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Thymic selection defines multiple T cell receptor Vβ 'repertoire phenotypes' at the CD4/CD8 subset level

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We describe here the use of a sensitive and accurate multiprobe Vβ RNase protection assay in characterizing the expression levels of 17 Vβ genes in separated CD4$^+$ and CD8$^+$ subsets of selected mouse strains. The IE-reactive Vβ genes (Vβs 11, 12, 5.1 and 16) showed various patterns of skewed subset expression in different strains, suggesting additional influences of IA, class I, and non-MHC genes in the selection process. Clonal deletion of Vβ11- and Vβ12-bearing T cells, among others, was skewed strongly towards the CD4$^+$ subset in many IE$^+$ mouse strains, supporting the notion that negative selection can cause incomplete, subset biased, Vβ clonal deletions. Broad analysis in separated CD4$^+$ and CD8$^+$ subsets gave improved resolution of Vβ repertoire selection, and revealed significant strain and/or subset specific skewing for additional Vβ genes; with consistent bias towards higher expression of Vβ7 and Vβ13 in the CD8$^+$ subset, and Vβ15 in the CD4$^+$ subset of most mouse strains. The influence of diverse non-MHC ligands in Vβ repertoire selection was further illustrated by the identification of unique Vβ repertoires for six different MHC-identical (H2^k) strains. Such polymorphisms in TCR repertoire expression may help to define better disease susceptibility phenotypes.

Key words: RNase protection assay/T cell receptor/thymic selection/tolerance

Introduction

The antigen specific T cell receptor (TCR) consists of a 90 kd heterodimeric glycoprotein composed of an α- and a β-chain, each encoded by variable (V, D and J) gene elements that rearrange during early thymic development (Wilson *et al.*, 1988; Strominger, 1989). Pairing and cell surface expression of polymorphic α- and β-chains first occurs in immature cortical thymocytes at the CD4$^+$8$^+$ (double positive) stage of differentiation (Fowlkes and Pardoll, 1989), producing a diverse TCR repertoire. In a process unique to T cell development, this repertoire is subsequently modified in the thymus by both positive and negative selection. Specifically, negative selection eliminates self-reactive T cells from the repertoire, and positive selection is the process whereby T cells are selected for maturation only if their TCRs preferentially recognize antigen in association with self-MHC molecules. Positive selection also directs thymocyte maturation into CD8$^+$4$^-$ or CD4$^+$8$^-$ subsets, depending on whether interaction of the TCR is with MHC class I or class II antigen presenting molecules, respectively (Teh *et al.*, 1988; Sha *et al.*, 1988a,b; Kisielow *et al.*, 1988a).

Previous studies using Vβ specific monoclonal antibodies (mAb) have demonstrated correlations between Vβ gene usage and reactivity with particular MHC molecules (or MHC−ligand complexes) during thymic selection. Using mAb KJ23, Kappler *et al.* (1987a) found that Vβ17a TCRs react preferentially with IE molecules, and that negative selection in IE$^+$ mice results in the clonal deletion of Vβ17a-bearing T cells (Kappler *et al.*, 1987b). Similarly, other mAb studies have shown that products of the *Mls*^{a} locus cause tolerance related clonal deletion of Vβ6- (MacDonald *et al.*, 1988a) and Vβ8.1- (Kappler *et al.*, 1988) bearing T cells; *Mls*^{c} products deletion of Vβ3.1-bearing T cells (Pullen *et al.*, 1988); and IE plus undefined antigen(s), the clonal deletion of Vβ11-bearing T cells (Bill *et al.*, 1989). More recently, similar correlations have also been shown for positive selection, in the case of Vβ6-bearing CD4$^+$ T cells in mice expressing certain H2 alleles (MacDonald *et al.*, 1988b), for Vβ17a-bearing CD8$^+$ T cells by K^s molecules (Zuniga-Pflucker *et al.*, 1989) and CD4$^+$ T cells by IA^q molecules (Blackman *et al.*, 1989) and for Vβ14-bearing CD8$^+$ T cells by K^k molecules (Liao *et al.*, 1989).

While the available Vβ specific monoclonal antibodies have proved invaluable for correlations of Vβ gene usage and thymic selection, such analysis can be more comprehensively carried out at the RNA level by molecular hybridization with Vβ probes. Both Northern blotting (Vacchio and Hodes, 1989) and RNase protection analysis (Okada and Weissman, 1989) have been used successfully in studies of overall Vβ gene usage in unseparated T cell populations. Such studies have increased the reported number of IE related Vβ clonal deletions to include, in addition to Vβ11, Vβs 5, 12 and 16 (Okada and Weissman, 1989; Vacchio and Hodes, 1989); and they have also revealed clonal deletion of Vβ9 in *Mls*^{a} mice (Happ *et al.*, 1989) and Vβ7 in certain *Mls*-expressing strains (Vacchio and Hodes, 1989). TCR repertoire selection and expression may significantly influence immune responses, including the susceptibility to autoimmune diseases. Since CD4$^+$ and CD8$^+$ T cells are independently selected by different MHC molecules and, presumably, different non-MHC ligands, we have undertaken to characterize more completely the TCR Vβ repertoire expression at the CD4/CD8 subset level, using an accurate and sensitive multiprobe Vβ RNase protection assay (Okada and Weissman, 1989; Theofilopoulos *et al.*, 1989).

