

The T Cell Receptor Repertoire Influences V β Element Usage in Response to Myoglobin

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Summary

T cell clones recognizing the sperm whale myoglobin (SpWMb) epitope 110-121 in association with H-2^d major histocompatibility complex class II molecules display a very limited heterogeneity of T cell receptor (TCR) V β usage in DBA/2 mice. All clones previously tested used the same V β 8.2 gene segment and very restricted junctional regions. To investigate the significance of this observation in vivo, we immunized DBA/2 mice with the intact SpWMb protein or peptide 110-121. Only the V β 8⁺ T cells showed any significant response to the 110-121 epitope. The response to peptide 110-121 was then analyzed in mice which, either as a consequence of antibody depletion or through genetic deletion of TCR V β genes, lacked V β 8⁺ peripheral T cells. DBA/2 mice depleted of V β 8⁺ T cells by antibody treatment responded poorly to the 110-121 peptide, and only at high antigen concentrations. In contrast, DBA/2V β ^a mice (homozygous for a deletion of multiple V β gene segments including the V β 8 family) made a response at least as great as that made by DBA/2 mice, even though the DBA/2V β ^a mice had a very restricted TCR V β repertoire compared with DBA/2 mice. Mechanisms which might determine differences in the 110-121 specific response of DBA/2, DBA/2V β ^a and F23.1-treated DBA/2 mice are discussed.

The T cell receptor, a heterodimeric glycoprotein composed of an α and β chain expressed on the cell surface of T lymphocytes, is responsible for recognition of antigen fragments associated with proteins of the MHC (1-3). The α and β chains are encoded by a series of V, D, and J elements which recombine during thymocyte ontogeny to produce functional TCR genes (1). The three dimensional structure of the TCR has not yet been determined and the way in which the use of particular TCR gene segments determines specificity remains unknown. There have been several reports where use of particular TCR V α (4-9) or V β (4, 5, 7-10) gene segments correlated with antigen or MHC recognition. T cell clones responding to a given epitope often exhibit limited heterogeneity of rearranged V and J segments (4, 5, 9, 11-14). It is not possible, however, to make general correlations between the use of particular TCR gene segments and antigen/MHC specificity. In this study, we have asked whether the dominant TCR V β gene usage observed in the response to a well characterized epitope is determined by structural constraints or by more complex events affecting the available TCR repertoire.

Our analysis of TCR sequences from a panel of DBA/2 (H-2^d) T cell clones specific for sperm whale myoglobin

(SpWMb)¹ has shown that the T cell response to the epitope 110-121, restricted by the isotype hybrid molecule A β ^dE α ^d (Ruberti, G., K.S. Sellins, R.N. Germain, C.G. Fathman, and A.M. Livingstone, manuscript submitted for publication) appeared to exhibit a very restricted TCR V β usage (10, 14). Six independent A β ^dE α ^d restricted clones specific for residues 110-121 all used TCR V β 8.2, D β 2.1, and either J β 2.5 (1/6) or J β 2.6 (5/6). The use of V α was more heterogeneous in that at least three different V α and 4 J α segments were used by these six T cell clones (14). Analysis of these clones on nested sets of overlapping synthetic peptides, on peptides substituted at various positions within the 110-121 sequence, and on allogeneic APCs, revealed differences in antigen fine specificity that could clearly be correlated with differences in the VJ or VDJ junctional regions of the α and β chains, respectively (reference 14; A. Livingstone, manuscript in preparation).

To ask whether this very limited heterogeneity was truly representative of the response in vivo, we analyzed the 110-121 T cell response of lymph node cells from primed DBA/2

¹ Abbreviations used in this paper: Mls, minor lymphocyte stimulating; PPD, tuberculin purified derivative; SpWMb, sperm whale myoglobin.

mice stimulated once in vitro. Our results confirmed the predominant use of the V β 8 gene element in the T cell response to 110-121 of DBA/2 mice immunized with either SpWMb or peptide 110-121.

Two different approaches were used to ask whether removal of V β 8⁺ T cells could abrogate or alter the T cell response to this epitope. First, DBA/2 mice were depleted of peripheral T cells expressing V β 8 by administration in vivo of the mAb F23.1, specific for T cell receptors bearing any of the three TCR V β 8 gene elements (15). These F23.1 treated mice were able to respond to the 110-121 peptide but only at fairly high antigen concentrations. The second approach involved the breeding of mice lacking TCR V β 8 genes. The majority of inbred mouse strains have a full complement of TCR V β genes. A few inbred strains and some wild mice, however, share a deletion on chromosome 6 including V β 5, 8, 9, 11, 12, and 13 (16, 17). Strains with the full complement of TCR V β genes have been given the haplotype V β ^b, while strains carrying this deletion have been designated V β ^a. H-2^d mice homozygous for V β ^a were generated by crossing the V β ^a locus from SWR onto the DBA/2 strain. Although DBA/2V β ^a mice have a very restricted TCR V β repertoire, they made an excellent response to peptide 110-121. We discuss possible explanations for the dominant V β 8 response in DBA/2 mice, and for the contrasting results with F23.1 treated and DBA/2V β ^a mice.

Materials and Methods

Animals. DBA/2, C57BL/6 and SWR mice were obtained from the Jackson Laboratory (Bar Harbor, ME) or from the National Cancer Institute, (Bethesda, MD). D2.GD mice from the Jackson Laboratory were bred in the Department of Laboratory Animal Medicine, Stanford University (Stanford, CA). Males from 8- to 12-wk-of-age were used for all experiments.

