Brief Definitive Report

Self Tolerance to T Cell Receptor Vβ Sequences

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Summary

T cell tolerance to self is achieved by deletion or inactivation of clones recognizing peptides of self proteins presented by major histocompatibility complex molecules. A considerable fraction of self proteins accessible to the immune system is contributed by the system itself, for example, the receptors used for antigen recognition (antibodies and T cell receptors [TCRs]). Thus far, it has remained unclear, whether antigen receptors are subject to self tolerance, or on contrary, engage into network interactions implying immunity rather than tolerance. In this study, we demonstrate self tolerance to synthetic peptides corresponding to the first hypervariable region of the Vβ 8.1 and Vβ 8.2 TCR proteins. We also show that the tolerogenic synthetic peptide corresponds to a fragment produced by processing of the Vβ protein, and conversely, that a Vβ peptide not produced by processing is also not subject to self tolerance. Thus, the rules of tolerance seem to apply to antigen receptors, at least to their germline-encoded portions, in a similar fashion as to other self proteins. This finding has important implications for studies of natural and artificially induced immune networks.

Discrimination between self and nonself is a salient feature of the immune system. At the T cell level, this is achieved by inactivation and/or deletion of clones recognizing peptides of self proteins presented by MHC molecules (1–5). Part of the diverse array of self proteins accessible to the immune system is provided by the system itself, for example, the receptors (antibodies and TCR) used for antigen recognition. Nevertheless, self tolerance to antigen receptors has not been demonstrated thus far. In contrast, network interactions implying immunity rather than tolerance to self receptors have been postulated and reported (6–9). We therefore designed an experimental model that could, in principle, answer the question of whether self tolerance to antigen receptors operates under physiological conditions. Our approach has been to investigate the immunogenicity of peptides that are unique to TCR Vβ proteins that are expressed in certain mouse strains but not in others. The defect in expressing these proteins can be twofold: first, a large genomic deletion of 50% of Vβ genes from chromosome 6 referred to as the TCR-Vβ^a allele, carried by several inbred mouse strains (10), and second, clonal deletion of T cells expressing particular Vβ proteins by endogenous superantigens (1, 2) early in ontogeny in the thymus. Mice expressing or deleting a particular Vβ were immunized with synthetic peptides derived from that Vβ sequence, and a correlation was sought between the pattern of responsiveness and the expression of the Vβ protein. We report here self tolerance to sequences corresponding to the first hypervariable region of the Vβ 8.1 and Vβ 8.2 proteins.

Materials and Methods

Mice and Immunizations. Mice were obtained from The Jackson Laboratories (Bar Harbor, ME), except strain B10.Q-Vβ^a (obtained from Dr. C. David, Mayo Clinic, Rochester, MN). F_1 hybrids were bred in our facilities. Mice, >8 wk of age, were injected with 35 nmol of peptide antigen in CFA in the hind footpads and tailbase.

Peptides. Peptides were prepared by solid-phase synthesis and purified by reverse-phase HPLC; their homogeneity was confirmed by analytical HPLC, amino acid analysis, and fast atom bombardment mass spectroscopy.

Soluble Single-chain TCR. The TCR Vα4 and Vβ8.2 genes were isolated from T hybridoma 1934.4 (11) by PCR using oligonucleotides as described (12). The V genes were cloned into plasmid vectors designed for expression and secretion of immunoglobulin domains by *Escherichia coli* (13). For the single-chain construct, the COOH terminus of Vα was joined to the NH_2 terminus of Vβ by a $(Gly_4 Ser)_3$ linker. A COOH-terminal His_6 tag was inserted to allow affinity purification on Ni^{2+}-NTA agarose columns. The strategy for construction of plasmids with single V genes and joined VαVβ genes was described previously (14). The single chain TCR construct was secreted by recombinant *E. coli* at 0.5–1 mg/liter, and the Vα construct at 2 mg/liter (the Vβ construct alone was poorly secreted; references 12, 14). Circular dichroism analyses of the