Results

Multiprobe Vβ RNase protection assay

The multiprobe Vβ RNase protection assay quantitates Vβ mRNA species by means of saturation hybridization to

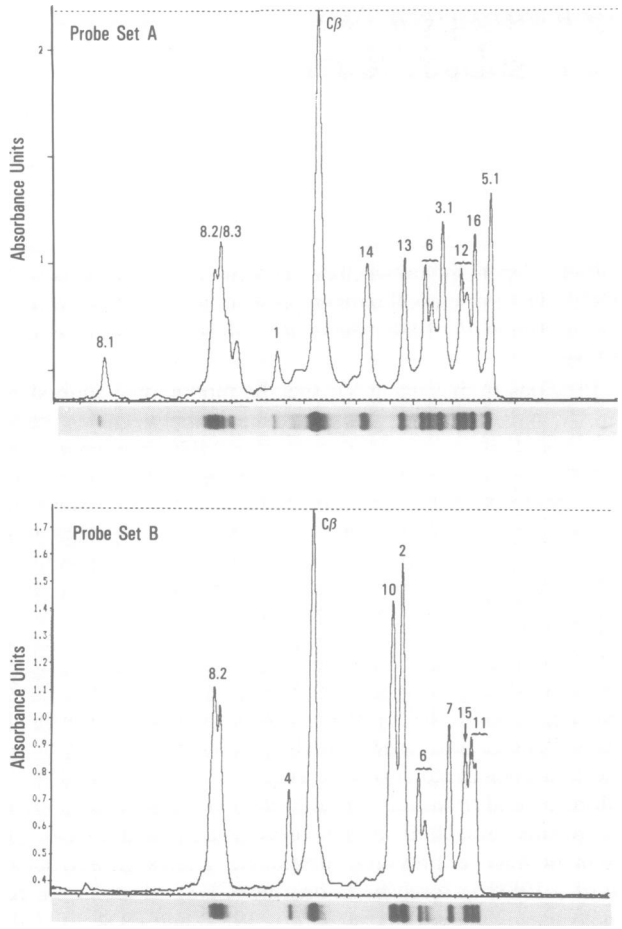

Fig. 1. RNase protection analysis of 17 different Vβ gene transcripts in two probe sets. Shown are the autoradiographs of protected probe fragments and corresponding densitometric tracings obtained on analysis of 2 μg total RNA from immature $CD4^+8^+$ thymocytes of C57BL/6 ($V\beta^b$ haplotype) mice. Vβ designations are according to established nomenclature (Wilson *et al.*, 1988) and are indicated above peaks.

specific labeled Vβ probes, which are then resolved on polyacrylamide gels, autoradiographed and analyzed for signal strength by densitometry. Our assay system (Figure 1) consists of probes for 17 Vβ genes [Vβs 5.2, 9, 17 and Vβ19 (Louie *et al.*, 1989) are missing] combined into two probe sets (A and B), based on extensive testing to determine the best probe lengths and combinations. As shown, analysis of unselected $CD4^+8^+$ thymocytes from the $V\beta^b$ haplotype C57BL/6 strain revealed all of the expected protected probe bands corresponding to the known Vβ genes (Wilson *et al.*, 1988).

To test the reproducibility and linearity of the assay, titrations were carried out using admixed RNA samples constructed so that the levels of individual Vβ mRNA species either remained constant, or changed linearly over the range of values expected in normal TCR repertoire analysis. A representative experiment (Figure 2) shows the triplicate analysis of C57BL/6 thymocyte RNA aliquots, using probe set B (Vβs 11, 15, 7, 6, 2, 10, 4 and 8.2) to which were added increasing amounts of a mixture of T cell line derived RNA containing Vβ4, 6 and 15 mRNAs. The autoradiographic results (Figure 2A) were quantitated by densitometric scanning (not shown), and the peak heights for each Vβ plotted against the amount of added cell line RNA (Figure 2B). As illustrated, the expected titration results were obtained with a high degree of reproducibility (SEM values of $\pm 5-10\%$) and linearity over the entire range measured.

Vβ mRNA levels in unselected thymocytes and splenic T cells of representative Mls-expressing strains

To test the ability of our protection assay to detect well characterized Vβ clonal deletions, and to provide baseline Vβ expression values for comparative purposes, analyses of unselected double positive thymocytes of $V\beta^b$ haplotype C57BL/6 mice and of peripheral total T cells from representative *Mls*-expressing mice, were quantitated (Table I). In the unselected repertoire, we found that Vβ genes are expressed unequally, ranging from a low of 1.5% for Vβ9 to a high of 15.1% for Vβ8.2. Similar results have been reported by others (Okada and Weissman, 1989). Analysis of two other $V\beta^b$ haplotype strains, C3H/HeJ and AKR/J, gave comparable percentages (data not shown), consistent with the uniform expression levels expected prior to thymic selection.

In the case of *Mls*-expressing strains, all known Vβ clonal deletions were readily detected in peripheral T cells by protection assay, as reported previously (Theofilopoulos *et al.*, 1989): i.e. Vβs 6, 8.1 and 9 for Mls^a, and Vβ3.1 for Mls^c, and all these Vβs for Mls^d mice. Compared with the Vβ levels in unselected $CD4^+8^+$ thymocytes, the data with peripheral T cells also revealed significant variations in the expression levels of most other Vβ genes (see below), indicating that Vβ gene usage levels might be responding to numerous distinct repertoire selection phenomena. Since $CD4^+$ and $CD8^+$ T cells are independently selected by different MHC molecules and, presumably, different non-MHC ligands, we initiated a survey of separated $CD4^+$ and $CD8^+$ subsets of > 25 mouse strains, anticipating that such analysis would provide better definition of Vβ selection phenomena. The principal patterns observed were largely represented in sets of B10 and A strain congenic mice, together with a panel of strains of various genetic backgrounds, described below.

CD4/CD8 subset bias and strain distribution of the IE dependent Vβ11 and Vβ12 clonal deletions

Previous investigation of the frequency of Vβ11-bearing thymocytes in IE^+ mice (Bill *et al.*, 1989) provided the first example of a tolerance related Vβ clonal deletion that was clearly only partial in certain stains, among which were B10 congenic mice expressing the IE^d allele (Bill *et al.*, 1989) as well as DBA/2 mice (IE^d) (Bill *et al.*, 1989) and A/J mice (IE^k) (Tomonari and Lovering, 1988; Vacchio and Hodes, 1989). To characterize Vβ11 expression further in these and other strains, we analyzed separated $CD4^+$ and $CD8^+$ T cells and calculated the percentage of Vβ11 mRNA present. The results (Figure 3A) revealed a common pattern of nearly complete Vβ11 deletion in the $CD4^+$ subset, but variable and incomplete deletion in the $CD8^+$ subset, seen in the IE^k strains CBA/Ca, C3H, AKR and CBA/J mice, as well as in IE^d B10.BDR2 mice. This pattern explains the partial nature of Vβ11 clonal deletion in these mice and, together with similar observations by others analyzing Vβ17a expression in different IE^+ haplotypes (Kappler *et al.*, 1989) appears to document a novel phenomenon associated with some tolerance related Vβ clonal deletions, i.e. that of variable and incomplete