Generation of the DBA/2V β ^a Line. DBA/2 (H-2^d, V β ^b) mice were crossed with SWR (H-2^a, V β ^a) mice and the F₁ progeny crossed back to DBA/2. PBLs were typed by cytofluorometric analysis (see below) using mAbs specific for MHC and TCR V β gene products. The mAb 1D9 binds to I-A^a but not to I-A^d (18) and therefore distinguished between H-2^{d/d} and H-2^{d/q} progeny. V β ^{b/b} and V β ^{a/b} mice were identified by double staining with allophycocyanin-labeled anti-Ly-1 (reference 19, a generous gift from Dr. Alan Stall, Stanford University, CA) and FITC-labeled F23.1 (15). V β ^{b/b} mice, like the parental DBA/2 strain, had about twice as many Ly-1⁺, F23.1⁺ cells (i.e., V β 8⁺ T cells) as (DBA/2 \times SWR)F₁ mice and the V β ^{a/b} backcross progeny, as a consequence of allelic exclusion of TCR V β genes (20). Backcross progeny typed as H-2^{d/d}, V β ^{a/b}, were intercrossed. The progeny were typed with anti-Ly-1 and F23.1 as described above, and could be categorized as V β ^{a/a}, V β ^{a/b}, or V β ^{b/b}. The DBA/2V β ^a line was established by brother/sister mating of V β ^{a/a} intercross progeny.

Antigens. The major chromatographic component (#10) was prepared from type II myoglobin from sperm whale skeletal muscle (Sigma Chemical Co.) by ion-exchange chromatography, as previously described (21). The 110-121 SpWMb peptide was synthesized using standard solid phase methods. Tuberculin purified protein derivative (PPD) was purchased from Connaught Laboratories (Willow Dale, Ontario, Canada).

Cell Lines. RT 10.3 C1 (22) I-E^d L cell transfectant was a

generous gift of R. Germain (National Institutes of Health [NIH], Bethesda, MD).

Cell Staining for TCR V β : Cytofluorometric Analysis. DBA/2, SWR and DBA/2V β ^a mice were sacrificed, and their peripheral lymph node cells (5×10^5) were stained in HBSS (Gibco Laboratories) with saturating amounts of mAb as previously described (23, 24). Sources and specificities of the antibodies were: anti-V β 2 (B20.6, Dr. B. Mallisen, INSERM-CNRS, Marseille, France), -V β 3 (KJ25) (25), -V β 4 (KT4) (26), -V β 6 (RR4-7) (27), -V β 7 (TR310) (28), -V β 8 (F23.1) (15), -V β 8.2 (F23.2) (29), -V β 14 (14.2) (30); biotinylated Ly-1 (Becton Dickinson and Co., Mountain View, CA); fluorescein conjugated goat anti-mouse, anti-rat, anti-hamster Ig, and Texas Red-streptavidin (Caltag Laboratories, San Francisco, CA). After staining, fluorescence was analyzed on a highly modified dual laser FACS IV[®] (Becton Dickinson and Co.). Controls included cells stained with secondary reagents alone and the background values were subtracted. Ficoll-enriched T cells from DBA/2, and DBA/2V β ^a 110-121 SpWMb T cell lines, were analyzed with the same anti-TCR V β mAbs.

Preparation of CD4⁺V β 8⁺ and CD4⁺V β 8⁻ Lymph Node T Cells from Sperm Whale Myoglobin (SpWMb) and SpWMb 110-121 Immunized DBA/2 Mice. 7 d after immunization, the draining inguinal lymph nodes of 5-6 mice were removed, and the pooled cell suspension (5×10^7) was stained with 10 μ g biotinylated anti-CD4 Ab GK1.5 (31) and with 10 μ g fluorescein-conjugated F23.1 or F23.2. Cytofluorometric analysis was performed as described above and 98-99% pure CD4⁺V β 8⁺ or CD4⁺V β 8.2⁺ and CD4⁺V β 8⁻ or CD4⁺V β 8.2⁻ T cells were sorted for further analysis.

F23.1 Antibody Depletion in DBA/2 Mice. On day 0 and after 48 h 500 μ g mAb F23.1 was injected i.p. into DBA/2 mice. Depletion was assayed on Ficoll-enriched, red blood cell depleted, PBMCs before injection at 24 and 72 h, and day 10 following the first mAb injection by two-color staining with GK1.5 and F23.1 as described above. V β 8-depleted mice were immunized 72 h after the first injection of F23.1, with the SpWMb 110-121 peptide as described above.

Proliferation Assays. T cell proliferative responses to SpWMb and the peptide 110-121 were determined as follows: sex and age matched DBA/2, DBA/2V β ^a, and F23.1 depleted DBA/2 mice were immunized in the base of the tail with the appropriate Ag (50-100 μ g) emulsified in CFA (Gibco Laboratories). 7 d after the immunization, draining lymph node cells (5×10^5) were cultured in flat-bottom 96-well microtiter plates with various concentrations of Ag. The cultures were pulsed with 1 μ Ci of [³H]thymidine per well on day 3 of culture and harvested 16 h later. The SDs were within 10% of the mean value. All experiments were repeated at least three times with similar results. To determine MHC restriction, lymph node cells were preincubated with 1-2 μ g/ml of anti-I-E α , 14.4.4S (32) and anti-I-A β ^d, MKD6 (33) (a generous gift from D. Sachs, NIH, Bethesda, MD) protein A purified mAbs for 2 h. Control wells included cells without Ag to determine background proliferation; cells with PPD (50-100 μ g/ml) to assess priming efficiency and allogeneic MHC Ag (0.5×10^6 irradiated C57BL/6 (3,000 rad) spleen cells) for proliferative responses.

4-5 $\times 10^4$ sorted V β 8⁺ or V β 8.2⁺/CD4⁺ and V β 8⁻ or V β 8.2⁻/CD4⁺ SpWMb or SpWMb 110-121 primed DBA/2 lymph node cells were cultured with 5×10^5 irradiated (3,000 rad) spleen cells without antigen or with SpWMb, SpWMb 110-121, or PPD as described above. To determine MHC restriction, lymph node cells were incubated with autologous DBA/2 spleen cells that express both I-A^d and I-E^d or with D2.GD spleen cells

which bear only I-A^d MHC class II molecules. Blocking experiments with anti-class II mAbs were carried out as described above. All assays were performed in RPMI 1640 (Whittaker M.A. Bio-products, Walkersville, MD) supplemented with 10 mM Hepes (Gibco Laboratories) 10% FCS or 0.5% normal mouse serum where indicated, 2 mM L-glutamine and 5 × 10⁻⁵ M 2-ME (Sigma Chemical Co.).