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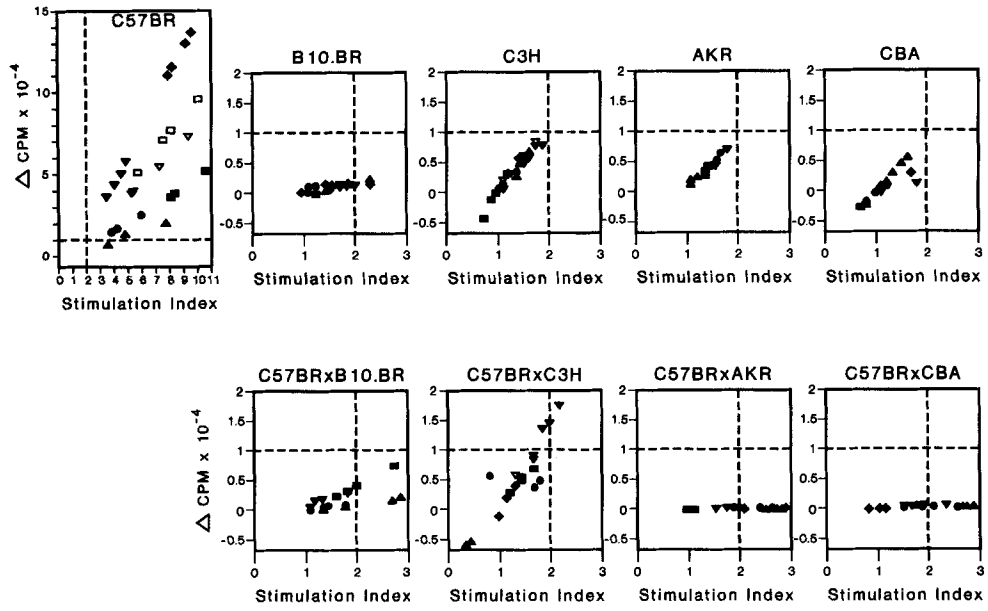

Figure 1. Response pattern of H-2^k mouse strains to peptide Vβ 8.1/18-31. In vitro proliferative response of T cells from mice of the indicated strains to different concentrations (3.5–30 μM) of the peptide is shown. Each symbol represents the dose-response curve of an individual mouse. For nonresponder strains, the enlarged lower left corner of coordinates is shown only (no responses occurred outside this range). Δcpm is the difference, and stimulation index is the ratio between cpm in cultures with and without antigen. As a positive control, the response of each mouse to the purified protein derivative of tuberculine was monitored. The ranges of responses (*Δcpm/S.I.*) to 50 μg/ml of PPD were the following: C57BR, 173799/33–274514/27; B10.BR, 92040/69–225288/53; C3H, 386000/20–500000/26; AKR, 269000/31–404000/38; CBA, 178200/99–344800/67; (C57BR×B10.BR)F1, 96752/68–291519/20; (C57BR×C3H)F1, 323500/31–339865/25; (C57BR×AKR)F1, 131604/340–150060/485; (C57BR×CBA)F1, 117704/430–176098/554.

secreted molecules indicate that they are folded into β-pleated sheet structures similar to those of immunoglobulin V domains (14). For stimulation of T cells, the soluble TCR constructs were used at 2–8 μM final concentration (corresponding to 50–200 μg/ml of single chain VαVβ).

T Cell Lines, Clones, and Proliferation Assays. 9 d after immunization, the regional lymph nodes were removed aseptically, and single cell suspensions were prepared. Cells (2.5 × 10^5 per well) were cultured in HL-1 medium (Ventrex, Portland, Maine) in flat bottom 96-well microtiter plates with or without peptide antigen for 3 d. Proliferation was measured by [^{3}H]TdR incorporation (1 μCi per well) in triplicates during the last 16 h of culture. Cell lines were generated by culturing T cells (5 × 10^6/ml) in RPMI 1640 medium with 10% FCS and 3.5 μM of peptide for 1 wk, and restimulating weekly with peptide, APC (2.5 × 10^6 cells per ml), and 2 ng/ml of recombinant IL-2. The stable lines were cloned by limiting dilution as described previously (15). Proliferative response was tested by culturing T cells from lines (5 × 10^4 per well) or cloned T cells (2 × 10^4 per well), and syngeneic irradiated spleen cells (5 × 10^5 per well) as APC in RPMI 1640 plus 5% FCS or human AB serum, with or without different concentrations of antigen for 3 d. Incorporation of [^{3}H]TdR was measured during the last 6 h of culture.

