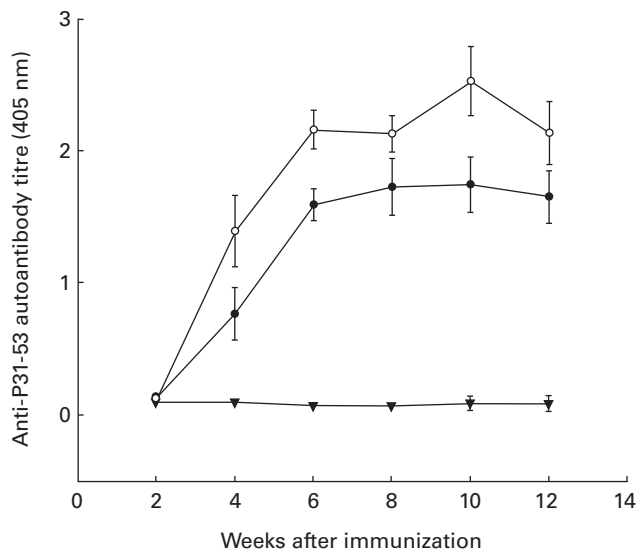


Fig. 5. Serum titres of IgG antibodies to peptide P₃₁₋₅₃ in groups of Lewis rats immunized with the peptide in Freund's complete adjuvant (FCA) to induce Heymann nephritis. Groups were immunized with 300 µg of P₃₁₋₅₃ in FCA (●; *n* = 4), or with 600 µg of P₃₁₋₅₃ (○; *n* = 4). Control rats were immunized with FCA alone (▼; *n* = 4).

stimulation (Table 3). By three rounds and six rounds of P₃₁₋₅₃ stimulation, eight out of 14 (57%) and six out of six clones (100%) had, respectively, the same CDR3 sequence as that seen in the T cell line studied after nine rounds of peptide stimulation. These results demonstrate that repeated stimulation with P₃₁₋₅₃ creates a clonal expansion of T cells bearing TCR Vβ18 segments with an antigen-specific CDR3 sequence.

As shown in Table 2, analysis of CDR3 sequence encoding Vβ4 RT-PCR products showed that 10 out of 10 clones had a common CDR3 sequence after nine rounds of P₃₁₋₅₃ stimulation *in vitro*. Ten Vβ14 clones examined had three different CDR3 sequences after nine rounds of P₃₁₋₅₃ stimulation *in vitro*.

Of particular interest is the striking similarity in the sequences of charged amino acids in the CDR3 regions of Vβ18 and Vβ4 in

the T cell receptors we had sequenced after nine rounds of P₃₁₋₅₃ stimulation *in vitro*. The CDR3 loops of Vβ18 and Vβ4 both contained a feature motif: positively charged residues (arginine) and negatively charged residues (aspartic acid) encoded N region addition/deletions and two hydrophobic residues (glycine) encoded D regions. Interestingly, a similar N region-encoded arginine (or the structurally similar histidine) was found in the CDR3 loops of Vβ14. An over-representation of another negatively charged residue (glutamic acid) in the CDR3 loops of Vβ18, Vβ4 and Vβ14 was contributed by the Jβ segment due to the nature of the joining process. These charged amino acids at the CDR3 region of these three predominant TCR Vβ subfamilies may contribute to the specific ability of these cells to recognize P₃₁₋₅₃.

Assessment of disease activity

Anti-peptide P₃₁₋₅₃ autoantibodies (IgG) were detectable in the sera of all rats immunized with P₃₁₋₅₃ at 4 weeks and throughout the course until the rats were killed at 12 weeks (Fig. 5). The site specificity of these anti-peptide P₃₁₋₅₃ autoantibodies was verified by staining normal rat kidney sections by indirect immunofluorescence. Autoantibodies against P₃₁₋₅₃ reacted with an epitope of RAP present in the brush border region of the proximal renal tubules.

We also analysed kidney sections from rats immunized with P₃₁₋₅₃ at the time of sacrifice stained with FITC-labelled goat anti-rat IgG by direct immunofluorescence staining. The results showed that minimal faint staining for rat IgG was present on the glomerular basement membrane (Fig. 6a). The fluorescent signal was slightly higher than the control staining for endogenous rat IgG usually present in normal rat kidney sections (Fig. 6b). None of the rats immunized with P₃₁₋₅₃ developed proteinuria.