DBA/2Vβ^a 110-121 T Cell Lines. SpWMb 110-121 specific T cell lines were established from individual SpWMb 110-121 primed DBA/2Vβ^a mice as previously described (21). Briefly, 2–3 × 10⁶ lymph node cells from immunized mice were cultured for 10 d in the presence of 5 μM SpWMb peptide 110-121 and propagated by periodic cycles of stimulation and rest (21). 2 × 10⁴ rested and Ficoll-enriched T cells were tested in proliferation assays as described above.

Results

The Predominant In Vivo Response to Peptide 110-121 Involves Vβ8⁺ T Cells. In our original analysis of the DBA/2 response to SpWMb, we identified six independent T cell clones specific for the 110-121 epitope. While this epitope overlaps considerably with the I-A^d restricted 106-118 epitope, first identified by Berzofsky and colleagues (34–36), the two could be easily distinguished using myoglobin from different species and overlapping sets of synthetic peptides (reference 14; A. Livingstone, manuscript in preparation). Experiments using antigen presenting cells from intra-MHC recombinant mouse strains, and inhibition with mAbs, originally suggested that the 110-121 clones were I-E^d restricted (10, 14). A more extensive analysis with mAbs and with class II-transfected L cells has now shown that these clones are restricted by the isotype mixed Aβ^dEα^d molecule (Ruberti, G., K. Sellins, R.N. Germain, C.C. Fathman, and A.M. Livingstone, manuscript submitted for publication).

All six of the 110-121 specific clones expressed TCR β chains using Vβ8.2, Dβ2.1, and either Jβ2.5 (1/6) or Jβ2.6 (5/6) (14). To see whether the use of the TCR Vβ8.2 gene segment to recognize the 110-121 epitope accurately reflected the specificity of the T cell response in vivo, DBA/2 mice were immunized with either the intact protein or with the 110-121 peptide. 98–99% pure populations of CD4⁺/Vβ8⁺, CD4⁺/Vβ8.2⁺ or CD4⁺/Vβ8⁻, CD4⁺/Vβ8.2⁻ T cells were sorted from the draining lymph nodes, and assayed against SpWMb or peptide 110-121 presented by DBA/2 APCs. In contrast to longterm T cell culture, very little selection for T cell clones occurs under these conditions. The results (Fig. 1–3) showed that the response to peptide 110-121 lay almost entirely within the CD4⁺/Vβ8⁺ population. CD4⁺/Vβ8⁻ from mice immunized with SpWMb made little response to SpWMb, and no response at all to peptide 110-121 (Fig. 1). CD4⁺/Vβ8.2⁻ cells made a slightly stronger response to SpWMb, but again, there was little, if any proliferative response to peptide 110-121 (Fig. 2). In mice immunized with peptide 110-121, the response also lay entirely in the CD4⁺/Vβ8⁺ population (Fig. 3). In each experiment, the CD4⁺/Vβ8⁻ and CD4⁺/Vβ8⁺ populations made comparable responses to PPD. In multiple experiments, no significant difference in the magnitude of the

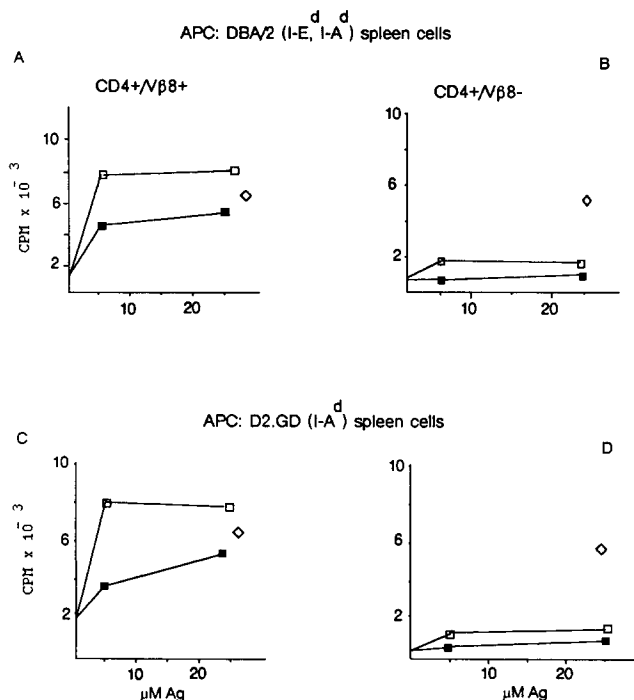


Figure 1. Selection for Vβ8⁺ cells in the 110-121 SpWMb response in vivo (I). After immunization of five DBA/2 mice with SpWMb in CFA, the pooled local lymph nodes (LN) were removed and LN cells were sorted by fluorescence-activated cell sorting into Vβ8⁺ (A and C) and Vβ8⁻ (B and D) helper T cell populations using labeled antibodies as described in Material and Methods. Both populations were stimulated with SpWMb (■), 110-121 SpWMb (◼), or PPD (◇) and syngeneic 3,000 rad irradiated DBA/2 (A and B) or D2.GD (C and D) spleen cells. The results shown are the mean values of triplicate measurements and are a representative example of three independent experiments.

PPD or allo (anti-C57Bl/6) response was observed between the Vβ8⁺ and Vβ8⁻ populations (data not presented).

