

# THE MHC MOLECULE I-E IS NECESSARY BUT NOT SUFFICIENT FOR THE CLONAL DELETION OF V $\beta$ 11-BEARING T CELLS

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The MHC-restricted TCR consists of a 90-kD heterodimeric glycoprotein composed of an  $\alpha$  and a  $\beta$  chain that are encoded by somatically rearranging gene segments (for review, see references 1–3). Receptor diversity and antigen/MHC specificity are generated by rearrangement of the known variable elements (variable [V], diversity [D], and joining [J] segments), as well as by the addition of *N*-region nucleotides. While the  $\alpha/\beta$  receptor confers both antigen recognition and MHC restriction (4, 5), no general rules for the relative contribution of specific variable elements to antigen or MHC specificity have emerged. Early work demonstrated that the same V $\alpha$  and V $\beta$  gene segments can encode a receptor with specificity for antigen plus a class II MHC gene product or with specificity for a class I alloantigen (6). The same V $\beta$  domain has been shown to participate in a TCR specific for cytochrome *c*/I-E<sup>k</sup> as well as in a TCR reactive to hen eggwhite lysozyme/I-A<sup>b</sup> (7). On the other hand, surveys of sperm whale myoglobin-reactive T cells (8) and of cytochrome *c*-reactive T cells (9, 10) have been consistent with the idea that the V $\alpha$  gene segment encodes a TCR's antigen specificity while the V $\beta$  domain confers its MHC specificity. However, studies of additional cytochrome *c*-specific T cells have shown that in some cases, the  $\alpha$  chain influences the MHC specificity of a particular TCR (11). Thus, no simple correlation has emerged.

Even though it has been difficult to establish a precise relationship between a V domain and a pattern of MHC restriction, TCR V region domains have been shown to confer an affinity for MHC-encoded gene products. Blackman et al. (12) found that the random pairing of a BW5147-derived  $\alpha$  chain and a  $\beta$  chain derived from a normal T cell generates an MHC-reactive (anti-D<sup>d</sup>) TCR. Kappler et al. (13) have shown that the participation of the V $\beta$ 17a domain in a TCR predisposes to I-E reactivity and that T cells bearing V $\beta$ 17a<sup>+</sup> TCRs are eliminated in I-E<sup>+</sup> strains of mice (14). Two other V $\beta$  elements (V $\beta$ 8.1 and V $\beta$ 6) have been shown to confer broad specificity to the Mls-1a gene product (15–17). We report here the production of an

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mAb, RR3-15, which recognizes TCRs encoded by the  $V\beta 11$  gene segment. Using this antibody, we find that, as with  $V\beta 17a^+$  T cells, T cells that express  $V\beta 11$ -encoded TCRs are eliminated in the thymus of I-E-expressing strains of mice. Surprisingly, it has been difficult to demonstrate reactivity of T cells expressing  $V\beta 11$ -encoded antigen receptors for I-E molecules. Finally, experiments with B6  $\times$  DBA/2 recombinant inbred mice suggest that I-E expression is necessary but not sufficient for the clonal elimination of  $V\beta 11^+$  T cells.

### Materials and Methods

*Mice.* All mice were purchased from The Jackson Laboratories, Bar Harbor, ME, or bred in the facilities of the National Jewish Center for Immunology and Respiratory Medicine, Denver, CO. Fisher rats were purchased from The Jackson Laboratory.

*Cell Lines and Antibodies.* To generate the RR3-15 mAb, a Fisher rat received four bi-weekly immunizations, each consisting of  $2 \times 10^7$  OH6 cells, injected intraperitoneally. The cytotoxic T cell clone OH6 has been described previously (18). It was derived from C57BL/6 and is specific for H-Y in the context of H-2D<sup>b</sup>. 1 mo after the fourth immunization,  $2 \times 10^7$  OH6 cells were injected intravenously, and 3 d later, the rat was killed and its spleen cells were fused to P3X63.Ag8.653 cells using established procedures. The hybridomas were cultured in HAT-containing medium and supernatants from growing hybrids were assayed as described (18). Briefly, culture supernatants were tested for their ability to facilitate lysis of labeled P815 cells by the OH6 T cell clone. Lysis depends on the presence in the culture supernatant of an antibody that can bridge target and killer cells by binding to the Fc receptor on P815 target cells and to the TCR on cytotoxic OH6 cells. In this manner, the mAb RR3-15 was identified.

The OH2 cytotoxic T cell clone and the mAb MR5-2 have been described (18). OH2 is also specific for H-D<sup>b</sup> plus the H-Y antigen. MR5-2 recognizes TCRs containing  $V\beta 8.1$  or 8.2, including the TCR of OH2, which contains  $V\beta 8.1$ . The T cell hybrid KL was obtained by fusing OH6 cells to the C58 rat thymoma as described (19). T cell hybrids K12 and K13 were generated by fusing Con A-stimulated T cell blasts from B10.A(4R) to C58 as above. The resultant hybrids were panned using RR3-15-coated plates, and two hybrids positive for surface staining with RR3-15 were obtained and cloned by limiting dilution.

*Cytotoxicity Assays.* Cytolytic activity was measured by the <sup>51</sup>Cr release assay (20). Briefly,  $10^4$  <sup>51</sup>Cr-labeled P815 cells (100  $\mu$ l) were added to dilutions of CTL cells in V-bottomed microtiter plates (100  $\mu$ l). After incubation at 37°C for 3.5 h, the plates were centrifuged and 100  $\mu$ l of supernatant was collected for measurement of <sup>51</sup>Cr release.

*Radioiodination and Immunoprecipitation.* Cell surface proteins were labeled by lactoperoxidase-catalyzed radioiodination, immunoprecipitated using RR3-15 or RR3-18 and Staphylococcus protein A, and analyzed on one-dimensional SDS polyacrylamide gels under reducing and nonreducing conditions as described (18).

*Hybridization Analyses.* Total cellular RNA was isolated by the method of Cheley and Anderson (21) or by cell lysis in guanidine isothiocyanate and ultracentrifugation over a cesium chloride cushion (22).  $3 \times 10^5$  cell equivalents or 0.5  $\mu$ g of total cellular RNA were blotted onto nitrocellulose filters that were hybridized with <sup>32</sup>P-labeled probes for TCR variable region genes as described (23).