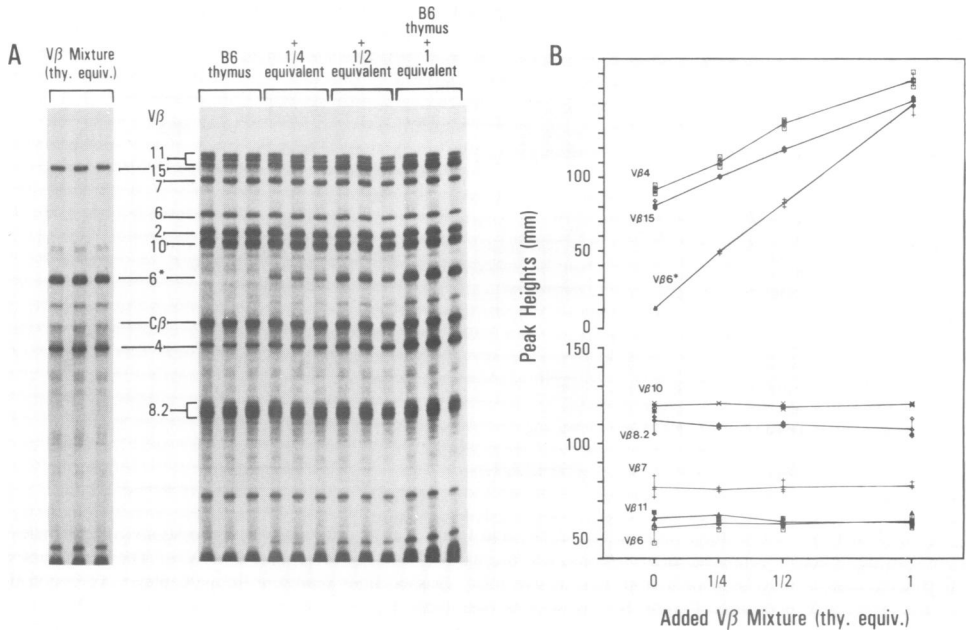

Fig. 2. Reproducibility and dose response of RNase protection assay. (**A**) Autoradiograph showing triplicate analysis of 2 μg C57BL/6 thymocyte RNA with increasing amounts (**right panel**) of admixed RNA sample consisting of cell line derived Vβs 4, 6* and 15 transcripts (**left panel**). Vβ6* refers to the allelic form expressed in the corresponding SJL cell line, which gives a different protected band from that of C57BL/6 derived Vβ6. 'Thy equivalent' refers to the approximate amount of mixture giving a signal for each Vβ in the range of that expected from normal thymocyte RNA. (**B**) $X-Y$ plot of peak heights for each Vβ analyzed in (A) (densitometric profiles not shown).

cross- (or opposite-) subset deletion. In contrast, certain strains in our study, e.g. B10.A(2R) and MRL/n, were found to delete Vβ11-bearing T cells efficiently in both $CD4^+$ and $CD8^+$ subsets. As expected, IE^- strains of the C57BL background failed to delete Vβ11-bearing cells.

Vβ11 expression in A strain mice was also found to be somewhat reduced, in agreement with others (Vacchio and Hodes, 1989); however this reduction did not display the same CD4/CD8 subset pattern as described above (Figure 3A). That is, the characteristic finding of nearly complete deletion in the $CD4^+$ subset was not observed; instead, variable and generally modest reductions in the $CD4^+$ and $CD8^+$ subsets accounted for the lower overall Vβ11 levels detected. Moreover, in contrast to the situation in B10 congenic mice, analysis of IE^+/IE^- semi-congenic A-strain mice, A.TL (*skkd*) and A.TH (*ss.d*), revealed no major effect of IE positivity on Vβ11 levels in the $CD4^+$ subset. Another strain, DBA/2 (*dddd*), also reported to have reduced overall Vβ11 levels (Bill *et al.*, 1989), did not show significant reduction in our study in either the $CD4^+$ or $CD8^+$ subset (Figure 3A). Since efficient $CD4^+$ subset deletion, in particular, was not observed, we conclude that A-strain and DBA/2 mice probably lack the putative non-MHC ligand(s) responsible for IE mediated Vβ11 clonal deletion.

In addition to the IE related clonal deletion of Vβ11 in many strains, we observed an effect in A-strain congenic mice suggesting differential Vβ11 selection by class I MHC genes as well. Thus, A.AL (*kkkd*) mice showed reproducibly higher levels of Vβ11 in the $CD8^+$ subset than the congenic A.TL mice (*skkd*), consistent with either more efficient positive selection by K^k or negative selection by K^s. Although evidence of dominance in F_1 mice is required to differentiate formally positive from negative selection, it seems likely that positive selection is involved here, since Vβ11 levels in both strains are quite high relative to the unselected Vβ11 level. Finally, expression of Vβ12 in B10 and A-strain mice (Figure 3B) was found to follow closely the pattern described for Vβ11; in partial, but not complete, agreement with the results of others (Vacchio and Hodes, 1989) (see Discussion). It should be noted, however, that in several other strains tested, the $CD4^+$ subset deletion

Table I. Frequency of Vβ gene expression in unselected $CD4^+8^+$ thymocytes and in peripheral T cells from *Mls*-expressing mice[1]