Results

Pattern of T Cell Responsiveness to Peptide Vβ 8.1/18-31. We have selected several 14–amino acid–long sequences that are unique to Vβ proteins not expressed in certain mouse strains caused by genomic deletion or somatic deletion of the corresponding T cells by endogenous superantigens (1, 2, 10). The corresponding synthetic peptides (mostly from Vβ 6 and Vβ 8.1) were tested for immunogenicity in mouse strains expressing or deleting the respective TCR Vβ. We have identified one peptide from the first hypervariable region of Vβ 8.1, peptide 8.1/18-31, which induced a T cell response in H-2^k mice. The pattern of responsiveness to this peptide is shown in Fig. 1. Of the five H-2^k strains tested, only C57BR mounted a T cell response to 8.1/18-31. It should be noted that this is a strain with Vβ8.1 deleted from the germ line (10). The remaining strains (AKR, B10.BR, CBA, C3H), as well as all (responder × nonresponder)F$_1$ hybrids were nonresponders (i.e., responsiveness was recessive). T cell lines from the nonresponder strains remained nonresponder after in vitro restimulation with antigen and IL-2 (data not shown), suggesting that 8.1/18-31–specific T cell precursors might have been absent. The response of C57BR T cells to 8.1/18-31 was not decreased upon mixing with C3H (nonresponder) T cells primed with the same peptide, indicating that suppression was not involved in nonresponsiveness (data not shown). We also tested representative strains of the AKXL recombinant inbred series. The progenitor strains of AKXL are AKR carrying H-2^k on chromosome 17 and a full array of Vβ genes on chromosome 6, as well as C57L that is H-2^b and carries the Vβ^a allele on chromosome 6 (i.e., 50% of Vβ genes including Vβ8 deleted; references 10, 16). According to the data in Table 1, the response clearly segregated with the presence of H-2^k, and the absence of Vβ 8.1 expression in these strains. To address the role of TCR more directly in the observed nonresponsiveness, we tested a TCR congeneic strain with the Vβ^a allele on B10.Q background (17), and a TCR-β–deficient strain expressing no $\alpha\beta$ TCR at all (18), in comparison to the wild-type strains of origin. These strains were crossed with C57BR to provide H-2^k required for the response. The data demonstrated that responses occurred only in the Vβ^a congeneic and the TCR-β–deficient strain but not in the wild-type controls (Table 1). Collectively, the data have demonstrated that the mechanism un-

Table 1. *Response Pattern of AKXL Recombinant Inbred Strains, TCR Vβ Congeneic, and TCR Vβ-deficient Strains to Peptide Vβ 8.1/18-31*

Strain	H-2	Vβ 8.1 + 8.2 expression‡	Δcpm range*	S.I. range*	*n*
AKXL-6	k	–	5,632–25,590	4.0–51.0	5
AKXL-8	k	–	3,522–13,400	3.2–4.3	4
AKXL-38	k	–	10,235–20,847	3.1–18.0	6
AKXL-13	k	+	16–10,580	1.1–2.0	7
AKXL-21	k	+	289–7,622	1.2–1.9	6
AKXL-7	b	–	156–2,418	1.0–1.2	3
C57L§	b	–	363–3,156	1.0–1.2	3
(B10Q.Vβ^a × C57BR)F1	(q × k)	–	60,842–104,361	4.8–26.0	4
(B10.Q × C57BR)F1	(q × k)	+	1,679–17,312	1.1–2.0	5
(TCRVβ$^-$ × C57BR)F1	(b × k)	–	27,651–144,900	3.7–22.0	5
(C57BL/6 × C57BR)F1	(b × k)	+	60–16,744	1.0–2.4	5

* Highest and lowest secondary in vitro proliferative response of lymph node cells from the number of immunized animals (*n*) tested after 10 d of stimulation with 5 μm of 8.1/18-31 and 2 ng/ml of rIL-2 at antigen concentrations from 2.5 to 10 μM.
‡ Tested by FACS® analysis (Becton Dickinson and Co., Mountain View, CA) of spleen cells using the Vβ 8.1 + 8.2–specific mAb KJ 16.
§ One of the parental strains of the AKXL series. Responsiveness of the other parental strain AKR is shown in Fig. 1. Ranges of positive control responses to 25 μg/ml of PPD (Δcpm/S.I.) were the following: AKXL-6, 97728/39–153321/30; AKXL-8, 96773/20–162257/15; AKXL-38, 94631/6.2–165018/10; AKXL-13, 112369/22–124541/21; AKXL-21, 101732/27–107182/27; C57L (to 50 μg/ml PPD), 186203/25–286677/21; (B10.Q-Vβ^a × C57BR)F1, 76130/19.3–248692/24.5; (B10.Q × 57BR)F1, 77622/13.4–223548/18.9; (TCRVβ × C57BR)F1, 105920/14.0–174947/29.2; (C57BL/6 × C57BR)F1, 72501/12.5–204494/19.0.

derlying nonresponsiveness to peptide 8.1/18-31 is self tolerance to Vβ 8.1.