*Immunofluorescent Staining.* T cell hybrids, thymocytes, and nylon wool-nonadherent lymph node cells were analyzed for expression of RR3-15 using indirect immunofluorescent staining. The primary reagent was either culture supernatant or biotinylated RR3-15, and the secondary reagent was fluorescein-conjugated goat anti-rat Fc (Rockland, Inc., Gilbertsville, PA) or phycoerythrin conjugated with streptavidin (Becton Dickinson & Co., Mountain View, CA), respectively. Thymocytes were precultured for 8 h in complete tumor medium (CTM) at  $5 \times 10^6$ /ml to increase the level of TCR expression on the immature, cortical cells (14). Thymocytes were preincubated with 5% normal mouse serum (BALB/c) to reduce background. The level of I-E<sup>d</sup> expression was measured on unfractionated lymph node cells using a biotinylated mAb 14.4.4., followed by phycoerythrin-conjugated streptavidin as above. The

presence of CD4 on T cell hybridomas was determined using the mAb GK1.5, followed by goat anti-rat Fc as above. Samples were analyzed on an Epics C flow cytometer (Coulter Electronics Inc., Hialeah, FL).

*IL-2 Assays.* T cell hybridomas were stimulated with cells or antibodies and the amount of IL-2 released was measured using HT-2 indicator cells, as previously described (24).

## Results

*Generation of the RR-3-15 Antibody.* We were interested in generating an mAb to the antigen receptor on the cytolytic T cell clone OH-6. This clone is specific for the male antigen, H-Y, and is D<sup>b</sup> restricted. Fisher rats were immunized with OH6 cells as described in Materials and Methods and spleen cells were fused with the B cell hybridoma fusion parent, P3X63Ag8.653. The supernatants from HAT-resistant B cell hybridomas were tested for their ability to mediate lysis of labeled P815 cells by cytolytic OH6 T cells, as described previously (18, 25, 26). The principle of this assay is that an anti-TCR mAb can bridge TCR-bearing OH6 cells with Fc receptor-bearing P815 cells and mediate lysis of the P815 cells, even though P815 cells do not bear the nominal antigen recognized by the OH6 cell line. The sensitivity of the assay resides in the fact that anti-TCR and anti-CD3 mAbs are highly efficient at activating the cytolytic T cells to kill P815. Similar activation with anti-Thy-1 mAbs, for example, requires much higher antibody concentrations (data not shown).

Using this assay, we identified a B cell hybridoma producing the mAb RR3-15. This mAb reproducibly mediates the killing of P815 cells by the immunizing clone, OH6, and the specificity of this antibody is demonstrated by the data in Table I. RR3-15 mediates the killing of P815 by OH6 but not by unrelated OH2 cells. On the other hand, the anti-V $\beta$ 8 mAb, MR5-2, mediates the killing of P815 by OH2 but not by OH6 cells. Thus, the RR3-15 antibody is specific for a determinant present on OH6 but absent from OH2 cells. That this antibody recognizes a determinant on the OH6 TCR is shown in Fig. 1. Lysates of radiolabeled surface proteins from OH6 cells were subjected to precipitation with the RR3-15 mAb and Staphylococcus protein A. Precipitated proteins were analyzed by SDS-PAGE and autoradiography. The major precipitated protein under nonreducing conditions (without 2-ME) is

TABLE I  
*Cytolytic Activity of OH2 and OH6 on P815 Cells*

	Antibody	E/T ratio			
		30	10	3	1
OH6	None	2	1	2	2
	MR5-2	1	2	3	2
	RR3-15	56	52	58	40
OH2 (V $\beta$ 8.1)	None	4	4	2	1
	MR5-2	47	37	36	28
	RR3-15	7	6	4	3

OH6 and OH2 are cytotoxic T cell clones specific for H-Y/H-2D<sup>b</sup>. MR5-2 is an mAb specific for TCR variable regions V $\beta$ 8.1 and V $\beta$ 8.2. In the absence of nominal antigen, lysis of P815 cells results from bridging by anti-TCR mAbs of P815 cells via Fc receptors and T cell clones via their TCR.

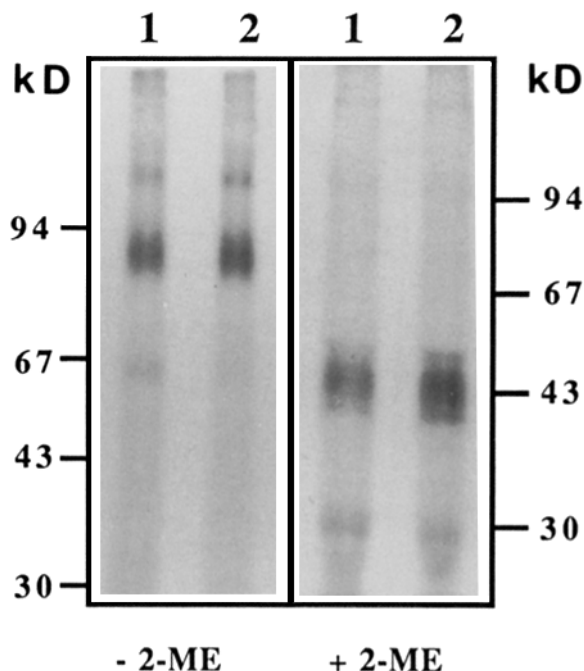


FIGURE 1. RR3-15 precipitates TCR from the T cell clone OH6. SDS-PAGE analysis of surface-labeled proteins immunoprecipitated from the OH6 T cell clone. Lane 1, immunoprecipitation with RR3-15; lane 2, immunoprecipitation with RR3-18, an anticonotypic mAb specific for the TCR of OH6. Gels were run under nonreducing conditions (-2-ME) or under reducing conditions (+2-ME).

a 90-kD species that appears as a 43-kD species under reducing conditions (with 2-ME). This is characteristic of the disulfide-linked heterodimeric, ( $\alpha/\beta$ ) antigen receptor on T cells. The data in Fig. 1 also show that a similar protein can be precipitated from OH-6 cells using an anticonotypic antibody, RR3-18.