Vβ gene	Unselected thymocytes[2] ($CD4^+8^+$)	Peripheral T cells[3]			
		Mls^b	*Mls*^{a}	*Mls*^{c}	*Mls*^{d}
1	5.1	2.7	3.6	3.4	3.9
2	6.3	8.2	10.6	8.7	10.5
3.1	2.8	2.3	9.7	0.2	0.4
4	3.2	2.2	3.8	1.9	4.3
5.1	3.8	1.9	1.0	0.9	0.9
6	4.2	3.6	0.3	7.6	0.4
7	3.2	2.6	1.1	4.4	2.2
8.1	6.9	11.0	1.0	16.3	1.9
8.2	15.1	17.3	25.3	24.2	28.1
8.3	10.7	13.9	9.7	6.8	11.1
9	1.5	n.d.	0.1	n.d.	0.1
10	8.2	7.5	11.1	7.2	11.1
11	5.1	3.9	1.0	1.2	1.1
12	6.4	4.6	1.5	1.1	1.7
13	5.3	5.3	3.4	2.8	3.5
14	6.5	7.8	8.8	6.4	9.8
15	2.4	2.1	6.6	5.1	8.7
16	3.3	1.8	0.3	0.2	0.3

[1]As percentage of total Vβs.
[2]Calculated from the densitometric profiles for double positive thymocytes of C57BL/6 mice in Figure 1. Two other Vβ^b haplotype strains, C3H/HeJ and AKR/J, were also tested with very similar results (SEMs < 10%). Vβ9 was analyzed separately.
[3]Strains used: *Mls*^{b}, C57BL/6; *Mls*^{a}, AKR/J; *Mls*^{c}, C3H/HeJ; *Mls*^{d}, CBA/J.

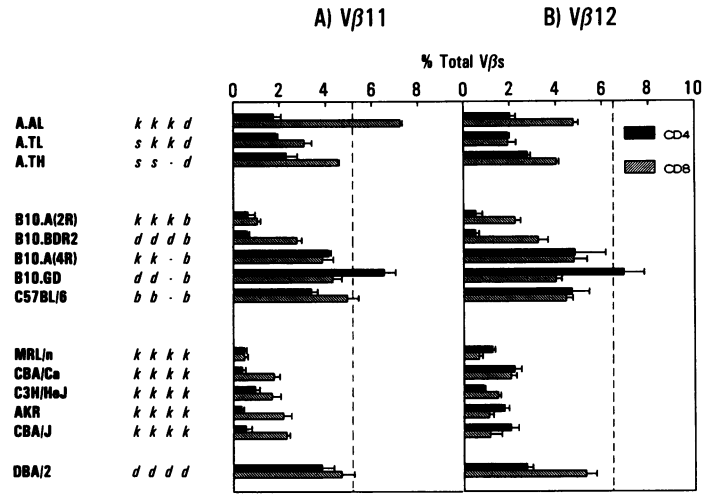

Fig. 3. Frequency of Vβ11- and Vβ12-bearing T cells in the $CD4^+$ and $CD8^+$ subsets of selected mouse strains. Frequencies were determined by peak height quantitation as described in *Materials and methods*, and are expressed as percentage of total Vβs. The error bars show the SEM values for two to three independent assays of RNA pooled from two to five mice. Dashed lines represent the percentage expression determined for the corresponding Vβ in the unselected repertoire of $CD4^+8^+$ thymocytes (see Table I).

was generally less complete for Vβ12- than for Vβ11-bearing cells. These results suggest that Vβ11 and Vβ12 clonal deletions may be controlled by the same, or similar, non-MHC ligand(s).

Partial clonal deletions of Vβs -5.1 and -16 suggest both IE and non-IE influences

We detected significant reduction (compared with the unselected level of 3.9%) in the expression of Vβ5.1 in both the $CD4^+$ and $CD8^+$ subsets of all IE^+ strains tested (Figure 4A), with expression ranging from 0.4 to 1.5% in the $CD4^+$ subset, and 0.3 to 1.7% in the $CD8^+$ subset. Interestingly, however, IE^- mice of the C57BL background (C57BL/6, B10.A(4R) and B10.GD) also showed substantially reduced Vβ5.1 levels (0.6−1.4%) in the $CD4^+$ subset, but not uniformly low levels (1.9−3.8%) in the $CD8^+$ subset. Thus, a role for non-IE MHC genes in Vβ5.1 selection also appears likely. These findings are in general agreement with the recent report of Liao *et al.* (1990). In addition, we found one IE^- strain, A.TH, which did not show reduced levels of Vβ5.1 in the $CD4^+$ (2.4%) or $CD8^+$ (3.9%) subsets, further suggesting a role for IA and/or non-MHC genes in Vβ5.1 usage.

With regard to Vβ16-bearing T cells, Figure 4B shows that they were efficiently deleted in both the $CD4^+$ and $CD8^+$ subsets in most IE^+ strains tested. Exceptions to this finding were B10.A(2R), B10.BDR2 and CBA/CaJ mice, where the IE mediated deletion was only partial, implying the absence of some of the appropriate non-MHC ligand(s) for Vβ16 deletion in these strains. Among IE^- strains, A.TH mice showed unselected (i.e. high) levels of Vβ16 T cells. However, IE^-B10.A(4R) and B10.GD mice showed reduced levels in both $CD4^+$ and $CD8^+$ subsets, thereby suggesting a role for IA as well, similar to the situation with Vβ5.1 selection.

Significant strain-to-strain and subset-to-subset skewing is a common feature of many Vβ genes

The CD4/CD8 skewing observed for the above Vβ genes was, in fact, found to be generally true for many (if not all) Vβ genes tested, with as yet undefined MHC and background

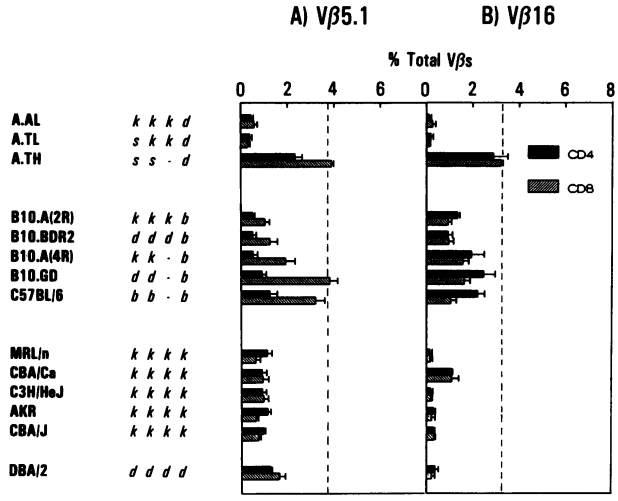

Fig. 4. Frequency of Vβ5.1- and Vβ16-bearing T cells in the $CD4^+$ and $CD8^+$ subsets of various mouse strains. See legend to Figure 3 for details.