These sorted populations were also assayed on D2.GD spleen cells. This strain has a recombinant MHC haplotype bearing a defective Eα gene (37–38). It therefore cannot express I-E^d or Aβ^dEα^d class II molecule. The response in every case was comparable with that seen on DBA/2 spleen cells, suggesting that a substantial part of these responses was restricted by I-A^d, or possibly by Aα^dEβ^d. However, the 110-121 response of unsorted lymph node cells from DBA/2 mice primed with either the peptide 110-121 or with whole myoglobin was significantly inhibited by the monoclonal antibody 14.4.4S (specific for Eα chains) as well as by the Aβ^d specific antibody MKD6 (data not shown). Thus at least part of the DBA/2 response to peptide 110-121 was restricted by class II molecules involving the Eα^d chain (i.e., I-E^d or Aβ^dEα^d).

Sperm Whale Myoglobin (SpWMb) 110-121 T Cell Responses in Anti-Vβ8 Antibody-Treated DBA/2 Mice. The experiments described above confirmed previous results showing that the majority of the response to peptide 110-121 was contained within the Vβ8⁺ population. We therefore asked whether DBA/2 mice that lacked Vβ8⁺ T cells could respond to peptide 110-121. Our first approach was to remove Vβ8⁺ peripheral T cells by treatment in vivo with the anti-Vβ8

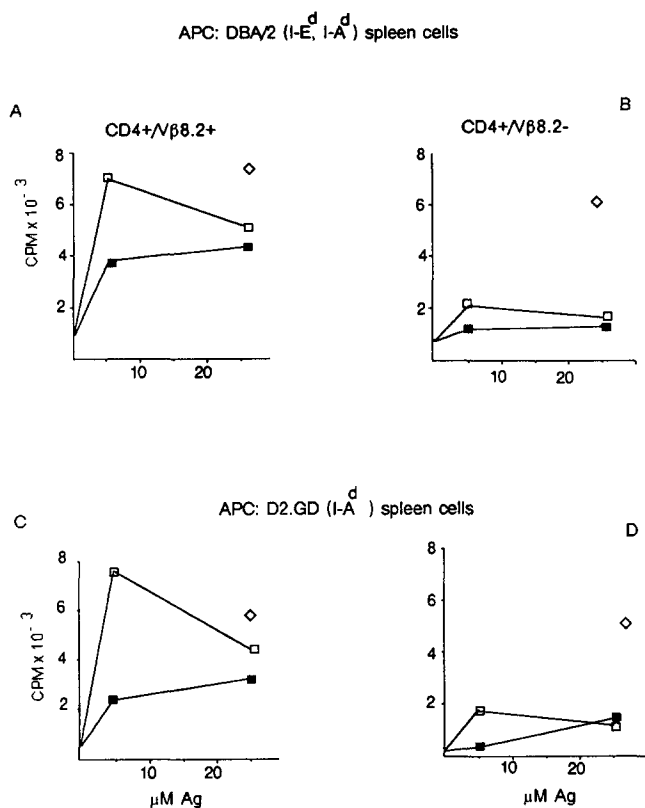


Figure 2. Selection for Vβ8⁺ cells in the 110-121 SpWMb response in vivo (II). Five DBA/2 mice were primed with SpWMb and the Vβ8.2⁺ (A and C) and Vβ8.2⁻ (B and D) helper T cell populations were analyzed as described in Fig. 1 in the presence of SpWMb (□), 110-121 SpWMb (■), or PPD (◇) on DBA/2 (A and B) and D2.GD (C and D) APCs.

specific mAb, F23.1, which depletes Vβ8⁺ T cell from the peripheral blood (15). Depletion of F23.1⁺ peripheral T cells is 99% complete 3 d after i.p. administration of a 1 mg dose and is still at this level on day 10 (Fig. 4). In three experiments, Vβ8 depleted DBA/2 mice were primed with the 110-121 peptide and proliferation of their individual or pooled lymph node T cells was analyzed in comparison with the normal DBA/2 primed lymph node T cell response. As shown in Fig. 5, F23.1 depleted mice responded to the 110-121 pep-

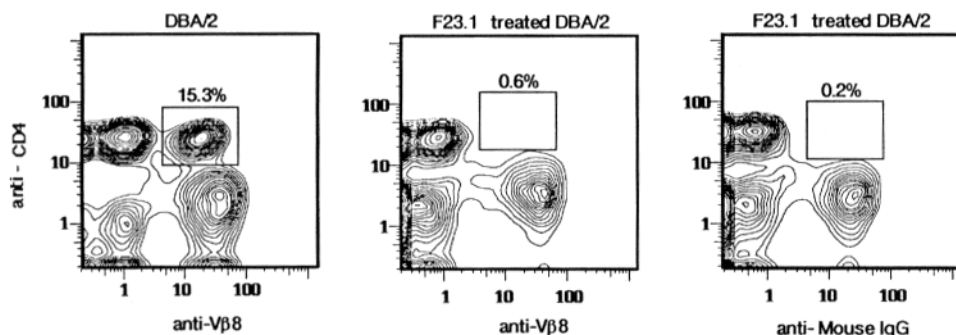


Figure 4. Depletion of Vβ8⁺, CD4⁺ T cells with mAb F23.1 in vivo. In (A) and (B) anti-CD4 vs. anti-Vβ8 (F23.1) FACS analysis is plotted. The boxed regions represent CD4⁺ F23.1⁺ cells. In (C), anti-CD4 is plotted vs. anti-mouse Ig to demonstrate clearance of surface F23.1 (a mouse IgG 2a-positive antibody). Depletion of CD4⁺ F23.1⁺ cells was assayed on PBLs. At 72 h, the depletion of F23.1 peripheral T cells is 99% complete (the boxed areas) 3 d after i.p. administration of a 1 mg dose and is

still at this level at day 10. Two-color staining data are provided as "contour plots" in which the levels of green and Texas-red fluorescence per cell define their location on a two-dimensional surface. Each contour line represents a 5% frequency of cells with a given fluorescence intensity.