*The Determinant Recognized by the RR3-15 mAb Is Encoded by the V $\beta$ 11 Gene Segment.* Cytofluorographic analysis demonstrated that the determinant detected by the RR3-15 mAb is present on  $\sim 5\%$  of C57BL/10 peripheral T cells but on an undetectable fraction of SWR and C57L peripheral T cell (Table II). Since SWR and C57L mice carry an allele of the  $\beta$  chain gene complex that has deleted the V $\beta$ 5, V $\beta$ 8, V $\beta$ 9, V $\beta$ 11, V $\beta$ 12, and V $\beta$ 13 structural genes (27, 28), we considered the possibility that the RR3-15 mAb recognizes a determinant encoded by one of these gene segments. We had available to us a large collection of random T cell hybridomas in which we had determined the expression of particular V $\alpha$  and V $\beta$  gene segments by hybridization analysis of hybridoma RNA (23); the data in the lower portion of Table III demonstrates that none of the 10 hybridomas expressing the V $\beta$ 5, V $\beta$ 8, V $\beta$ 9, V $\beta$ 12, or V $\beta$ 13 gene segments expressed the epitope recognized by the RR3-15 mAb. In contrast, 31 of 39 hybrids that were shown to express V $\beta$ 11 RNA express the RR3-15 determinant. The hybridomas that express the RR3-15 epitope coexpress a variety of different V $\alpha$  gene families. Therefore, it seems likely that the RR3-15 determinant is encoded by the V $\beta$ 11 gene segment, and its expression is not dependent on the presence of particular V $\alpha$ , J $\alpha$ , D $\beta$ , or J $\beta$  gene segments. This is similar to other mAbs with reactivity for other v $\beta$  gene products (13, 16, 17, 26, 29, 30). The eight hybrids expressing V $\beta$ 11 RNA but not the RR3-15 determinant probably

TABLE II  
*Expression of V $\beta$ 11<sup>+</sup> T Cells in Various Mouse Strains*

Strain	H-2				Percent of peripheral T cells expressing V $\beta$ 11
	K	A	E	D	
B10	b	b	-	b	4.6 $\pm$ 0.1
B10.M	f	f	-	f	4.5 $\pm$ 0.1
B10.Q	q	q	-	q	6.3 $\pm$ 0.5
B10.S (7R)	s	s	-	d	3.5 $\pm$ 0.7
B10.D2	d	d	d	d	1.7 $\pm$ 0.8
B10.BR	k	k	k	k	0.7 $\pm$ 0.1
B10.PL	u	u	u	u	0.2 $\pm$ 0.1
C57L	b	b	-	b	0.04 $\pm$ 0.03
SWR	q	q	-	q	0.1
DBA/2	d	d	d	d	1.6 $\pm$ 0.1
CBA/Ca	k	k	k	k	0.5 $\pm$ 0.1
CBA/J	k	k	k	k	1.2 $\pm$ 0.1
C3H/HeJ	k	k	k	k	0.9 $\pm$ 0.1
(B10 $\times$ B10.BR)F <sub>1</sub>	b/k	b/k	b/k	b/k	0.8 $\pm$ 0.1
BDF1	b/d	b/d	b/d	b/d	1.3 $\pm$ 0.1
B10.A (4R)	k	k	-	b	5.0 $\pm$ 0.6
B10.A (2R)	k	k	k	b	0.5 $\pm$ 0.3
B10.A (3R)	b	b	b/k	d	1.57
B10.MBR	b	k	k	q	0.78, 0.59
B10.TL	s	s/k	k	d	0.56
B10.HTT	s	s	s/k	d	0.45

V $\beta$ 11 expression is determined by immunofluorescent staining with RR3-15 as described in Materials and Methods. H-2 genotype for recombinant loci is indicated according to chromosomal position relative to the centromere of chromosome 17 (K, I-A $\beta$ , I-A $\alpha$ , I-E $\beta$ , I-E $\alpha$ , D). For most strains, at least three mice were examined and the mean value  $\pm$  SEM is given. For strains where only one or two mice were examined, the individual values are given.

transcribe rearranged V $\beta$ 11 genes that are out-of-frame. Alternatively, some V $\beta$ 11-encoded  $\beta$  chains may not pair with particular  $\alpha$  chains to form surface heterodimers.

The expression of V $\beta$  gene segments was also determined in several T cell hybrids known to express the RR3-15 epitope. RNA was prepared from a T cell hybridoma (KL) obtained by fusing the immunogen, OH6, with the rat thymoma, C58, and from two other RR3-15<sup>+</sup> T cell hybridomas (K12 and K13). It can be seen in Fig. 2 that all three RR3-15<sup>+</sup> T cell hybrids transcribe the V $\beta$ 11 gene. These data, along with those from Table III demonstrate that all 34 T cell hybridomas expressing a surface molecule detected by the RR3-15 mAb transcribe the V $\beta$ 11 gene. An example of an RR3-15<sup>+</sup> T cell line that does not express V $\beta$ 11 RNA has not yet been found. Since we have not exhaustively examined V $\beta$ 11<sup>+</sup>, RR3-15<sup>-</sup> T cells, the possibility cannot be excluded that the V $\beta$ 11 domain does not express the RR3-15 determinant in the context of certain combinations of V $\alpha$ , J $\alpha$ , D $\beta$ , or J $\beta$  segments. Nevertheless, the simplest interpretation of these data is that the RR3-15 determinant is present on the V $\beta$ 11 gene product and that all V $\beta$ 11-encoded TCRs express this epitope.

