A Truncated T Cell Receptor Repertoire Reveals Underlying Immunogenicity of an Antigenic Determinant

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Summary

Induction of T cell responses to an antigenic peptide that is known to bind a major histocompatibility complex molecule is a function of either the T cell receptor (TCR) repertoire or regulatory influences by CD8 or CD4 regulatory T cells. We have tested the hypothesis that a lack of 10 TCR Vβ gene segments in Vβa mice may result in an incomplete repertoire of regulatory T cells involved in maintaining peripheral tolerance. Such a hole in the repertoire of regulatory cells could result in expression of T cell responses to antigenic determinants that normally remain undetected in mice with a wild-type repertoire of TCR Vβ gene segments. We show here that H-2d mice respond to the peptide 74-96 of hen egg-white lysozyme (HEL) when they are of Vβa haplotype at their TCR locus. The wild-type (Vβb) H-2d mice with their complete set of 20 TCR Vβ gene segments fail to respond to HEL 74-96. The 74-96-specific T cell responsiveness was revealed in the wild-type (Vβb) mice when they were treated in vivo with anti-CD8 antibody, implicating the existence of regulatory cells that prevent expression of T cell responses specific for peptide 74-96. This is a demonstration that holes in the regulatory T cell repertoire can, in certain circumstances, become beneficial to the host, for example, in susceptibility against pathogens.

Due to the loss of 10 T cell receptor Vβ gene segments from their germline repertoire, Vβa mice possess a truncated TCR repertoire (1, 2). We have previously shown that mice with such a deletion in their TCR Vβ genes (Vβ truncated haplotype, Vβa) are unable to respond to two antigenic determinants (sperm whale myoglobin [SWM]1 110-121/Eae4Aβ and myelin basic protein [MBP] 1-11/Ae4) (2). These experiments indicated that there is an absolute limit to the flexibility inherent in the TCR repertoire: the absence of specific TCR Vβ gene segments resulted in holes in T cell responsiveness to the above-mentioned determinants (2, 3).

One mechanism for maintenance of peripheral tolerance to self- and foreign antigens involves regulatory cells that inhibit the function of antigen-specific T cells such that there is no response upon immunization with specific peptide or protein antigens despite the existence of T cells that can recognize their determinants. Peripheral tolerance to self-peptides exists to prevent autoreactivity by T cells that have escaped deletion during thymic selection (4–6). Peripheral tolerance to foreign antigens is likely to exist to prevent immune pathology caused by the persistence of certain activated T cells, for example in several parasitic systems (7). Peripheral tolerance or suppression to foreign antigens could also exist as a fortuitous consequence of the cross-reactivity of self-directed regulatory cells. Thus, (a) many foreign antigen determinants associated with self-MHC molecules are structural mimics of MHC-restricted self-determinants and thus should be susceptible to the same regulation as self-antigens (8), and (b) certain foreign and self-determinants induce T cells bearing similar or identical TCR V region gene segments (3, 9). TCR-centered regulatory cells that recognize specific TCR determinants may therefore be able to regulate both foreign and self-antigens through TCR-targeted circuitry (for reviews see references 9 and 10).

We hypothesized that regulatory cells controlling the expression of immune responsiveness toward certain antigenic determinants might also have a limited T cell repertoire. Thus, a lack of 10 TCR Vβ gene segments in Vβa mice could possibly give rise to holes in the repertoire of the regulatory cells, analogous to the holes in the repertoire of SWM 110-121/Eae4Aβ and MBP 1-11/Ae4-specific CD4 T cells that we had previously reported. The outcome of such a hole in the repertoire of regulatory cells would be the expression of T cell responsiveness to normally nonimmunogenic determinants.

We show in this report that a strong T cell response, specific for peptide 74-96 of hen egg-white lysozyme (HEL),
can be induced in H-2d mice only when they lack 10 TCR gene segments, presumably encoding a TCR β chain on a potent regulatory T cell. None of the three strains of H-2d mice (BALB/c, B10.D2, and F₂ [BALB/c × SJL]) with an intact repertoire of 20 TCR Vb gene segments responds to the peptide HEL 74-96. However, two different H-2d recombinant inbred mouse strains ([C3]9, [C3]8) with the Vb5, truncated set of TCR Vb gene segments show very strong T cell responses to this peptide. Furthermore, although 74-96-specific long-term CD4+ T cell lines and clones can be readily derived from Vb5 mice, it was not possible to obtain such a long-term T cell line from BALB/c (Vb5) mice (data not shown). We also show that in vivo treatment with anti-CD8 antibodies of nonresponsive, wild-type Vb5 mice, allows expression of T cell responsiveness to HEL 74-96. These results reveal a hole in the regulatory repertoire of CD8 T cells in Vb5 mice that usually controls the response to HEL 74-96.