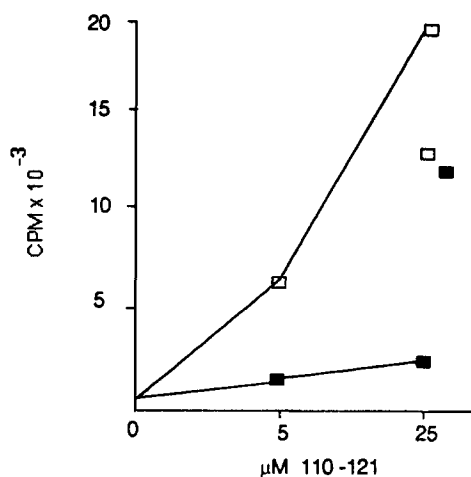


Figure 3. Selection for Vβ8⁺ cells in the 110-121 SpWMb response in vivo (III). Five DBA/2 mice were primed with the 110-121 SpWMb peptide in CFA. Vβ8⁺ (□) and Vβ8⁻ (■) populations were sorted as described in Fig. 1 and the response to SpWMb peptide 110-121 was analyzed. Vβ8⁺ (□) and Vβ8⁻ (■) PPD responses were included to assess priming efficiency.

tide but the response was very poor compared with that of the untreated controls and could only be seen at high peptide concentrations; lymph node cells from F23.1-treated mice needed at least five times more antigen than cells from untreated mice to give comparable responses.

Generation and Characterization of the DBA/2Vβ^a Line. An alternative way to look at the 110-121 response in DBA/2 mice lacking Vβ8⁺ peripheral T cells involved the generation of a DBA/2 mouse line that carried a genetic deletion of the Vβ8 gene family. Most inbred mice strains have a full complement of TCR Vβ gene segments (haplotype Vβ^b), but several strains have a large deletion (haplotype Vβ^a) involving the TCR Vβ5, 8, 9, 11, 12, and 13 gene segments (16). DBA/2 has the Vβ^b haplotype while the SWR strain carries the deleted haplotype Vβ^a. The breeding of the DBA/2Vβ^a line is described in detail in Materials and Methods. Briefly, SWR mice were mated to DBA/2, and the (SWR × DBA/2)F₁ progeny were backcrossed to DBA/2. The backcross progeny were typed for MHC and Vβ haplo-

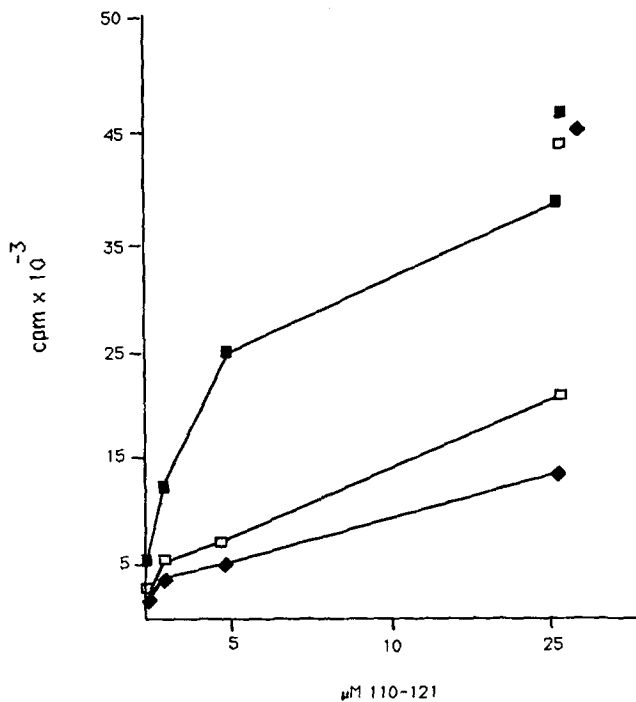


Figure 5. Proliferative response of DBA/2 (■) and F23.1 depleted DBA/2 mice (□, ◆). Five mice were primed with the 110-121 SpWMb peptide and their pooled lymph node cells assayed in the presence of different antigen concentrations of the 110-121 peptide. F23.1 depleted mice were treated with two different antibody preparations: (◆) concentrated F23.1 from tissue culture supernatant and (□) protein-A purified F23.1 mouse ascites. This is a representative example of three experiments in which pooled and individual lymph node cells were analyzed. The SDs were within 10% of the mean values. Complete media supplemented with 0.5% fresh mouse serum was used in these assays.

types by cytofluorometric analysis of PBLs. H2^{d/d}, Vβ^{b/a} progeny were intercrossed and the Vβ^{a/a} progeny selected as the parent of a DBA/2Vβ^a line. This line still carried a substantial genetic contribution from the SWR strain. How-

Table 1. Peripheral Expression of Vβ Domains in DBA/2, SWR, and DBA/2Vβ^a Mice

Vβ	Percentage of positive T cells (mean ± SE)		
	DBA/2	SWR	DBA/2Vβ ^a
Vβ2	8.0 ± 0.2	14.9 ± 0.8	15.8 ± 0.8
Vβ3	0.6 ± 0.1	2.4 ± 0.4	0.2 ± 0.1
Vβ4	9.2 ± 0.3	9.7 ± 0.3	19.1 ± 0.5
Vβ6	0.4 ± 0.2	8.1 ± 0.8	0.4 ± 0.2
Vβ7	0.4 ± 0.2	5.3 ± 0.8	0.6 ± 0.2
Vβ14	9.9 ± 0.4	9.2 ± 0.8	15.7 ± 0.1

Frequency of Vβ gene element expression among Ly-1⁺ T cells. Staining and flow cytometric analysis were carried out on peripheral lymph node cells as described in Materials and Methods; the background values were subtracted.

ever, both the inbred DBA/2 strain and the DBA/2Vβ^a line demonstrably shared a number of loci known to have important effects on the intrathymic selection of the T cell repertoire. Both lines expressed I-E^d molecules, and also the minor lymphocyte stimulating (Mls) Mls-1^a and Mls-2^a antigens. All of these antigens cause intrathymic deletion of T cells expressing particular TCR Vβ genes during development of the T cell repertoire. Cytofluorometric analysis of TCR Vβ expression on peripheral lymph node cells (Table 1) showed that both lines had low numbers of Vβ3, Vβ6, and Vβ7 cells, consistent with deletion by Mls-1^a and Mls-2^a (25, 27, 28, 39, 40, 41), while Vβ2, Vβ4, and Vβ14 expression was higher in DBA/2 Vβ^a mice than in DBA/2 mice.