TABLE III  
*RR 3-15 Expression Among Selected Hybridomas*

Hybridoma	V $\beta$	V $\alpha$	RR 3-15
1 HB 27	11	3	-
1 HB 36	11	3 and 11	+
1 HB 88	11	3	+
1 HB 133	11	3	-
1 HB 183	11	-	+
1 HB 184	11	5	+
2 HB 49	11	3 and 8	+
3 HB 68	11	-	+
3 HB 120	11	3	+
3 HB 123	11	4 and 13	+
4 HB 32	11	13	+
4 HB 40	11	3	+
4 HB 42	11	8	-
4 HB 56	11	4	+
4 HB 81	11	-	-
4 HB 99	11	10 and 11	+
1 Q 11	11	-	+
1 Q 53	11	3	+
1 Q 59	11	8	-
1 Q 62	11	-	+
2 Q 34	11	2 and 4	-
2 Q 36	11	-	+
3 Q 39	11	-	+
3 Q 96	11	-	+
4 Q 41	11	-	+
5 Q 4	11	2 and 3	+
5 Q 5	11	2	+
5 Q 12	11	13	+
5 Q 65	11	4	+
5 Q 68	11	4	+
5 Q 78	11	3 and 8	-
5 Q 79	11	-	+
5 Q 125	11	-	+
1 BR 92	11	8	+
1 BR 168	11	5 and 13	-
2 BR 118	11	3 and 5	+
4 BR 10	11	3	+
4 BR 25	11	3 and 13	+
4 BR 37	11	10	+
2HB51.8	5	10	-
4HB48.8	5	3 and 11	-
4Q53.5	5	10	-
1Q16.7	5	8	-
18BBM-4	8	-	-
17BBM52	8	3 and 8	-
1HB111	9	3	-
16BBM53	12	8	-
5Q26	13	3	-
2BR51	13	2	-

TCR V region RNA expression was determined by hybridization with <sup>32</sup>P-labeled V-specific probes to V $\beta$ 2-17 and V $\alpha$ 2-8 and V $\alpha$ 10,11, and 13 as described (23). HB, Q, and BR hybrids were derived from Con A-stimulated peripheral T cells from B10, B10.Q, and B10.BR mice, respectively. BBM hybridomas were derived from B10 mice and are I-Abm12 reactive. Hybrids were stained with labeled RR3-15 antibody and analyzed in an Epics C flow cytometer as described in Materials and Methods. For V $\alpha$  expression, (-) indicates that the hybridoma expresses RNA from the V $\alpha$ 1 family or from a V $\alpha$  family for which we have no probe.

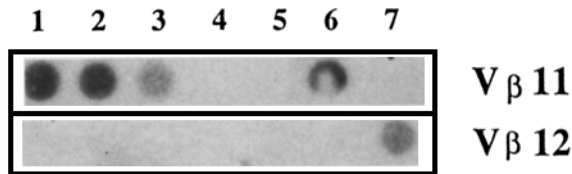


FIGURE 2. RR3-15<sup>+</sup> T cell hybrids express V $\beta$ 11 RNA. (1) KL, RR3-15<sup>+</sup>; (2) KI2, RR3-15<sup>+</sup>; (3) KI3, RR3-15<sup>+</sup>; (4) 4Q53.5, RR3-15<sup>-</sup>; (5) 4HB 48.8, RR3-15<sup>-</sup>; (6) 3HB19-control for V $\beta$ 11; (7) 3HB51 control for V $\beta$ 12. 0.5  $\mu$ g of total cellular RNA from KL, KI2, and KI3 or 3  $\times$  10<sup>3</sup> cell equivalents of total cellular RNA from the other hybrids were blotted onto nitrocellulose filters which were hybridized with <sup>32</sup>P-labeled probes for V $\beta$ 11 and V $\beta$ 12.

*The Frequency of V $\beta$ 11<sup>+</sup> T Cells in Mice Is Dependent on the MHC Haplotype.* To examine the effect of particular MHC haplotypes on the frequency of V $\beta$ 11<sup>+</sup> T cells, we subjected lymph node T cells from a panel of mouse strains to cytofluorographic analysis using the RR3-15 mAb. The data in Table II show that B6 (H-2<sup>b</sup>), B10 (H-2<sup>b</sup>), B10.M (H-2<sup>f</sup>), B10.Q (H-2<sup>q</sup>), and B10.S(7R) (H-2<sup>s/d</sup>) mice express the largest fraction of V $\beta$ 11<sup>+</sup> T cells (3.5–6.3%). On the other hand, the fraction of V $\beta$ 11<sup>+</sup> T cells in B10.D2 (H-2<sup>d</sup>), B10.BR (H-2<sup>k</sup>), and B10.PL (H-2<sup>u</sup>) is significantly lower (0.2–1.7%). Other H-2<sup>d</sup> and H-2<sup>k</sup> strains (DBA/2, CBA/Ca, CBA/J, and C3H/HeJ) have a relatively low frequency of V $\beta$ 11-bearing T cells (0.5–1.6%). The number of V $\beta$ 11<sup>+</sup> T cells in C57L (H-2<sup>b</sup>) and SWR (H-2<sup>q</sup>) mice is negligible (0.04–0.1%). Given that the V $\beta$ 11 gene is deleted from the genome of these two strains, these values probably represent the true background in these cytofluorographic experiments.

Crossing a strain that carries a high frequency of V $\beta$ 11-bearing T cells with a strain that carries a low frequency of V $\beta$ 11-bearing T cells produces F<sub>1</sub> mice that express a low frequency of V $\beta$ 11<sup>+</sup> T cells (see Table II). The frequency of T cells with V $\beta$ 11-encoded TCRs in [B10  $\times$  B10.BR]F<sub>1</sub> mice (0.8%) is similar to that seen in B10.BR mice (0.7%). Similarly, the frequency of V $\beta$ 11<sup>+</sup> T cells in [B6  $\times$  DBA/2]F<sub>1</sub> mice (1.3%) is similar to that seen in DBA/2 mice (1.6%). Thus, the phenotype of infrequent expression of V $\beta$ 11<sup>+</sup> T cells is dominant, relative to the phenotype of frequent expression of V $\beta$ 11<sup>+</sup> T cells in F<sub>1</sub> mice. Considering that the frequency of T cells with V $\beta$ 11-encoded antigen receptors is low in [B10  $\times$  B10.BR]F<sub>1</sub> mice, it is likely that a protein encoded by the MHC of B10.BR (H-2<sup>k</sup>) mice is depressing the level of V $\beta$ 11<sup>+</sup> T cells. To examine this point further, we compared the expression of V $\beta$ 11-bearing T cells in MHC recombinant mice, and the most relevant MHC-recombinant strains are B10.A(4R) and B10.A(2R). The frequency of V $\beta$ 11<sup>+</sup> T cells is high (5.0%) in B10.A(4R) but low (0.5%) in B10.A(2R) mice, yet the only genetic difference between these two strains is that B10.A(2R) mice express an I-E protein while B10.A(4R) mice do not. Thus, it seems likely that the majority of T cells expressing the V $\beta$ 11 domain as part of their surface antigen receptor are clonally deleted in I-E-bearing mice. Consistent with this idea is the fact that the frequency of V $\beta$ 11<sup>+</sup> T cells is generally high in mice that carry I-E<sup>-</sup> haplotypes (H-2b,f,s[7R], and q) but generally low in mice that carry I-E<sup>+</sup> haplotypes (H-2d, -k, and -u).