Cross-reactivity of Vβ 8.1/18-31–specific T Cells with Vβ 8.2/18-31. Of the nonresponding strains tested, CBA and AKR are peculiar in that they do carry the Vβ 8.1 gene, but the protein is expressed only in very small amounts because of clonal deletion of Vβ 8.1$^+$ T cells by the endogenous superantigen Mls-1^a (Mtv-7) (1, 19). Thus, the reasons for nonresponsiveness of these two strains remained to be clarified. The failure of CBA and AKR mice to respond to 8.1/18-31 suggested that self tolerance to the peptide operated, although the corresponding tolerogen was not present in large amounts, except in the thymus for a short period of ontogeny. However, the fine specificity of T cells primed to 8.1/18-31 suggested another explanation. As shown in Table 2, these T cells fully cross-reacted with the corresponding Vβ 8.2 peptide, although they were antigen specific, in that they failed to respond to hen egg white lysozyme (HEL) 46-61, an unrelated peptide to which H-2^k mice are high responders (20). The observed cross-reactivity is not surprising, taken the high degree of sequence homology between the 8.1/18-31 and 8.2/18-31 peptides (they differ only in three amino acid positions). Thus, although the role of residual Vβ 8.1 protein (left after somatic deletion by Mtv-7) in tolerance induction

Table 2. *Cross-reactivity of Vβ 8.1/18-31–specific T Cells with Vβ 8.2/18-31*

Peptide in culture*	Sequence	Proliferative response (Δ/cpm/S.I.)	
		Line 1‡	Line 2‡
Vβ 8.1/18-31	KVTLSCHQTNNHDY	29662/4.8	128157/7.8
Vβ 8.2/18-31	KVTLSCNQTNNHNN	83889/11.7	337551/19.0
HEL/46-61	NTDGSTDYGILQINSR	−988/0.9	−6084/0.7

* Used at 19 μM.
‡ T cell lines were derived from 8.1/18-31 immune C57BR mice. Nine lines were altogether tested with the same results.

Table 3. *MHC Restriction of 8.1/18-31-specific T Cell Clones*

APC Strain	H-2 loci K A E D	Proliferative response (Δcpm/S.I.) by clones* No. 3	No. 46	No. 22	No. 67
C57BR	k k k k	234519/2174	53875/196	103703/382	117879/614
B10.A(4R)	k k b b	243368/526	32143/83	10724/40	65913/388
B10.AQR	q k k d	221479/669	34766/144	nt	nt
B10.T(6R)	q q q d	1208/5	646/5	nt	nt
A.TL	s k k d	nt	nt	22984/144	56469/317
A.TH	s s s d	nt	nt	237/1.7	111/0.8

* Clones 3, 46, and 22 were derived from strain AKXL-38 and clone 67 from C57BR mice. Using a panel of anti-Vα and anti-Bβ antibodies (specific for Vα 2,3.2,8,11, and Vβ 2,3,4,5.1+5.2,6,7,8.1 + 8.2,9,10,11,12,13,14,17a), clone 3 typed Vβ7 Vα-blank, clone 22 Vβ14 Vα-blank, and clones 46 and 67 Vα-Vβ-blank by immunofluorescence. All clones were TCRαβ$^+$, CD4$^+$, and CD8$^-$.

is not ruled out formally, the lack of response in CBA and AKR mice is readily explained by cross-tolerance to Vβ 8.2 that is expressed in these strains.

Characterization of T Cells Responding to Vβ 8.1/18-31. We investigated the type of T cell response induced by the Vβ 8.1 peptide. The MHC restriction of 8.1/18-31-specific T cell clones was determined using spleen cells from the appropriate intra-H-2 recombinant strains as APC. The data in Table 3 demonstrate mapping of the MHC restriction to I-A^k. All clones characterized thus far were CD4$^+$ and αβ TCR$^+$ (data not shown). Polyclonal T cell lines were also CD4$^+$ and I-A^k restricted, as shown by inhibition or depletion with the relevant mAb (anti-L3T4, and 14V18; data not shown). The 8.1/18-31-specific T cells had no demonstrable cytotoxic activity against syngeneic T or B cell blasts or B cell lymphoma in the presence of the peptide (data not shown).