Materials and Methods

Mice. Mice either were bred at our own laboratory or were obtained from The Jackson Laboratory (Bar Harbor, ME).

Antigens and Peptides. Hen egg lysozyme was purified as described earlier (2). Peptide 74-96 was synthesized in bulk and HPLC purified in bulk by Macromolecular Resources (Pt. Collins, CO) in two batches. The sequence of HEL 74-96 (NLCl-NIPCSALLSSDTASVNCAlK) includes three cysteine residues. One batch of 74-96 was synthesized with the usual cysteine residues, whereas the other batch contained a replacement by α-amino butyric acid instead of cysteine in each of the three positions. The pepscan series of truncated and extended peptides within the sequence 69-104 were made by synthesis on pins followed by cleavage into 96-well plates (11).

Antibodies. Anti-CD8 antibody (clone 53-6.7, a rat IgG2a antibody) and an isotype control antibody, rat IgG2a (rgG2a), were obtained from Pharmingen (San Diego, CA). FITC-conjugated murine anti-CD8 antibody (clone 53-6.7) and FITC-conjugated isotype control antibody were obtained from Becton Dickinson & Co. (San Jose, CA).

In Vivo Depletion of CD8 T Cells. 125 μg of anti-CD8 antibody in PBS was injected intraperitoneally into BALB/c mice. The control groups were injected either with 125 μg of rat IgG2a (Pharmingen) in PBS or with PBS alone.

Immunofluorescence. Immunofluorescence on LN or splenic cells was performed and analyzed as described earlier (2).

Immunization. Mice were immunized with 7 nmol of peptide or protein antigen in the hind footpads with an emulsion of CFA (H37Ra; Difco Laboratories, Detroit, MI). On day 9, LN cells (LNC) from draining LN were harvested and used in antigen-induced proliferation assays or as a source of T cells to generate T cell hybridomas.

Generation of T Cell Hybrid. T cell hybridomas were generated from mice immunized with peptide 74-96 as described before (2) except that LNC were harvested and bulk cultured with the peptide 74-96 for only one cycle of stimulation before hybridization. Thus, LNC cultured for 6 d with peptide 74-96 were further cultured for 3 d with rIL-2. T cell blasts were then purified by Ficoll-Hypaque density centrifugation and fused with a variant of BW5147, BW/α-β− (2, 11) as a fusion partner, as described before. The resulting hybrids were seeded at less than one cell per well in 96-well plates; the positive wells were expanded and tested for their ability to produce IL-2 using HT-2 cells (IL-2 dependent) in response to peptide 74-96 and HEL. The antigen-reactive hybrids were further expanded and analyzed for function.

Specificity Assays. For antigen-primed LNC, LNC proliferation assays were performed as described earlier (2). Briefly, 4 × 10⁶ (unless otherwise indicated) LNC from individual mice were cultured with 14, 7, or 3.5 μM concentrations of peptide 74-96 or HEL in 0.2 ml/well of HL-1 medium (Ventrex Laboratories Inc., Portland, ME), supplemented with 2 mM glutamine, 100 U/ml of penicillin, and 100 μg/ml streptomycin. All cultures were done in triplicate. Proliferation was measured by addition of 1 μCi of [3H]thymidine for the last 18 h of a 5-d culture, and the incorporation was assayed by scintillation counting.

T Cell Hybrid. 10⁶ T hybridoma cells were cultured with various concentrations of the peptide 74-96, or the pepscan peptides within the sequence 70-104, or HEL, with 5 × 10⁶ irradiated BALB/c spleen cells as APC in 0.2 ml of DMEM (Flow Laboratories, Irvine Ayrshire, Scotland) supplemented with 2 mM glutamine, 0.05 mM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum (HyClone, Logan, UT), as described (2, 11). All cultures were performed in triplicate. The supernatants collected 24 h later were assayed for IL-2 activity on the IL-2/IL-4-dependent cell line, HT-2. 10⁶ HT-2 cells were cultured with medium alone or supernatants for 48 h. Proliferation was measured by adding 1 μCi [3H]thymidine during the last 18 h of culture and incorporation assayed by liquid scintillation counting.