*Mature V $\beta$ 11<sup>+</sup> Thymocytes Are Absent from I-E-bearing Mice.* We examined the V $\beta$ 11-bearing thymocytes in young adult B10.A(4R) (I-E<sup>-</sup>), B10.A(2R) (I-E<sup>+</sup>), and C57L (V $\beta$ 11 gene-deleted) mice. As shown in Fig. 3, V $\beta$ 11 thymocytes expressing low levels of TCR (RR3-15 dull cells) are present in B10.A(4R) (I-E<sup>-</sup>) and in B10.A(2R) (I-E<sup>+</sup>)

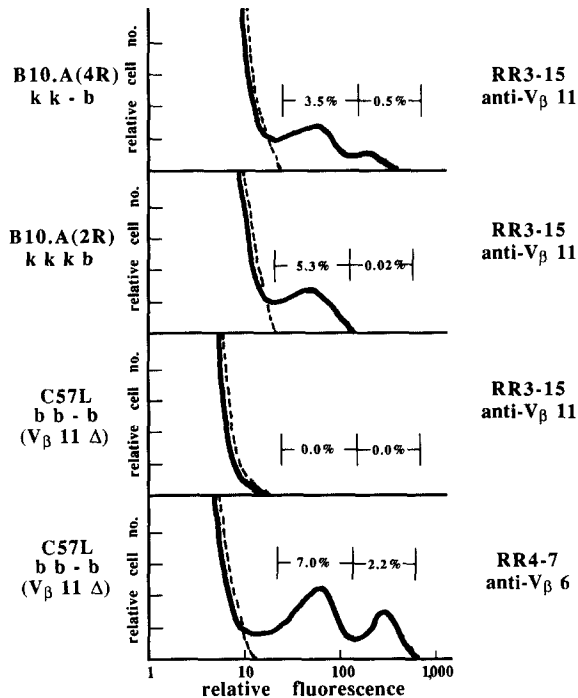


FIGURE 3. Absence of Vβ11 expression among mature but not immature thymocytes in I-E+ mice. Fluorescence histograms of thymocytes from B10.A(2R) and B10.A(4R) or, as a negative control, C57L adult mice. RR3-15 and RR4-7 were biotinylated and phycoerythrin-conjugated streptavidin was used as a secondary staining reagent. Indicated percentages of Vβ11+ T cells were obtained by curve subtraction. Dashed lines indicate staining by secondary reagent alone. Thymocytes were cultured for 8 h before staining to increase the level of TCR on immature cells as described (14).

mice; however, Vβ11+ thymocytes expressing high levels of TCR (RR3-15 bright cells) are present only in B10.A(4R) (I-E<sup>-</sup>) mice. As expected, both RR3-15 dull and RR3-15 bright cells are absent from C57L (Vβ11 gene-deleted) mice. As a control, thymocytes from C57L mice were stained with an mAb, RR4-7 (17), specific for the Vβ6 gene product. Clearly, Vβ6+ thymocytes (TCR dull and bright) are present in this strain. Thymocytes with low levels of surface TCRs are characteristically double positive (CD4<sup>+</sup> and CD8<sup>+</sup>), cortical, and functionally immature, while thymocytes with high levels of surface TCRs are characteristically single positive (CD4<sup>+</sup> or CD8<sup>+</sup>), medullary, and functionally mature (31). Our findings suggest that the presence of I-E prevents the appearance of the majority of Vβ11+ mature thymocytes and Vβ11+ peripheral T cells.

*Most Vβ11+ Hybridomas Are not Reactive with I-E-bearing Splenocytes.* Since I-E mediates the clonal deletion of Vβ11+ T cells, we have studied the reactivities of 25 T cell hybridomas that express Vβ11-encoded surface receptors. These hybrids were stimulated with I-E<sup>k</sup>-bearing splenocytes or CH12 (I-E<sup>k</sup>) lymphoma cells; surprisingly, we found only three (12%) that could be stimulated to produce IL-2. That all 25 hybrids were capable of IL-2 production was demonstrated by their response to stimulation with the anti-Vβ11 antibody, RR3-15 (data not shown). Since it is possible that the I-E reactivity of Vβ11+ hybridomas is dependent on the presence of surface CD4 molecules, we have studied a set of six Vβ11+ hybrids that express CD4 surface molecules (see Table IV). All six hybridomas secrete IL-2 when stimulated with a crosslinked form of the anti-TCR antibody, RR3-15, yet only one hybrid (5Q5) can be strongly stimulated with I-E-bearing splenocytes from B10.BR (I-E<sup>k</sup>)



TABLE IV  
Specificity of CD4<sup>+</sup> V $\beta$ 11<sup>+</sup> T Cell Hybridomas

Hybridoma	IL-2 released when stimulated with:			Surface expression	
	RR3-15 (anti-V $\beta$ 11)	B10.BR(H-2 <sup>k</sup> ) splenocytes	B10.D2(H-2 <sup>d</sup> ) splenocytes	V $\beta$ 11	CD4
	<i>U</i>				
1HB36	640	<10	<10	+	+
1HB184	160	<10	<10	+	+
3HB123	320	<10	10	+	+
4HB56	320	<10	10	+	+
5Q4	80	10	10	+	+
5Q5	640	160	20	+	+

or B10.D2 (I-E<sup>d</sup>) mice. Given the clonal deletion of  $\sim 90\%$  of V $\beta$ 11<sup>+</sup> T cells from I-E<sup>k</sup>-bearing mice (see Table II and Fig. 3), the rarity of V $\beta$ 11<sup>+</sup> hybridomas reactive with I-E<sup>k</sup>-bearing splenocytes seems paradoxical.