gene effects presumably governing expression levels. Figure 5 shows protection assay densitometric profiles revealing various examples of such novel skewing effects for Vβs 2, 7, 13 and 15. These effects include: (i) The two *Mls*^{a} strains tested, AKR (Figure 5A and B) and CBA/J (not shown), exhibited the illustrated Vβ2 skewing, i.e. high expression in the $CD8^+$ subset, low in the $CD4^+$ subset; whereas the *Mls*^{b} C57BL/6 mice (Figure 5C and D) showed the opposite pattern. (ii) Vβ7 expression was strongly reduced in both T cell subsets in these *Mls*^{a} mice (Figure 5A and B), and moderately to strongly reduced in the $CD4^+$, but not the $CD8^+$, subset of the *Mls*^{b} C57BL/6 strain (Figure 5C and D). (iii) Skewing of Vβ15 towards higher expression in the $CD4^+$ subset (Figure 5A and B) was a common feature of many strains. Of particular note, Vβ15 expression was strongly reduced in the $CD8^+$ subset of all C57BL strains tested, i.e. both IE^+ and IE^- B10 congenics as well as C57BL/6 mice (Figure 5C and D). Finally, (iv) Vβ13 levels also showed significant subset and strain variations, with bias towards high levels in the $CD8^+$ subset (≈ 11−

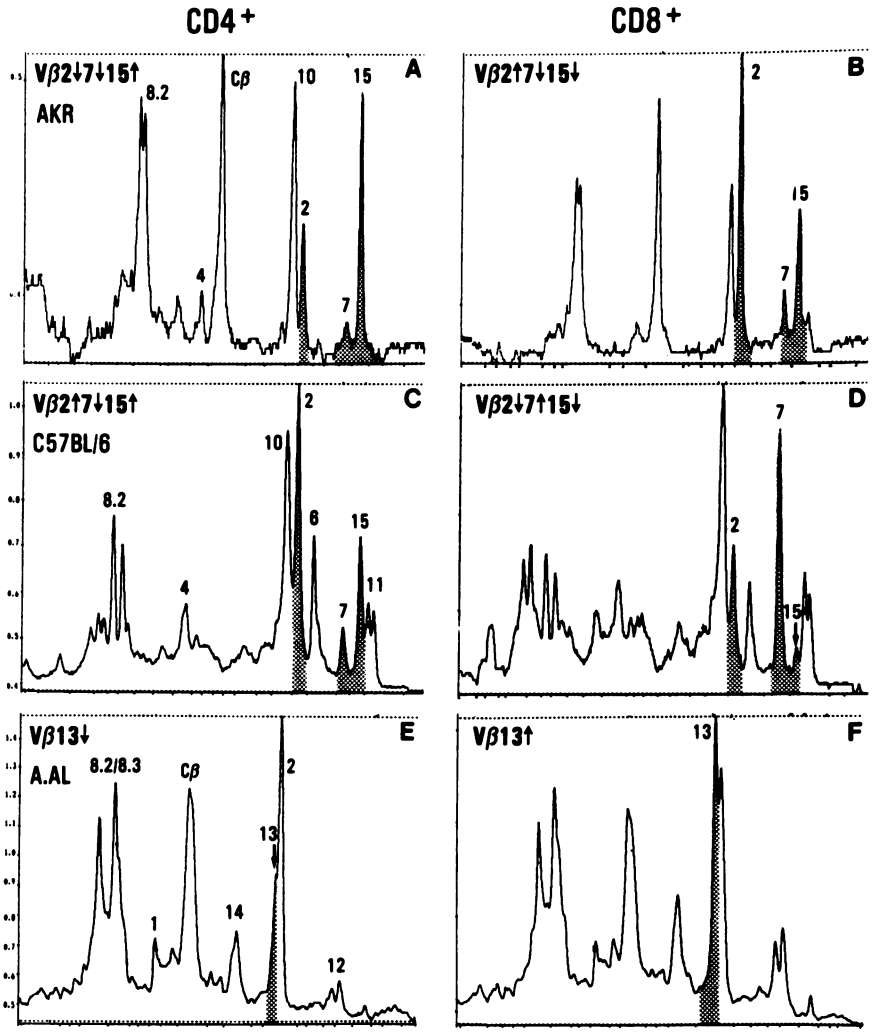

Fig. 5. Densitometric profiles of protection assays showing skewed subset expression of Vβs 2, 7, 13 and 15. Shown are representative examples of specific subset-to-subset and/or strain-to-strain skewing effects observed. **Left** panels are $CD4^+$, and **right** panels $CD8^+$ subsets of the following strains: (**A,B**) AKR/J (**C,D**) C57BL/6, (**E,F**) A.AL. Probe Set B was used for panels A–D, except that a Cβ probe was not included in panels C and D. For panels E and F, probe set A was used, except without the Vβ6 probe and with an added Vβ2 probe.

15%), compared with moderate levels (≈ 5–9%) in the $CD4^+$ subset, in K^k-expressing A-strain mice (Figure 5E and F) and K^k- or K^d-expressing B10 mice, (not shown). Of interest, other tested $H2^k$ or $H2^d$ strains showed lower levels of $CD8^+$ $V\beta13^+$ (≈ 4–7%) and $CD4^+$ $V\beta13^+$ (≈ 2–3%) cells, suggesting non-MHC influences on Vβ13 selection as well.