Results

We compared T cell responsiveness in HEL-immunized H-2d mice with and without a wild-type repertoire, to test the hypothesis that the lack of 10 TCR Vb gene segments in Vb5 mice could result in a hole in the repertoire of regulatory T cells and lead to a manifestation of T cell responsiveness to certain determinants that are normally nonimmunogenic. For these initial experiments, we used the wild-type (Vb5), H-2d haplotype BALB/c mice and the RI, H-2d haplotype mice, (C3)8 and (C3)9, that have a truncated (Vb5) repertoire at their TCR locus. The (C3)9 and (C3)8 mice have been described earlier (2). A pepscan analysis (using a series of overlapping peptides that walk the whole antigen [HEL] in single amino acid steps) of LNC from HEL-primed (C3)9 (H-2d, Vb5), and BALB/c (H-2d, Vb5) mice revealed that only Vb5 mice were able to respond to peptides in the 74-96 region of HEL (data not shown). We therefore selected peptide 74-96 (a tryptic peptide of HEL), to further study the differential responsiveness of Vb5 and Vb5 mice in this haplotype.

In the H-2d Haplotypes, Peptide HEL 74-96 Induces T Cell Responses Exclusively in Vb5 Mice. Fig. 1 shows the responses from individual mice of three different Vb5 strains (B10.D2, BALB/c, and F₂ [BALB/c × SJL], left), and two Vb5 strains ([C3]8 and [C3]9, right), H-2d mice primed with peptide HEL 74-96. As the RI (C3)8 and (C3)9 (Vb5, H-2d) mice were originally derived from BALB/c (Vb5) and SJL/J (Vb5) parents, we chose to use F₂ (BALB/c × SJL) Vb5 mice.
in order to have comparable strain backgrounds in the $V_{\beta}^a$ and $V_{\beta}^b$ groups. The results clearly show that none of the 16 different $V_{\beta}^b$ mice primed with peptide 74-96 could respond to the HEL peptide 74-96 when recalled with peptide 74-96 (Fig. 1 a, top left). The mean delta counts per minute (cpm of LNC incubated with the peptide 74-96 minus the cpm of cells incubated medium alone) of 21 $V_{\beta}^a$ mice tested individually is 128,000 and that of 16 $V_{\beta}^b$ mice is 5,850. With HEL (Fig. 1 a, bottom left) as the in vitro recall antigen, 3 of 10 BALB/c mice showed a low level of responsiveness. Surprisingly, each of the 21 $V_{\beta}^a$ mice, primed with 74-96, showed strong T cell proliferation to both peptide 74-96 and HEL used as in vitro antigens (Fig. 1 a, right). Similar results were obtained from an additional 10 $V_{\beta}^b$ and 10 $V_{\beta}^a$ mice (data not shown). HEL was used as a recall antigen to ensure that T cells induced by the peptide 74-96 could be stimulated by the determinant derived from natural, intracellular processing of HEL. A pepscan of HEL 74-96–primed $V_{\beta}^b$ and $V_{\beta}^a$ mice, recalled by sequential 15-mer peptides within HEL 69-104, is shown in Fig. 1 b. It seems clear that (a) only T cells from $V_{\beta}^a$ mice recognize the peptide 74-96 and (b) several determinant cores are recognized by T cells from $V_{\beta}^a$ mice, with peaks at 71-85, 74-88, and 79-93 (Fig. 1 b).

The responsiveness of (CJ)8 and (CJ)9 mice might have been owing to contribution of portions of the SJL genome found in each of these strains, other than within the $V_{\beta}^a$ region. However, this is unlikely because of the lack of

Figure 1. $V_{\beta}^b$ (truncated TCR repertoire) but not $V_{\beta}^a$ (wild-type TCR repertoire) mice of H-2b haplotype are able to respond to HEL 74-96. (a) 16 $V_{\beta}^b$ (B10.D2, BALB/c and [BALB/c X (CJ)9]F1) mice and 21 $V_{\beta}^a$ ([CJ]9 and [CJ]8), immunized with HEL peptide 74-96 in foot pads, were tested individually for proliferative responses to 74-96 and HEL using LNC as described earlier (2). The data are represented as mean cpm ± SE ($\times 10^{-3}$) from triplicate cultures of LNC from each mouse at the optimal concentration of 7 µM of p74-96 (top) or HEL (bottom). LNC tested at concentrations of either 14 or 3.5 µM of peptide or HEL gave similar results (data not shown). The mean ± SE values for medium controls (X) are presented in front of the bars for p74-96 and HEL (b). The PPD values for the first six $V_{\beta}^b$ mice were: (mean ± SE $\times 10^{-3}$) 210.8 ± 12.2, 203.2 ± 3.6, 228.0 ± 2.2, 193.4 ± 8.0, 159.8 ± 6.5, 531.7 ± 7.8. The PPD values for the remaining 10 $V_{\beta}^b$ mice and the $V_{\beta}^a$ mice were in the same range as those for $V_{\beta}^b$ mice (not shown). (b) LNC from three B10.D2 and three (CJ)9 mice immunized with p74-96 were pooled and tested for proliferative responses to 15-mer pepscan peptides (peptides covering the sequence HEL 69-104 in one amino acid step). The data are presented as the means of triplicate cultures as described in a.
74-96–specific T cell responses found in primed F₁(BALB/c × SJL) mice (Fig. 1 a, left).