*The MHC Molecule I-E Is Necessary but not Sufficient to Mediate the Clonal Deletion of V $\beta$ 11-bearing T Cells.* To confirm that the regulation of the number of V $\beta$ 11<sup>+</sup> T cells is mediated by MHC-encoded proteins, the frequency of V $\beta$ 11<sup>+</sup> cells was determined in 23 C57BL/6  $\times$  DBA/2 (BXD) recombinant inbred (RI)<sup>1</sup> strains. All loci in these strains are either B6 or DBA/2 in origin; consequently, the MHC in each of these strains is either H-2<sup>b</sup> (B6-derived) or H-2<sup>d</sup> (DBA/2 derived). The data in Fig. 4 show the frequency of V $\beta$ 11<sup>+</sup> T cells in these RI strains. The level of V $\beta$ 11-bearing T cells in each strain is quite reproducible in that the SEM of these measurements is small relative to the total fraction of V $\beta$ 11<sup>+</sup> cells. Thus, the frequency of V $\beta$ 11<sup>+</sup> T cells represents a reproducible phenotype in each of these strains. Considering the H-2<sup>b</sup> (I-E<sup>-</sup>) strains, the frequency of V $\beta$ 11-bearing cells is high ( $\sim 5.5\%$ ), consistent with the absence of clonal deletion in I-E<sup>-</sup> mice. On the other

<sup>1</sup> Abbreviations used in this paper: RI, recombinant inbred.

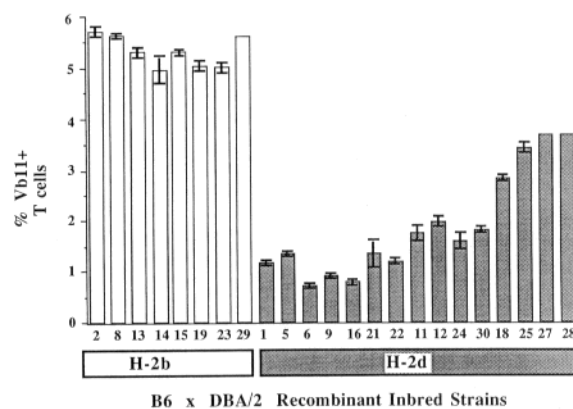


FIGURE 4. Frequency of V $\beta$ 11-bearing T cells in 23 strains of C57BL/6  $\times$  DBA/2 RI mice. The frequency of V $\beta$ 11<sup>+</sup> T cells was determined as described in the legend to Fig. 3. For most of the strains, the values in the figure represent the average of at least three animals. The SEM is indicated by the error bars. For strains 27, 28, and 29, only two animals were analyzed, and consequently, the SEM could not be calculated. The frequency of V $\beta$ 11<sup>+</sup> T cells was 3.2%, 3.7% for strain 27; 3.6%, 3.8% for strain 28; and 5.4%, 5.9% for strain 29. C57BL/6, DBA/2, and (C57BL/6  $\times$  DBA/2)F<sub>1</sub> mice carry  $5.6 \pm 0.2\%$ ,  $1.6 \pm 0.1\%$ , and  $1.3 \pm 0.1\%$  V $\beta$ 11<sup>+</sup> T cells, respectively.

hand, the frequency of  $V\beta 11^+$  T cells varies considerably among the H-2<sup>d</sup> (I-E<sup>+</sup>) strains. Some I-E<sup>+</sup> strains (Nos. 6, 9, and 16) carry a small fraction ( $\sim 0.8\%$ ) of  $V\beta 11^+$  T cells, while other I-E<sup>+</sup> strains (Nos. 25, 27, and 28) carry a much larger fraction ( $\sim 3.5\%$ ) of  $V\beta 11^+$  T cells. To rule out the possibility that these differences are due to quantitative differences in the degree of I-E expression between these strains, the amount of the I-E gene product was measured on lymph node B cells from several RI strains (Nos. 6, 9, 11, 18, 21, 24, and 25) that carry different levels of  $V\beta 11^+$  T cells (data not shown). The amount of I-E expressed on the surface of B cells in several of these RI strains is virtually identical. Thus, the variation in the frequency of  $V\beta 11^+$  T cells among I-E<sup>+</sup>, BXD RI strains cannot be explained by quantitative differences in the expression of the I-E gene product.

### Discussion

We have identified an mAb that recognizes a determinant on all or nearly all T cells expressing the  $V\beta 11$  domain as part of the TCR. Based on the following criteria, the antibody, RR3-15, recognizes an epitope encoded by the  $V\beta 11$  gene segment. RR3-15 precipitates a 90-kD heterodimer, characteristic of the TCR, from the immunizing T cell clone OH6. RR3-15 mediates the lysis of P815 cells by OH6 ( $V\beta 11^+$ ) but not by another ( $V\beta 8.1^+$ ) T cell clone, OH2. Of 34 T cell hybridomas that express the RR3-15 determinant all transcribe the  $V\beta 11$  gene segment (Table III and Fig. 2). RR3-15<sup>+</sup> T cells are undetectable in SWR and C57L mice (see Table II); these strains carry an allele of the  $\beta$  chain gene complex that has deleted the  $V\beta 11$  structural gene (27, 28). Of 10 T cell hybrids expressing  $V\beta 5$ ,  $V\beta 8$ ,  $V\beta 9$ ,  $V\beta 12$ , or  $V\beta 13$  RNA, none express a surface molecule recognized by the RR3-15 mAb. This is notable since SWR and C57L mice are deleted for these  $V\beta$  genes as well. Taken together, these data strongly suggest that the TCR determinant recognized by the mAb, RR3-15, is part of the  $V\beta 11$  domain. It is expected that an epitope encoded by a single  $V\beta$  gene would be present on  $\sim 5\%$  of T cells as there are 21 functional  $V\beta$  gene segments in mice. Since  $\sim 5\%$  of T cells from B10, B10.Q, and B10.M mice are RR3-15<sup>+</sup>, it seems likely that expression of  $V\beta 11$  gene is both necessary and sufficient for the expression this epitope. However, we can not rule out the possibility that some combinations of  $V\alpha$ ,  $J\alpha$ ,  $D\beta$ , and  $J\beta$  genes segments do not permit the expression of the RR3-15 determinant on a few  $V\beta 11$ -encoded TCRs.