Background gene influences create unique Vβ repertoire phenotypes among a panel of H2k strains

The demonstrated skewing of Vβ gene usage at the CD4/CD8 subset level can potentially identify phenotypes representing characteristic patterns of Vβ repertoire expression, reflecting the constellations of MHC and non-MHC factors which influence TCR selection in different genetic backgrounds. One important aspect in this regard is the extent of diversity imparted to the Vβ repertoire by non-MHC components. Table II summarizes CD4/CD8 expression levels for the Vβ genes which showed the most distinctive variations among the six MHC-identical ($H2_k$) strains tested. For the purpose of describing phenotypes, it is useful to define any Vβ level in a particular strain as being either high (*h*) or low (*l*) with respect to its range in the peripheral repertoire of the mouse population as a whole. As shown

Table II. Identification of unique TCR Vβ repertoire phenotypes among H2k mouse strains[a]

Strain	$CD4^+$ subset						$CD8^+$ subset					
	Vβ3.1	-6	-11	-13	-15	-16	Vβ3.1	-6	-11	-13	-15	-16
B10.BR	3.4	4.9	0.5	2.8	4.4	1.0	**2.7**	**6.1**	**3.0**	7.4	1.1	**1.1**
MRL/n	5.9	5.4	0.5	3.0	7.6	0.1	**4.0**	**6.0**	0.5	3.8	**5.5**	0.2
CBA/Ca	6.6	5.4	0.4	2.8	7.8	1.1	**6.8**	**6.8**	**1.8**	4.6	**7.2**	**1.1**
AKR/J	10.4	0.3	0.4	1.9	7.5	0.3	**8.4**	0.3	**2.2**	6.4	**4.7**	0.2
CBA/J	0.4	0.4	0.5	2.1	9.2	0.3	0.4	0.4	**2.3**	6.3	**7.6**	0.3
C3H/HeJ	0.2	7.9	0.9	2.5	5.3	0.2	0.1	**7.0**	**1.7**	3.3	**4.7**	0.2

[a]Values represent the mean percentage expression for each Vβ from at least two independent assays of pools of two to five mice. SEMs were < 10% of mean values in all cases. For the purpose of describing phenotypes, the level of each Vβ in a particular strain can be defined as being either high (h) or low (l) with respect to its range in the peripheral repertoire of the mouse population as a whole. Values in the high range for a given Vβ are shown in **bold**, *underlined*. Thus, in this illustration, phenotypes are identified using the $CD8^+$ subset expression levels for Vβs 3.1, 6, 11, 13, 15 and 16, respectively, as follows: (a) B10.BR (hhhllh); (b) MRL/n (hhllhl); (c) CBA/a (hhhlhh); etc. These particular values were chosen for conciseness of presentation; however other values in the table could also be used for the same purpose, e.g. the $CD4^+$ subset values for Vβs 3.1, 6 and 16 make the same point as the chosen $CD8^+$ subset values.

for the more informative $CD8^+$ subset, the distribution of high (*h*) (*bold type* in Table II) and low (*l*) values for each Vβ gene delineates unique Vβ repertoire phenotypes for each of the strains listed. Such analysis, though still in its early stages, nevertheless illustrates the significant effects of background gene induced Vβ repertoire modifications in the mouse.

Discussion

Recent evidence supports the notion that TCR repertoire selection may play an important role in controlling immune responsiveness (Vidovic and Matzinger, 1988) and susceptibility to autoimmune diseases (Reich *et al.*, 1989). To understand this putative role in systematic and organ specific autoimmune diseases better, we have focused on the need for a broad method of Vβ repertoire analysis suitable for disease correlation studies. Toward this goal, we describe here the development of a sensitive and accurate multiprobe Vβ RNase protection assay system, and its application to the analysis of Vβ gene expression in separated $CD4^+$ and $CD8^+$ subsets in a panel of congenic and mixed background mouse strains. This assay system was found to facilitate the analysis of murine Vβ expression levels in multiple samples. Moreover, it requires as little as 1 μg total RNA per sample ($\approx 10^6$ cells) and, as we demonstrate here by titration experiments, consistently detects clonal expansions or deletions representing only 10–20% relative change for a given Vβ. A similar approach to Vβ repertoire analysis was recently described by Okada and Weissman (1989).

The present application of this assay system to the analysis of separate $CD4^+$ and $CD8^+$ subsets has allowed improved resolution of Vβ repertoire selection phenomena, and extended previous investigations in three general ways: (i) we further define selection effects for the set of IE-reactive Vβ11, -12, -5.1 and -16 genes, and document 'subset biased' clonal deletion as a novel means of affecting TCR repertoire selection: (ii) we identify several new Vβ gene effects of both positive and negative selection, indicating the diversity of non-MHC ligands influencing this selection; and (iii) we provide a method of describing and cataloging Vβ repertoire phenotypes derived from such selection effects.

Our investigation further defines a number of Vβ selection phenomena, which complement and extend the results of previous studies, particularly those of Bill *et al.* (1989) and Vacchio and Hodes (1989) on the Vβ genes involved in IE mediated TCR repertoire selection: Vβs 5.1, 11, 12 and 16. These authors documented both the partial nature of the IE related Vβ11 and -12 clonal deletions as well as their dependence on non-MHC antigens. We found that this partial deletion was attributable to either: (i) complete $CD4^+$, but incomplete $CD8^+$, subset deletion in most strains exhibiting the effect; or (ii) little or no deletion in the $CD4^+$ subset in the case of A-strain and DBA/2 mice, which we interpret as probable lack of the deletion mediating background ligand(s) in these strains. Lack of the Vβ11 deleting ligand(s) in A-strain mice, and its presence in the C57BL/6 background, would explain the reported ability of (A/J × B6)F1s to delete Vβ11 more efficiently than the A/J parent (Vacchio and Hodes, 1989). Similarly, lack of the relevant ligand(s) in DBA/2 mice is consistent with the finding of Bill *et al.* (1989) that Vβ11-bearing cells are not uniformly deleted in all $H2^d$-expressing (IE^+) DBA/2 × B6 recombinant inbred strains.