DBA/2Vβ^a Mice Make a T Cell Response to Peptide 110-121. DBA/2 and DBA/2Vβ^a mice were immunized in parallel with peptide 110-121, and cells from the draining lymph nodes were assayed for proliferative response to this peptide. As shown in Fig. 6, the magnitude of the response made by DBA/2Vβ^a mice was at least as great as that made by DBA/2 mice. Two lines of evidence suggested that a significant proportion of this response might be restricted by I-A^d. First, the response to peptide 110-121 presented by D2.GD spleen cells was as strong as that seen with DBA/2 antigen presenting cells (Table 2). Second, in experiments where mAbs were used to block the response of DBA/2 Vβ^a T cells to peptide 110-121, MKD6 inhibited the response more effectively than 14.4.4S (Table 3); in similar experiments using these antibodies to inhibit the response of Aβ^dEα^d-restricted, 110-121 specific clones, 14.4.4S invariably blocked the response far more effectively than MKD6 (Ruberti, G., K.S. Sellins, R.N. Germain, C.G. Fathman, and A.M. Living-

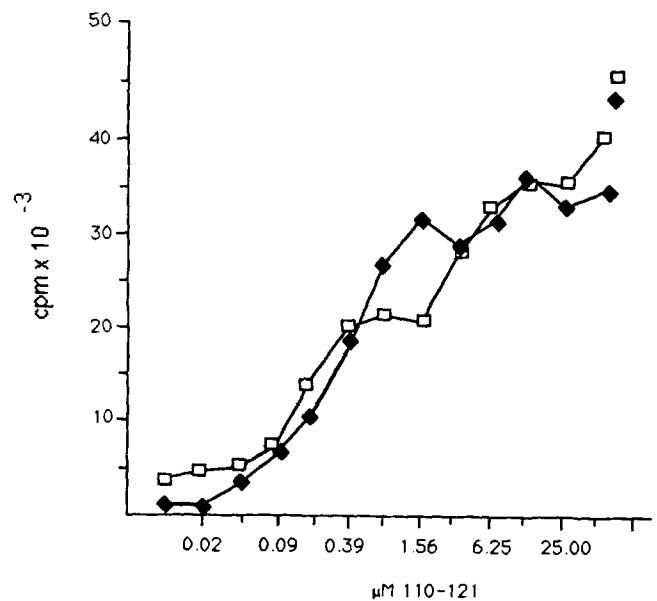


Figure 6. DBA/2Vβ^a mice response to the 110-121 SpWMb peptide. Proliferative response of pooled DBA/2 (◆) and DBA/2Vβ^a (□) lymph node cells to different concentrations of the 110-121 peptide and to PPD (◆, □). The data represent the mean of triplicate determinations and are representative of five experiments in which pooled and individual lymph node cells were assayed. The SDs were within 10% of the mean values.

Table 2. Proliferative Responses of DBA/2V β^a T Cell Lines on I-E $^{d+}$, I-A $^{d-}$, and on I-E $^{d+}$ I-A $^{d+}$ Antigen-Presenting Cells

		Ag				
		0 (μ M)	0.4 (μ M)	2 (μ M)	10 (μ M)	50 (μ M)
A	731	4,202	22,670	30,670	59,364	69,277
	735	7,680	27,762	40,141	73,411	90,722
B	731	4,357	25,323	32,201	54,510	62,308
	735	3,961	29,563	43,533	80,082	92,473
C	731	718	604	790	1,466	10,538
	735	716	706	680	1,489	12,705

Ficoll-enriched T cells were cultured with different APCs: DBA/2 spleen cells (I-A $^{d+}$ and I-E $^{d+}$) (0.5×10^5 c/well (A), D2.GD spleen cells (I-A $^{d-}$ and I-E $^{d-}$) (0.5×10^5 c/well), (B) RT 10.3 C1 (I-A $^{d-}$ and I-E $^{d+}$) transfectants ($0.5-2 \times 10^5$ c/well), (C) and the 110-121 SpWMb peptide as described in Materials and Methods. Each panel shows a representative example of several experiments on five individual T cell lines.

stone, manuscript submitted for publication). However, 110-121 specific T cell lines isolated from these mice did respond to the RT 10.3 C1 I-E d transfected fibroblast line (Table 2). In these experiments, responses were seen only at very high peptide concentrations, but such APCs are often very poor stimulators of T cell lines and clones, although they may stimulate T cell hybridomas quite effectively. To date, limiting dilution of five DBA/2V β^a 110-121 specific T cell lines has yielded only I-A d restricted T cell clones that recognize the 110-121 peptide presented by D2.GD spleen cells (data not shown). Together, these results suggest that only a small part of the DBA/2V β^a response recognizes the 110-121 peptide in the context of E α^d E β^d or A β^d E α^d class II MHC molecules.

Discussion

This study was undertaken to analyze the T cell response in vivo of DBA/2 mice to the 110-121 epitope of SpWMb

and to investigate the basis for the extremely restricted TCR repertoire observed in this response in vitro. Our original results, using a panel of independent T cell clones specific for three distinct epitopes on SpWMb, showed that 11/14 clones expressed a V β 8 gene segment (10). Moreover, the response to one particular epitope, defined by residues 110-121 and restricted by A β^d E α^d , showed extremely limited TCR β gene segment usage. All six independent clones specific for 110-121/A β^d E α^d used V β 8.2 and D β 2.1, together with either J β 2.5 or J β 2.6 (14). The experiments described in this paper analyzed the response to peptide 110-121 in vivo in normal DBA/2 mice and in DBA/2 mice unable to use the TCR V β 8 gene family.