The most striking aspect of our findings is that a majority of T cells with  $V\beta 11$ -encoded antigen-receptors are clonally deleted from I-E-bearing mice. In general, the frequency of  $V\beta 11^+$  T cells is much lower in I-E-bearing mice than it is in strains that do not express a functional I-E protein (see Table II). The most dramatic example of this effect can be seen when comparing the frequency of  $V\beta 11^+$  T cells in B10.A(4R) and B10.A(2R) mice. These two strains share the same genetic background (B10) and the same H-2K, H-2I, and H-2D genes; however, they differ in their expression of the I-E gene product (32). B10.A(4R) mice are genetically defective in their expression of an I-E gene product and 5% of their T cells express a  $V\beta 11$ -encoded TCR. On the other hand, B10.A(2R) mice express the I-E gene product but only 0.5% of their T cells express a  $V\beta 11$ -encoded TCR (see Table II). Thus, the frequency of  $V\beta 11^+$  T cells is inversely correlated with the presence of an I-E molecule. The effect of I-E is genetically dominant in that F<sub>1</sub> mice derived from I-E-expressing and I-E-nonexpressing parents express I-E and clonally delete the

majority of their  $V\beta 11^+$  T cells (see Table II). A comparison of the frequency of  $V\beta 11^+$  T cells in B10.D2(I-E<sup>d</sup>), B10.BR(I-E<sup>k</sup>), B10.PL(I-E<sup>u</sup>), B10.A(3R)(I-E<sup>b</sup>), and B10.HTT(I-E<sup>s</sup>) mice indicates that most if not all alleles of the I-E locus are capable (to varying degrees) of deleting  $V\beta 11^+$  T cells from the repertoire. The elimination of  $V\beta 11^+$  T cells encompasses both the CD4<sup>+</sup> and CD8<sup>+</sup> subsets, though the elimination of CD4<sup>+</sup> cells is more dramatic (data not shown). Two closely related but I-E-disparate strains, B10.A(2R) (I-E<sup>+</sup>) and B10.A(4R) (I-E<sup>-</sup>), were examined for the expression of  $V\beta 11$  on both mature and immature thymocytes (Fig. 3). Both strains contained immature thymocytes expressing the  $V\beta 11$  domain; however, in B10.A (4R) (I-E<sup>-</sup>) but not in B10.A(2R) (I-E<sup>+</sup>) mice, there were thymocytes expressing high density  $V\beta 11^+$  TCRs. Thus, the clonal deletion of  $V\beta 11^+$  T cells extends to mature thymocytes in I-E<sup>+</sup> mice.

These findings are remarkably similar to those reported to Kappler et al. (13, 14). They have determined that T cells bearing antigen receptors encoded by the  $V\beta 17a$  gene segment are clonally deleted from I-E-bearing mice. They have further demonstrated that mature (high density TCR)  $V\beta 17a^+$  thymocytes are absent from strains expressing I-E and that all I-E alleles examined mediate the clonal elimination of  $V\beta 17a^+$  T cells (14). These properties apply to  $V\beta 11^+$  T cells as well. Given that all allelic forms of the I-E protein mediate the clonal deletion of both  $V\beta 11^+$  and  $V\beta 17a^+$  T cells, it seems likely that these cells recognize an epitope that is common (public determinant) to all polymorphic forms of I-E. However, allelic differences between I-E molecules may determine the extent of clonal deletion. For example, I-E<sup>k</sup> deletes a larger fraction of  $V\beta 11^+$  and  $V\beta 17a^+$  T cells than does I-E<sup>d</sup> (Table II and reference 33).

One striking difference between  $V\beta 11$  and  $V\beta 17a$ -encoded TCRs is that a large fraction (~90%) of  $V\beta 17a^+$  hybridomas are strongly reactive to I-E when stimulated with I-E-bearing splenocytes or lymphomas (13). We have examined 25  $V\beta 11^+$  hybrids for their reactivity to I-E when stimulated with B10.BR (H-2<sup>k</sup>) spleen cells or CH12(H-2<sup>k</sup>) lymphoma cells and found only three potentially I-E-responsive hybridomas (data not shown). Since all 25 hybrids respond when their antigen receptors are crosslinked with the anti- $V\beta 11$  reagent, RR3-15, these hybrids have  $V\beta 11^+$  surface TCRs and are capable of secreting IL-2. Furthermore, we have examined six of these hybrids that express CD4 surface molecules and found only one to be potentially I-E reactive (see Table IV). Thus, it is difficult to argue that the absence of CD4 accessory molecules accounts for the lack of pronounced I-E recognition in most of these  $V\beta 11^+$  hybrids. The explanation for the lack of I-E recognition by hybrids bearing  $V\beta 11$ -encoded TCRs may be trivial. It is conceivable that the affinity of a  $V\beta 11^+$  TCR for the I-E ligand is low (accounting for the lack of I-E reactivity among  $V\beta 11^+$  hybrids) but that the in vivo clonal deletion of  $V\beta 11^+$  T cells requires only a weak interaction between the TCR and I-E. A second, perhaps more intriguing, hypothesis to explain this discrepancy is that splenocytes, although I-E bearing, do not present the actual ligand for  $V\beta 11$ -encoded TCRs and are not the tolerogenic cells in the mouse. This could be the case, if the actual ligand for  $V\beta 11^+$  T cells is an I-E molecule plus a self-peptide. This protein fragment may not be produced by splenocytes but is expressed by other I-E-bearing cells that mediate the clonal deletion. This idea has been proposed recently (34) and experiments are underway to resolve this issue.