Our data on the thymic selection of Vβ5.1-bearing T cells are in general agreement with the recent study of Liao *et al.* (1990). Thus, we and they find that: (i) all IE^+ mice analyzed show low Vβ5.1 levels in both the $CD4^+$ and $CD8^+$ subsets; (ii) most IE^- strains [e.g. B10.GD and C57BL/6 in this study; and C57BL/10,B10.A(18R) and B10.D2(R107) in the study of Liao *et al.*, 1990] show high $CD8^+$ subset levels; but some IE^- strains [e.g. B10.A(4R) and B10.M] in contrast, show reduced $CD8^+$ subset levels; and (iii) most strains, whether IE^+ or IE^-, show low $CD4^+$ subset levels. In addition, we describe here one IE^- strain, A.TH, which shows high Vβ5.1 levels in both the $CD4^+$ and $CD8^+$ subsets. Since IE positivity results in essentially complete Vβ5.1 deletion in both subsets, interpretations center on the selection effects occurring in IE^- mice. One interpretation, favored by Liao, *et al.*, suggests that the high $CD8^+$ subset levels in IE^- mice reflect preferential positive selection by class I MHC molecules; and low $CD4^+$ subset levels reflect failure of positive selection by class II molecules. Another possibility, however, is that IA mediated clonal deletion accounts for the low $CD4^+$ subset levels in most IE^- mice. As shown here for Vβ11, such IA mediated clonal deletion could in fact be selectively expressed in the $CD4^+$ subset, while the variable levels of $CD8^+V\beta5.1^+$ cells seen in various IE^- strains may reflect differential cross-subset deletion. The unique situation of high $CD4^+$ and $CD8^+$Vβ5.1 cell levels in A.TH (*ss-d*) mice may be due either to lack of the relevant non-MHC ligand or, alternatively, inability of the IA^s molecule to mediate clonal deletion. Further studies of appropriate recombinant strains and F1 crosses will be required to distinguish among these and other possible Vβ5.1 selection effects.

The phenomenon of 'subset biased' Vβ clonal deletions, i.e. strong deletion in the subset corresponding to the controlling MHC molecule, with variable and incomplete reduction in the opposite subset (as with Vβ11), is likely to be a major contributor, along with positive selection, to CD4/CD8 subset bias on the TCR Vβ repertoire, as suggested by the findings described in the present study. It also appears to represent a novel way in which negative selection can affect TCR repertoire expression. Previous investigations of *Mls* related Vβ clonal deletions (Kappler *et al.* MacDonald *et al.* 1988a; Pullen *et al.*, 1988), for example, revealed essentially complete deletion in both the $CD4^+$ and $CD8^+$ subsets. A similar conclusion was drawn from earlier studies of the IE related Vβ17a deletion (Kappler *et al.*, 1987b) [although recent analysis in mice expressing different IE^+ alleles has also detected incomplete deletion of $CD8^+V\beta17a^+$ cells in some strains (Kappler *et al.*, 1989)]. Together, these findings have led to the suggestion that tolerance related deletions occur at the double positive ($CD4^+8^+$) stage of thymocyte development; and evidence supporting this concept has been obtained by CD4 blocking experiments in both the Mls^a/Vβ6 (MacDonald *et al.*, 1988c) and IE/Vβ17a (Fowlkes *et al.*, 1988) systems, as well as in transgenic mice for class I restricted TCRs (Kisielow *et al.*, 1988b). It is not known whether further deletion of self-reactive cells continues into the single positive stage; however the acquisition of higher surface TCR density at this stage (Roehm *et al.*, 1984) may increase the affinity of receptor–MHC interactions, thereby triggering further deletion.

How can the mechanism of partial, 'subset biased' clonal deletions (of Vβ11, for example) be understood within the above context? Several possibilities might be considered based on different assumptions concerning the relative influence, stage of engagement, temporal and spatial order, and ligand involvement for both the positive and negative selection processes. One possibility is that 'subset biased' clonal deletions, like those for *Mls*, also begin at the double positive stage (either simultaneous with or subsequent to positive selection), but affect only a portion of $V\beta11^+$ cells; that is, those with sufficient IE−ligand affinity, contributed by the combination of associated TCR Jβ or Vα−Jα elements, to be autoreactive. Non-IE-reactive $V\beta11^+$ cells would mature in IE^+ mice into $CD8^+$ or $CD4^+$ thymocytes according to normal positive selection, with the precise make-up of this population varying according to the nature of the MHC class I and class II alleles and non-MHC ligands involved in the selection processes. The recent observation (Jones *et al.*, 1989) that the few residual $CD4^+V\beta11^+$ cells in IE^+ mice could be eliminated by anti-IA treatment (and thus represent IA selected cells) is consistent with this model. It should be noted, however, that in strains which completely delete Vβ11-bearing T cells [such as MRL/n and B10.A(2R) in our study], IE-reactivity must extend to essentially all $V\beta11^+$ TCRs and not just a given portion. The basis for this difference in various $H2^k$ haplotype-identical mice is not clear, but would seemingly involve a broader or more effective range of deleting ligands or, alternatively, less efficient positive selection of the $CD8^+$ subset in the latter mice.

Broad analysis of Vβ gene expression in separated $CD4^+$ and $CD8^+$ subsets revealed additional Vβ repertoire modifications, which in many cases would not have been apparent on analysis of total T cell RNA (Vacchio and Hodes, 1989). Some examples include high Vβ2 expression in the $CD8^+$, but low relative expression in the $CD4^+$, subset of AKR mice, and the converse in C57BL/6 mice; reduction of Vβ7 expression in the $CD4^+$, but not the $CD8^+$, subset of B6 mice; strong reduction of Vβ15 expression in the $CD8^+$ subset of C57BL strains of mice; and higher expression of Vβ13 among $CD8^+$ cells in K^k- or K^d-expressing A-strain or B10 mice, but not in other tested $H2^k$ or $H2^d$ strains. Other reported repertoire modifications, such as the overall deletion of Vβ7-bearing cells in many Mls^a and Mls^c mice (Vacchio and Hodes, 1989), and the strong positive selection of Vβ14 in $H2K^k$-expressing mice (Liao *et al.*, 1989), were also generally confirmed in our findings (data not shown). It should be noted that these represent only the most extreme repertoire modifications detected in our study; many more moderate, yet reproducible, Vβ clonal deletions and expansions. involving essentially all known Vβ genes, remain to be fully characterized. Such Vβ repertoire modifications, whether as a result of differential positive selection or the novel phenomenon of 'subset-biased' negative selection, are shown here to define numerous distinct phenotypes in the mouse. The identification of such phenotypes should assist in the further understanding and cataloging of the processes involved in Vβ repertoire selection. In this regard, the identification of unique TCR repertoire phenotypes among six $H2^k$ mouse strains in this study suggests that analogous repertoire polymorphisms will be found in comparing HLA-identical individuals who are discordant for HLA linked immune mediated diseases. Such Vβ polymorphisms, caused by other than HLA genes, may help to define disease susceptibility better, as well as to identify disease mediating T cell clonotypes.