Fig. 2 shows a titration of LNC from individual Vβ₃ or Vβ₅ mice primed with peptide 74-96. It is clear that at 10⁵, 4 x 10⁵ and 6 x 10⁵ cells per well, LNC from Vβ₃ (CxJ)9 mice showed markedly greater proliferative responses than Vβ₅ (BALB/c) mice.

BALB/c (H-2b) Mice Are Able to Process and Present Peptide 74-96 to T Cell Hybrids Derived from (CxJ)9 Mice and Specific for Various Determinants within the Peptide 74-96. Fig. 3 shows the stimulation of four representative 74-96–specific T cell hybridomas derived from (CxJ)9 mice using pepscan analysis.

As BALB/c splenic cells were used as APC in these experiments, it is evident that BALB/c APC are able to process and present each of these determinants to 74-96–specific T cells. Furthermore, each of these T cell hybridomas can be activated by HEL (data not shown), indicating the ability of BALB/c mice to generate all the determinants within peptide 74-96, from the intact protein or from the peptide. We would like to point out that even though it was possible to isolate 74-96–specific CD4 T cell hybridomas from Vβ₅ BALB/c mice, their cloning efficiency was more than 10,000 times lower than that of the cloning efficiency of 74-96–specific T cell hybridomas from (CxJ)9 mice under equivalent conditions (Nanda, N.K., unpublished data). Moreover, each of these hybridomas can be activated by HEL (data not shown), indicating the ability of BALB/c mice to generate all the determinants within peptide 74-96.

In Vivo Deletion of CD8 T Cells Reveals the Expression of Responsiveness to Peptide 74-96 in Vβ₅ Mice. To explore the possibility that a TCR Vβ₅ gene required by a regulatory population was the cause of the responsiveness of (CxJ)9 mice to 74-96, we sought proof for...
In Vivo: PBS rlgG2b Anti-CD8 HEL

Figure 4. V₃⁻ mice depleted of CD8 T cells are able to respond to p74-96. BALB/c (shown) and (C3H)F₁ (not shown) mice were injected intraperitoneally with either PBS alone or rat IgG2b in PBS, or anti-CD8 antibody in PBS, 4 d before immunization with p74-96. LNC from individual mice were tested in vitro for proliferation in response to p74-96 or HEL, 9 d after immunization as described in Fig. 1. LNC were simultaneously analyzed for presence of CD8 T cells by immunofluorescence (see Results). The data are expressed as described in Fig. 1. In vivo depletion of CD8 T cells in (C3H)F₁ mice resulted in a slight decrease (10-29%) in p74-96-specific responses of their LNC (data not shown). The decrease in proliferative responses in (C3H)F₁ mice could possibly be due to bystander stimulation of CD8 T cells and its effects on proliferative T cell responses.

Discussion

It is evident that constraints in T cell recognition would create gaps not only in the direct repertoire of responsive cells but also in the repertoire of regulatory cells (2, 3). Thus, the absence of 10 TCR V₃ gene segments in V₃⁻ mice resulted in a hole in the repertoire of CD4 T cell immune responses to two antigenic determinants, SWM 110-121/E₉⁻A₉⁻ and MBP 1-11/Aᵤ (2). We now show that V₃⁻ mice with a truncated repertoire of TCR V₃ gene segments also have a hole in the repertoire of their regulatory CD8 T cells that results in disclosing an underlying T cell immune responsiveness to HEL 74-96/A₄. The response to this determinant is absent in the wild-type mice displaying a complete repertoire of TCR V₃ gene segments. CD8 T cell ablation in these wild-type mice, however, results in expression of 74-96-specific T cell responses. Thus, lack of responsiveness in the V₃⁻ mice cannot be attributed to the absence of a 74-96-specific CD4 T cell repertoire or to a defect in antigen processing.