The first experiments confirmed that the very strong bias towards TCR V β 8 expression observed in the panel of T cell clones was also found in vivo. This V β 8 restricted response was seen not only in the response to peptide 110-121, but also in the response to whole myoglobin. These experiments

Table 3. Antibodies Blocking Studies on DBA/2V β^a T Cell Lines

		Ag						
		12×10^{-5} (μ M)	64×10^{-4} (μ M)	32×10^{-4} (μ M)	16×10^{-4} (μ M)	8×10^{-2} (μ M)	4×10^{-1} (μ M)	2×10^1 (μ M)
731		4,992	9,279	7,883	21,305	34,806	45,349	41,601
	+ 14.4.4S MAb	4,392	6,851	6,400	15,529	28,770	39,083	35,264
	+ MKD6 MAb	2,164	2,912	3,839	9,196	22,367	31,773	30,478
735					12,245	40,974	60,188	63,713
	+ 14.4.4S MAb				8,924	36,505	60,401	64,646
	+ MKD6 MAb				6,196	25,827	52,983	53,652

Ficoll-enriched resting T cells were cultured with syngeneic spleen cells and with different antigen concentrations of the 110-121 peptide as described in Materials and Methods. APCs were preincubated with 14.4.4S (anti-I-E α) or MKD6 (anti-I-A β^d) mAb (1-2 μ g/ml). Background 3 H incorporation in the absence of antigen ranged from $1-2 \times 10^3$ cpm/well and has been subtracted. This is a representative example of five experiments on five individual DBA/2V β^a -derived T cell lines.

also showed that I-A^d (and perhaps A α ^dE β ^d) in addition to the hybrid A β ^dE α ^d, could restrict T cell recognition of peptide 110-121. This result is of particular interest since Sette et al. (42-43) have shown that residues within the sequence 111-118 allowed peptide binding to I-A^d and I-E^d molecules. These current studies emphasize the importance of analyzing responses with T cells taken directly from the animal; the initial analysis, where clones were isolated after several rounds of in vitro restimulation, only gave A β ^dE α ^d restricted 110-121 specific T cell clones (Ruberti, G., K.S. Sellins, R.N. Germain, C.G. Fathman, and A.M. Livingstone, manuscript submitted for publication).

In the past few years, several groups have reported strong associations between expression of particular TCR V β domains and MHC restriction (4, 5, 7, 10). However, expression of V β 8 gene segments alone cannot be sufficient to determine the specificity of the restriction, since members of the V β 8 family are expressed in other antigenic systems by T cells with disparate MHC restriction specificities. The V β 8.2 gene segment, for instance, is used by both I-A, I-E, and class I restricted T cell clones and hybridomas. DBA/2 mice express I-E^d, Mls-1^a, and Mls-2^a antigens and so the majority of T cells expressing V β 3 (25, 39), V β 5 (44-46), V β 6 (27, 40), V β 7 (28, 41), V β 8.1 (29), V β 9 (41, 47), V β 11 (48, 49), and probably V β 12 and 16 (41, 50) were deleted during thymic maturation. Despite this limitation, the DBA/2 peripheral T cell repertoire includes T cells expressing gene segments from at least eight other V β families in addition to V β 8. The predominant use of TCR V β 8 gene segments in the DBA/2 response to peptide 110-121 could be explained in several ways. One possibility was that V β 8.2, perhaps in association with particular D β and/or J β segments, was the only available TCR V β gene element that could be used to construct a receptor recognizing peptide 110-121. If so, then DBA/2 mice lacking peripheral V β 8 T cells would be unable to make any 110-121-specific response. Alternatively, the predominance of V β 8⁺ T cells in the DBA/2 response to peptide 110-121 could be due to constraints imposed during rearrangement of TCR V β gene segments, or to as yet undetermined regulatory interactions during the establishment of the peripheral T cell repertoire. If so, V β 8⁻ DBA/2 mice might make a 110-121-specific response using an alternative TCR V β gene segment(s).

We, therefore, looked at the 110-121 response in DBA/2 mice depleted of V β 8⁺ T cells, by treatment with a V β 8-specific mAb and in DBA/2 mice carrying a genetic deletion of the entire TCR V β 8 family.

These two approaches gave very different answers. DBA/2 mice depleted of peripheral V β 8⁺ T cells by antibody treatment were able to make a 110-121-specific response, but this was considerably weaker than that seen in control mice, and could only be detected at relatively high antigen concentrations. As discussed above, this V β 8⁻ response was not seen at all in normal DBA/2 mice. One explanation is that in DBA/2 mice, V β 8⁻ T cells specific for 110-121 are present at low frequency, or have low affinity (or both) compared with V β 8⁺ 110-121-specific T cells. Immunization of untreated DBA/2 mice preferentially expanded the V β 8⁺ re-

sponse, effectively masking any V β 8⁻ component; in the absence of V β 8⁺ cells, the V β 8⁻ cells were expanded sufficiently to be detected. This explanation of data suggests the possibility that "high affinity" and/or more frequent T cell clone responses override potential lower affinity and/or lower frequency receptor(s) engagements.

The response in DBA/2 mice lacking TCR V β 8 gene elements was strikingly different. The DBA/2V β ^a mice produced by crossing the V β ^a locus from SWR onto the DBA/2 background had a very severely restricted TCR V β repertoire. As a consequence of the genetic deletion within the V β locus, they had no T cells expressing V β 5, V β 8, V β 9, V β 11, V β 12, or V β 13 gene elements; in addition, the majority of V β 3, V β 6, V β 7, V β 17a (51), and probably V β 16 T cells were deleted intrathymically during thymocyte maturation, since these mice expressed I-E^d, Mls-1^a, and Mls-2^a antigens. Despite this limitation, the DBA/2V β ^a mice made a normal T cell proliferative response to peptide 110-121 when compared to DBA/2V β ^b mice. However, preliminary data suggested that there was a shift of MHC restriction. The DBA/2V β ^a response to 110-121 appeared to be restricted almost entirely by I-A^d, although inhibition by the antibody 14.4.4S, and a very weak response to an I-A^d, I-E^d+ APC line suggested that a small component of this response might be restricted by I-E^d and/or perhaps by the hybrid molecule A β ^dE α ^d. Further analysis will be necessary to investigate the presence of T cell responses to hybrid molecules and the 110-121 peptide in DBA/2V β ^a mice and whether this Ag/MHC complex is recognized at all. Cytofluorometric analysis of 110-121-specific T cell lines from DBA/2V β ^a mice, using mAb, specific for V β 2, V β 3, V β 6, V β 7, and V β 14, failed to identify any dominant TCR V β gene usage.