The idea that the tolerizing ligand(s) for  $V\beta 11^+$  cells is an I-E/self-peptide complex is further supported by experiments examining the frequency of  $V\beta 11^+$  cells in C57BL/6  $\times$  DBA/2 (B $\times$ D) RI mice (see Fig. 4). All RI strains that carry the H-2<sup>b</sup> (I-E<sup>-</sup>) haplotype have a full complement of  $V\beta 11^+$  T cells, demonstrating the lack of clonal deletion in the absence of I-E expression. On the other hand, the degree of clonal deletion of  $V\beta 11$ -bearing T cells is not equivalent in all H-2<sup>d</sup>(I-E<sup>+</sup>), B $\times$ D RI strains even though the magnitude of I-E expression among these H-2<sup>d</sup> strains is similar. Some RI strains (Nos. 6, 9, and 16) show a strong deletion of  $V\beta 11^+$  T cells, while other RI strains (Nos. 18, 25, 27, and 28) display a much weaker elimination of  $V\beta 11$ -bearing T cells (see Fig. 4). Other RI strains display intermediate levels of clonal deletion. Taken together, these results demonstrate that expression of the class II molecule, I-E, is necessary but not sufficient to mediate the clonal deletion of  $V\beta 11^+$  T cells. It is likely that some genes in the B6 genetic background and other genes in the DBA/2 genetic background complement the I-E genes to mediate the clonal deletion of  $V\beta 11^+$  T cells. Furthermore, it is the segregation of these genes that most likely accounts for the varied extent of clonal deletion among these B $\times$ D RI strains. These background genes possibly encode self-proteins that generate peptides with I-E-binding properties. The implication of these findings is that the products of genes other than I-E control the extent of clonal deletion.

Given the plethora of  $V\beta$  domains that impart reactivity to I-E, Mls-1 or Mls-2 gene products (13, 15, 16, 17, 30), we have wondered whether  $V\beta$  or  $V\alpha$  domains exist that confer a generic reactivity to H-2 K, I-A, or D glycoproteins. In retrospect, it has been relatively easy to identify TCRs that are clonally deleted in the presence of I-E proteins because of the existence of I-E-expressing and nonexpressing strains of mice. Since no strains have been described that are nonexpressors of the H-2 K, I-A, or D gene products, it is experimentally difficult to identify these kinds of TCR domains. Nevertheless, it seems unlikely that V region domains exist that are pan-reactive with H-2 K, I-A, or D gene products because of enormous pressure against maintaining such V genes in the germline. A V domain that imparts reactivity for a gene product expressed in every member of a species and somatically eliminated from the repertoire would offer no protective value and be of no advantage to the organism nor to the species. In this context, it is conceivable that the  $V\beta 11$ ,  $V\beta 17$ ,  $V\beta 8.1$ ,  $V\beta 6$ , and  $V\beta 3$  TCR domains have evolved to have a high affinity for their ligands (I-E, Mls-1, Mls-2), because these ligands are not ubiquitously expressed in all mice.

The selective pressure to maintain V genes encoding self-reactive domains should be weak or nonexistent and the sequence of these genes might drift resulting in the appearance of pseudogenes. The  $V\beta 17b$  gene provides an illustration of this point. This  $V\beta 17$  allele carried by most strains of inbred mice is nonfunctional due to the presence of a termination signal at codon 88 (35). Since  $V\beta 17a$ -encoded receptors are presumably eliminated from a large proportion of outbred mice, the selective pressure to maintain this  $V\beta$  gene is weak. Thus, it is not surprising that one of the  $V\beta 17$  alleles has drifted and become a pseudogene.

We have previously observed a decrease in the frequency of  $V\beta 11^+$  T cells from B10.BR (I-E<sup>+</sup>) mice. By analyzing the RNA from large panels of random T cell hybridomas for the expression of 16  $V\beta$  and 10  $V\alpha$  gene families (23), we determined the frequency with which these V gene families are expressed among T cells from

three MHC congenic strains. From this analysis, we noticed a statistically significant decrease in the frequency of V $\beta$ 11-expressing hybrids in B10.BR (I-E<sup>+</sup>) compared with B10 (I-E<sup>-</sup>) and B10.Q(I-E<sup>-</sup>) mice. The cytofluorographic experiments described here with the anti-V $\beta$ 11 reagent, RR3-15, have independently confirmed our previous observations.

Our analysis of V gene expression among MHC congenic strains (23) also reveals a low frequency of T cells expressing the V $\beta$ 5.1, V $\beta$ 5.2, and V $\beta$ 12 gene segments in B10.BR (I-E<sup>+</sup>) mice relative to B10 (I-E<sup>-</sup>) and B10.Q(I-E<sup>-</sup>) mice. It is at least conceivable that T cells bearing these V $\beta$  domains are clonally deleted from I-E<sup>+</sup> mice as well. Thus, an I-E-bearing mouse may lose up to 20% (4 of 20 V $\beta$  domains) of its TCR repertoire due to the somatic deletion of I-E-reactive T cells. While this is costly, in terms of the size of the repertoire, a mouse that expresses I-E has another MHC protein that can present antigens and can positively select (I-E-restricted) T cells. On balance, I-E<sup>+</sup> and I-E<sup>-</sup> haplotypes may generate equally protective cell-mediated immunity.

### Summary

We have generated an mAb, RR3-15, that recognizes murine TCRs containing the V $\beta$ 11 domain. Using this antibody to stain peripheral T cells, we have demonstrated that V $\beta$ 11-bearing T cells are largely absent from strains of mice that express the class II MHC molecule, I-E. Studies with F<sub>1</sub> mice demonstrate that this effect is dominant, consistent with tolerance. The clonal deletion of V $\beta$ 11-bearing T cells appears to occur intrathymically, as immature but not mature V $\beta$ 11<sup>+</sup> T cells are present in the thymus of I-E-bearing mice. Examination of B6  $\times$  DBA/2 recombinant inbred strains demonstrates that the expression of I-E molecules is necessary for the clonal deletion of V $\beta$ 11-bearing T cells, but that other non-MHC genes control the clonal deletion process, as well. Paradoxically, only a small fraction of V $\beta$ 11<sup>+</sup> T cell hybridomas are I-E reactive.

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