Materials and methods

Mice and cell lines

Mice were obtained either from the Scripps Clinic breeding facility, or purchased from the Jackson Laboratory. Cell lines used in the titration experiments, and their strain derivations, were: A.11.11 (SJL), C4.17 (SJL), and B7 (CBA/Ca), for Vβs 4, 6 and 15, respectively; provided by Dr R.B.Clark (University of Connecticut School of Medicine) and Dr W.O.Weigle (Research Institute of Scripps Clinic).

Isolation of T cell subsets

Thymuses and spleens from 2−3 month old mice were used for the preparation of single-cell suspensions. Double positive ($CD4^+8^+$) thymocytes were prepared by staining with FITC-conjugated anti-CD8 (53−6.7) and PE-conjugated anti-CD4 (GK1.5) (Becton Dickinson, Mountain View, CA), followed by separation on a FACS IV flow cytometer. Single positive ($CD4^+8^-$ or $CD8^+4^-$) peripheral T cells were prepared from splenocytes by either: (i) two rounds of killing of the unwanted subset with the appropriate anti-CD8 or anti-CD4 mAbs plus complement; or (ii) staining and FACS separation. Purified T cell subsets isolated from pools of three to five mice were kept at −70°C until use.

Labeled hybridization probes

Murine Vβ probes were obtained from a lymph node cDNA library (Singer *et al.*, 1986), and subcloned into pGEM plasmids by standard procedures (Maniatis *et al.*, 1982). Protected probe lengths in nucleotides (nt) are as follows: probe set A: Vβ5.1 (364 nt), Vβ16 (317 nt), Vβ12 (284 nt), Vβ3.1 (250 nt), Vβ6 (222 nt), Vβ13 (197 nt), Vβ14 (163 nt), Vβ1 (112 nt), Vβ8.2/8.3 (93/93 nt), and Vβ8.1 (62 nt); probe set B: Vβ11 (324 nt), Vβ15 (307 nt), Vβ7 (293 nt), Vβ6 (222 nt), Vβ2 (203 nt), Vβ10 (190 nt), Vβ4 (119 nt) and Vβ8.2 (93 nt). A 134 nt Cβ probe and 60 nt Vβ9 probe were prepared similarly.

Transcription of RNA probes was done by pooling of linearized (*Hin*dIII or *Eco*RI) Vβ pGEM plasmid templates in two probe sets and adding 80−200 μg to a 5 μl Riboprobe system (Promega Biotec, Madison, Wisconsin) reaction with 75 μCi [^{32}P]UTP at 15 μM final concentration, followed by DNase treatment and purification per supplier's instructions. The Cβ probe was labeled in a separate reaction where the [^{32}P]UTP was at one-tenth the specific activity. The Vβ9 probe was labeled and used separately, since its small size caused it to be obscured by background in the probe sets.

RNase protection assay

Assays were performed essentially as described by Melton *et al.* (1984) with minor modifications. 1−2 μg aliquots of total T cell RNA were lyophilized and dissolved in 4 μl hybridization buffer (80% formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM PIPES, pH 6.7). Labeled probe was dissolved in hybridization buffer at $1-2 \times 10^6$ c.p.m./μl (adjusted to 2000 c.p.m./μl per UMP residue in the probe set) and 1 μl aliquots added to the RNA samples in sterile microfuge tubes. The solution was overlayed with liquid paraffin and incubated at 56°C for 12−16 h, sufficient to insure quantitative hybridization of probes to target mRNA sequences. Digestion of unhybridized probe was performed using 10 μl of digestion buffer (10 mM Tris pH 7.5/5 mM EDTA/0.3 M NaCl with RNase A at 50 μg/ml and RNase T1 at 50 U/ml) per μg of RNA for 1 h at 30°C. Digested samples containing 'protected' probes were phenol extracted, ethanol precipitated, dissolved in sample buffer, and electrophoresed in standard polyacrylamide sequencing gels. Autoradiography of the dried gel was on Kodak XRP film at −70°C with intensifying screens for 16−48 h.

Quantitation

Vβ quantitation was done on the basis of peak heights from densitometric scans of autoradiographs, with exposures controlled such that all bands fell within the linear range (0.1−1.5 OD units) of the LKB Ultroscan laser densitometer. In calibration experiments (see results and Figure 2), peak height was found to be proportional to added RNA and/or signal strength as determined by direct radioactivity counting. In the case of double or multiple bands, individual peaks were added to obtain final values. We found that the inclusion of Vβ8.2 and -8.3 probes in probe set A allowed accurate determination of Vβ8.1 levels by cross-hybridization. Vβ8.3 levels were quantitated by subtraction of Vβ8.2 values in probe set B from Vβ8.2 + 8.3 values in probe set A. Profiles from the two probe sets were normalized using a Cβ probe incorporated into each probe set and adjusted to one-tenth

the specific activity of the Vβ probes. Peak height values were corrected for the [^{32}P]uridine content of individual probes and expressed as percentage of total Vβs. In preliminary experiments (data not shown), it was determined that mAb derived (anti-Vβs 3, 6, 8.1, 8.2, 8.3 and 11) Vβ expression levels were generally in good relative agreement with our protection assay values; with the exception of Vβ8 subfamily levels, which are apparently overestimated by protection assay results in some strains.

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Note added in proof

Regarding Vβ11 and Vβ12 clonal deletions, Vacchio,M.S., Ryan,J.J. and Hodes,R.J. (1990) (*J. Exp. Med.*, **172**, 807–813) recently documented that the non-MHC ligands recognized by Vβ11$^+$ and Vβ12$^+$ T cells are multiple and overlapping, but not necessarily identical.