These experiments demonstrated very clearly that the use of TCR V β 8⁺ receptors in the DBA/2 response to peptide 110-121 was not determined by any structural requirement. Since DBA/2V β ^a mice were able to make a strong V β 8⁻ response to peptide 110-121, the absence of a comparable response in DBA/2 mice must be explained. All the TCR V β genes expressed on peripheral T cells in DBA/2V β ^a mice should in theory also be present in DBA/2 mice. Since the DBA/2V β ^a animals used in these experiments were only at the first backcross level, we cannot rule out the possibility that the 110-121 response in these mice is mediated by T cells positively selected by non-MHC antigens derived from the SWR strain. This seems rather unlikely, since the cotolerogens described to date cause deletion (45, 49) rather than positive selection. T cell receptor V β polymorphism between V β ^a and V β ^b strains have been observed by RFLP and sequence analysis (16, 52-53) and such germ-line encoded differences in the TCR V β repertoire might play a role in the SpWMb 110-121 immune responsiveness. It is unlikely that polymorphisms within TCR V α genotype (54-56) play a significant role in the dissimilarities between the DBA/2 and DBA/2V β ^a response to SpWMb 110-121. Although the V α genotype of the DBA/2V β ^a mice was not analyzed, the ability of multiple V α 's to pair effectively with V β 8.2 in the DBA/2 mice in response to SpWMb 110-121 peptide suggests that limitations imposed in V α use are less demanding. We are,

however, continuing to cross the $V\beta^a$ haplotype onto the DBA/2 background to create a congenic line to address these questions as well as a possible role of $J\beta$ and $J\alpha$ polymorphism in the 110-121 immune response.

An additional explanation is that those $V\beta 8^-$ cells responsible for the 110-121 specific response in the F23.1-treated DBA/2 mice are present at considerably higher frequency in the DBA/2 $V\beta^a$ mice, and are therefore able to make a much stronger response. Because peripheral T cells in DBA/2 $V\beta^a$ mice can express only about half the number of $V\beta$ gene expressed in DBA/2 mice, cells expressing a particular $V\beta$ gene would be expected to be about twice as frequent in DBA/2 $V\beta^a$ mice as in DBA/2 mice in the absence of selective pressures. Cytofluorometric analysis of TCR $V\beta$ expression in lymph node cells confirmed this expected level of expression for $V\beta$ families that could be analyzed by FACS. The percentage of T cells expressing $V\beta 2$, $V\beta 4$, and $V\beta 14$ was approximately twice that seen in DBA/2 mice (Table 1). We are currently investigating the possibility of restricted $V\beta$ use in these two models by comparing the fine specificity, MHC restriction and TCR $V\beta$ gene usage of the 110-121 response in F23.1 treated DBA/2 mice versus DBA/2 $V\beta^a$ mice.

It is also possible that mechanisms of TCR β gene segment rearrangement may favor the production of functional receptors using particular gene elements. For example, the deletion of about 10 TCR $V\beta$ gene segments in the $V\beta^a$ haplotype might affect the repertoire by allowing preferential $V\beta$ rearrangements not seen at high frequency in the $V\beta^b$ haplotype. Preferential usage of the 3'-most VH gene has been observed in VDJ rearrangement in murine and human B cell lineage early in ontogeny (57-59). B cells in both immature and adult rabbits preferentially use VH1 in VDJ rearrangement (60). Moreover, biased expression of JH-proximal VH genes occurs in the newly generated repertoire of neonatal and adult mice (61). The proximity of the VH1 to D and JH gene or the more effective combinatorial association with junctional regions have both been postulated to explain

the more frequent expression of the VH1 gene (60). It is not yet known whether, during thymic development before selection, there is a preferential rearrangement of particular VDJ β and VJ α gene element combinations or whether certain combinations are more affected by positive or negative selection pressures. Examination of β chain rearrangements in a large panel of T cell clones and hybridomas with different specificities has demonstrated that the majority of expressed β chains use a $J\beta 2$ gene segment (62). Moreover, a homogeneous junctional sequence of $V\alpha 14$ has been recently reported in naive mice (63). This may be a function of selective pressures or a mechanistic gene rearrangement which occurs before selection. If there is a "mechanistic" nonselective rearrangement of the VDJ β elements during thymic development which influences the peripheral repertoire, it is likely that the $V\beta^a$ haplotype, with the genomic deletion, may play a crucial role in the VDJ β combination frequencies and thus explain the equivalent SpWMb 110-121 epitope response in DBA/2 and DBA/2 $V\beta^a$ mice. Experiments are currently under way to address whether "mechanistic" rearrangements which precedes selective pressures may explain these data.

Several previous studies have analyzed responses where there is predominant use of a particular TCR $V\beta$ gene segment, to ask what happens when T cells expressing the $V\beta$ gene in question are not available (64-67). As in our study comparing DBA/2 and DBA/2 $V\beta^a$ mice, it was found that a response was made using alternative $V\beta$ gene segments. In these experiments, however, T cells expressing the preferred $V\beta$ gene segment were deleted intrathymically through recognition of Mls antigens. They thus compared T cell populations that were selected in Mls⁺ versus Mls⁻ thymic environments, and therefore potentially had quite distinct repertoires. The study described here compared peripheral T cell populations that had both matured in H-2^d, Mls-1^a, Mls-2^a environments, and therefore (with the reservation about SWR-derived antigens mentioned above) were subject to the same positive and negative selection.

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