This report is a first demonstration of expression of a CD4 T cell response to a peptide determinant in wild-type mice by simple in vivo ablation of CD8 T cells. CD8 T cells have been implicated in downregulation of immune responses in several systems over the last two decades (for reviews see references 13 and 14). Most of these studies involved complicated in vitro cell reconstitution experiments (13, 14) or adoptive transfer systems (15). Moreover, these studies focused on experimental inhibition of either T or B cell function, which is distinct from our system in which the V₃⁻, H-2₄ mice do not respond upon immunization with peptide 74-96 of HEL with CFA, a traditionally powerful immunization regimen. In addition, the expression of CD4 T cell responses in V₃⁻ mice, without having to deplete T cells, involved no experimental manipulation. Our results are aligned with more recent observations where the direct approach of in vivo CD8 T cell depletion was used in the murine experimental autoimmune encephalomyelitis (EAE) model (B10.PL, H-2₂ mice), and shown to result in inducing chronicity of disease as well as a lack of resistance to a second induction of disease in these mice (16). Similar results were obtained using mutant (CD8⁻⁻) mice that lack the CD8 gene and therefore CD8 T cells. CD8⁻⁻ mice undergo more relapses of EAE than wild-type mice immunized by MBP (17). These experiments showed that CD8 T cells, under normal circumstances, downregulate the function of autoimmune CD4 T cells and thus prevent relapses or reinduction of EAE.
Why do V\(_{\gamma}\)\(_{4}\)-H-2\(^d\) mice respond to immunization with peptide HEL 74-96 without having been depleted of CD8 T cells? As discussed above, one reason could be the contribution of some genes (other than the TCR genes) from the SJL parent of the RI(CxJ)9 and (CxJ)8 mice (as these mice were derived from BALB/c and SJL grandparents). This appears unlikely as (BALB/c × SJL)F\(_1\), H-2\(^{bs}\) mice (expressing all genes contributed by the SJL parent) are unable to respond to peptide 74-96. Another reason could be that the (CxJ)9 and (CxJ)8 mice express quantitatively smaller amounts of a self-superantigen-like gene product which, when expressed in larger amounts in BALB/c mice, results in the deletion of HEL 74-96-specific CD4 T cells. The absence of 74-96-specific T cell deletion in (CxJ)9 and (CxJ)8 mice could conceivably enable them to respond to HEL 74-96. This is very unlikely because BALB/c mice are able to respond to HEL 74-96 after having been depleted of CD8 T cells. Our results favor the explanation that the lack of T cell responses to HEL 74-96 in wild-type V\(_{\beta}\)\(_{4}\) mice and their expression in V\(_{\beta}\)\(_{8}\) mice is solely determined by the TCR V\(_{\beta}\) locus: it is the lack of 10 TCR V\(_{\beta}\) gene segments underlying the existence of a regulatory contingent, that leads to responsiveness in V\(_{\beta}\)\(_{4}\) mice without having to deplete them of CD8 T cells. These CD8 T cells able to downregulate HEL 74-96-specific T cells are present in the wild-type mice and are missing in (CxJ)9 and (CxJ)8 mice because of the absence of a critical TCR V\(_{\beta}\) gene segment in these mice. It is likely that further examples of neo-responses to determinants on other antigens will be detected in such mice.

Our results present evidence that deletion of multiple TCR V\(_{\gamma}\) gene segments in an individual can not only result in a lack of immunogenicity, as shown earlier, but also in a gain of immunogenicity to an antigenic determinant, as shown in the current report (also discussed in 2). During evolution, a balance between these opposite repercussions from a loss of TCR V\(_{\gamma}\) gene segments may have resulted in expression of different numbers of deletion ligands (self-superantigens) in different strains of mice, leading to a spectrum of possibilities of loss of TCR V\(_{\gamma}\) segments across the species (18, 19), which in turn results in gain or loss of immunogenicity to different antigenic determinants in different strains. In addition, these results provide direct evidence that peripheral tolerance to specific antigenic determinants can be mediated by CD8 T cells and can be overcome by in vivo depletion of CD8 T cells.

What is the mechanism by which CD8 T cells prevent the expression of HEL 74-96-specific CD4 T cell responses in wild-type mice? The answer is unclear. We have now succeeded in isolating cloned CD8 T cell hybridomas specific for HEL 74-96 from wild-type BALB/c mice (Nanda, K. et al., unpublished observations). Our preliminary results have revealed that these CD8 T cells are unique in possessing a distinct set of requirements for activation as compared with conventional, cytotoxic CD8 T cells. For example, irradiated splenic APC are unable to activate these CD8 T cells. Whether or not, and how these cells downregulate HEL 74-96-specific CD4 T cells, remains to be learned.

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