DISCUSSION

In this study we have shown that a synthetic pathogenic P₃₁₋₅₃ recognized by antibodies eluted from the glomeruli of rats with HN also contains a T cell epitope. After immunization with P₃₁₋₅₃, five T cell lines from primed regional LN cells manifested

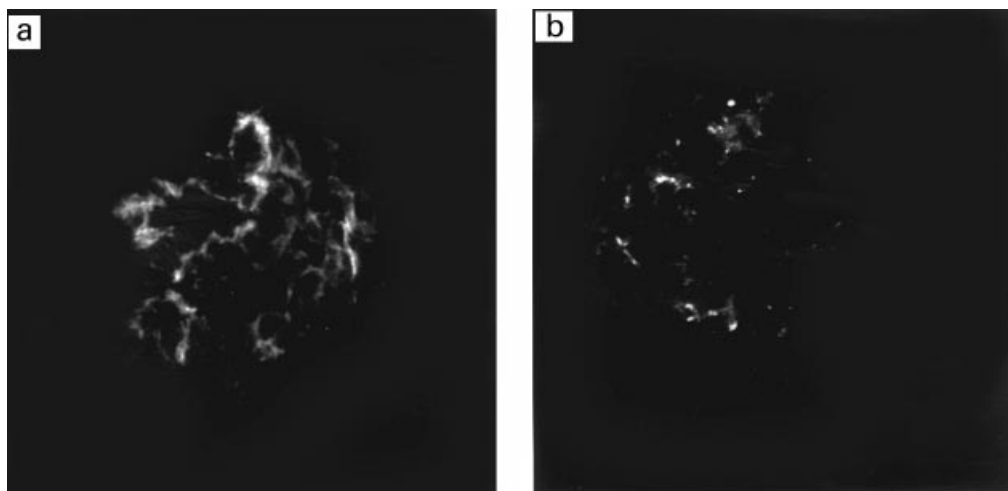


Fig. 6. FITC-labelled anti-rat IgG staining by directed immunofluorescence on frozen kidney sections from a rat immunized with P₃₁₋₅₃ (a) and normal rat as a control (b) (mag. × 400). Faint staining for rat IgG (a) is seen in the capillary loops 12 weeks after immunization.

a strong T cell proliferation in response to P₃₁₋₅₃. This proliferative response was specifically inhibited by RT1.B¹ MHC class II antibody, suggesting that P₃₁₋₅₃ was presented in the context of RT1.B¹ molecules to T cells. This result is consistent with the predictions of Kerjaschki [9], in that the 23 amino acid P₃₁₋₅₃ contained two putative RT1.B¹ MHC class II binding motifs specific for Lewis rats which comprise a serine residue separated from a glutamic acid residue by five intervening amino acids (S x x x x E) [13]. T cell recognition in ID-inducing P₃₁₋₅₃ was clearly restricted by RT1.B¹ MHC class II.

The peptide-specific T cell lines expressed the CD4 determinant and had a significant increase in mRNA expression for cytokines IL-4 and IL-10, suggesting that the cell lines were of CD4⁺ Th2 phenotype. Th2 cells are helpers for B cells. They trigger resting B cells to secrete antibodies directed at specific antigens, and are particularly effective in promoting IgG1 and IgE responses [21]. We have demonstrated that these CD4⁺ Th2 cells are able to trigger syngeneic naive B cell activation for anti-peptide immunoglobulin autoantibody production *in vitro*. CD4⁺ Th2 cells may play an important role in the pathogenic process of autoantibody production involved in initiation of HN *in vivo*.

Since CD4⁺ Th cells are strong autoantibody inducers [11,12], characterization of their TCR should provide important information regarding the relationship between the CDR3 of the TCR and the resulting antigenic peptide specificity. Interestingly, anti-P₃₁₋₅₃ autoantibody-inducing CD4⁺ Th2 cells used a restricted set of V β 18 (54%), V β 4 (10%) and V β 14 (10%) TCRs after nine rounds of stimulation with peptide. Sequencing analysis of TCR CDR3 regions of these three predominant V β segments from the T cell line specific for P₃₁₋₅₃ showed striking features: a motif of charged residues was found in the V-D-J junctional regions of highly restricted V β 18 and V β 4 in TCR (bold letters in Table 2). The CDR3 region from a currently proposed TCR structure is thought to contact the antigenic peptides bound within a groove on the molecules of MHC during conventional antigenic peptide recognition [22,23]. Recent studies show that amino acid charge substitutions in the antigenic peptide induce T cell responses with reciprocal charge alterations in the junctional regions of the TCR β -chain [24]. Our CDR3 sequencing of these three predominant TCR V β genes suggest that a charged amino acid motif at the CDR3 region of TCR in the specific antibody-inducing CD4⁺ Th2 cell line may contribute to the specific ability of these cells to recognize the T epitopes within P₃₁₋₅₃ of RAP.

In this study we have shown a synthetic pathogenic P₃₁₋₅₃ of RAP contains a T cell epitope, and we have also characterized RAP P₃₁₋₅₃-specific T cell lines. In future studies we plan to identify T cell epitopes in megalin/gp330 and their corresponding TCR. We hope that these experiments may eventually lead in turn to the development of rational immunotherapeutic interventions for the treatment of HN, and eventually of human membranous glomerulopathy.

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