Reactivity of Vβ Peptide-specific T Cells with a Soluble TCR Construct. To establish a direct link between the T cell response to peptide 8.1/18-31 (and 8.2/18-31) and the corresponding Vβ protein, we have investigated whether peptide-primed T cells could recognize a soluble TCR construct (12, 14), Vα4/Vβ8.2, presented by syngeneic APC. The results in Table 3 demonstrate that this is indeed the case: both cloned and polyclonal T cell lines have recognized peptides 8.1/18-31, 8.2/18-31, and the soluble Vα4/Vβ8.2 construct equally well, but failed to respond to the control Vα4 construct. Thus, the immunogenic/tolerogenic peptide appeared to be produced by processing of the Vβ8.2 protein. Based on these results, it was conceivable that the detectability of tolerance may be critically dependent on the identity of the synthetic peptide with the one produced by processing of the relevant protein. This hypothesis was investigated by using an extended

Table 4. *Reactivity of Vβ Peptide-specific T Cells to a Soluble TCR Construct*

Antigen*	Proliferative response (Δcpm/S.I.)‡ Clone 46§ 8.1/18-31 AKXL-38	Line 3§‖ 8.1/18-31 C57BR	Line D§¶ 8.1/15-32 C57BR	Line 4§ 8.1/15-32** C3H
sol.Vα4/Vβ8.2	36204/178	23169/4.8	−534/0.9	−286/0.9
sol.Vα4	394/2.0	−155/1.0	−1688/0.6	−884/0.7
8.1/18-31	53875/196	16618/3.8	3694/1.8	−468/0.8
8.2/18-31	77663/282	12497/3.1	3437/1.8	128/1.0
8.1/15-32	34733/127	13658/3.3	31955/8.6	22993/9.0

* Proliferative response to 5 μM of peptide and 8 μM of soluble TCR construct is shown.
‡ In the presence of irradiated C57BR splenic APC.
§ The specficity and strain of origin of T cell lines are as indicated.
‖ Two cell lines were tested with the same results.
¶ Two additional 8.1/15-32-specific lines generated from C57BR and B10.BR mice, respectively, gave the same results.
** Peptide 8.1/15-32 has an NH_2-terminal Thr-Gly-Gly and a COOH-terminal Met addition to 8.1/18-31.

peptide of the same TCR region, Vβ 8.1/15-32. As shown in Table 4, both C57BR mice and C3H mice (the latter nonresponder to 8.1/18-31) were able to respond to 8.1/15-32. However, T cells recognizing the extended peptide failed to respond to either the short peptide or the soluble TCR construct. Thus, the extended peptide was probably not produced by processing, and consequently, was not subject to self tolerance. These results provide direct support to the hypothesis that tolerance to the Vβ 8.1 and Vβ 8.2 proteins is the cause of nonresponsiveness to the 18-31 Vβ peptides in strains expressing these proteins as self.

Discussion

The results presented herein have demonstrated self tolerance to a germline-encoded variable portion of αβ TCRs. The detection of tolerance seemed to depend on the identity of the probing synthetic TCR peptide with the one produced from processing the relevant TCR protein. Probably because of this requirement, tolerance to synthetic TCR peptides has not been demonstrated thus far, but several laboratories were able to show T cell responses to long (usually 20mer) synthetic TCR peptides (9, 21–24). Our finding, if generalizable, demonstrates that antigen receptors, with the possible exception of their somatically diversified portions (i.e., somatic mutations in antibodies, and the third hypervariable region of TCR), are subject to tolerance, similarly to other self proteins accessible to the immune system. The somatically diversified sequences of antigen receptors may escape self tolerance, because they may not attain the concentration required for tolerance induction (25). Thus, potential network interactions could only be directed to such sequences, provided that they are presented by the individual's MHC molecules.

These results also have implications for the therapeutic use of synthetic TCR peptides in autoimmune diseases (9, 21, 22). The reported beneficial effect of TCR peptides in autoimmune disease models has been explained by the induction of a downregulatory immune response against T cells that express the relevant receptor and are involved in autoimmune pathology (9). Our present results suggest that a T cell response to a TCR peptide can only occur when the immunizing peptide is not identical with a naturally processed tolerogenic TCR fragment. Consequently, a treatment with synthetic TCR peptides can have different outcomes. First, when the synthetic peptide corresponds to a tolerogenic fragment, no response and no therapeutic effect will ensue. Second, when the fragment corresponding to the immunogen is not produced at all by processing, a response will result, but it is likely to remain without therapeutic consequence. Indeed, this possibility has been demonstrated experimentally by Sun (26). Third, when the fragment is produced in subtolerogenic amounts by processing, the corresponding synthetic peptide will induce an immune response that could affect the cells naturally presenting this sequence, in the first place, the T cells expressing the appropriate receptor. When the latter cells are involved in autoimmune pathology, a response to them could modify the disease in two possible ways: downregulation with a beneficial effect (9, 22), or upregulation leading to exacerbation (27). Future research will have to identify ways to direct the anti-TCR response toward beneficial effects.

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