

Genetic Analysis of Low V β 3 Expression in Humans

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Summary

While studying the T cell receptor (TCR) repertoire of normal individuals, we found that more than 20% of adults have low levels of circulating V β 3.1⁺ T cells in both CD4 and CD8 populations. A similar frequency was found in fetal cord blood samples, suggesting that in most cases, the V β 3.1^{low} phenotype is inherited. In support of this conclusion, children expressing low levels were only found in families where one of the parents expressed this phenotype. In two large families, genetic studies showed that low expression was a recessive trait and dependent on inheritance of particular TCR VB gene complexes. Family members with the low phenotype, however, expressed VB3.1 genes with normal sequences and expressed normal levels of receptor per cell. Results from these families suggest that up to 50% of normal individuals may carry a VB3.1 allele that is defective in its ability to rearrange effectively. In another large family, low expression in one individual was shown not to be determined by genes within the TCR VB gene or major histocompatibility complexes, suggesting a different mechanism for low V β 3.1⁺ T cells. Overall, our results describe novel mechanisms that result in low levels of V β 3.1⁺ T cells in a relatively large subset of the normal human population.

Studies in mice have shown that different mechanisms may lead to low levels of expression of particular TCR V β genes (now referred to as TCRBV or VB genes). For example, a deletion within the VB complex of some strains leads to the complete absence of T cells bearing certain V β s (1–3). Missing V β subsets in mice can also be the result of polymorphism in specific VB structural genes (3–5). In addition to these mechanisms, expression of endogenous mouse mammary tumor virus (MMTV) superantigens or exposure to bacterial superantigens can lead to dramatic reductions in the percentage of T cells bearing target V β s (for a review see reference 6). Although usually present in both the CD4⁺ and CD8⁺ populations, T cell subset deletions mediated by superantigens are not complete. Thus, unlike mice carrying VB gene deletions or structural gene mutants, there is expression of residual cells bearing the specific V β s involved in recognition.

Thus far, studies in humans have only convincingly shown low V β expression related to alterations in the corresponding structural VB gene. For example, coding region changes account for low levels of V β 6.7a⁺ T cells because of a lack of mAb binding to the V β 6.7b product (7). Relatively frequent nonfunctional alleles of the VB6.1 (TCRBV6S1) and VB18 loci have also been described (8–10). There are no descriptions in humans of TCR repertoire deletions related to en-

dogenous superantigen expression. In this report, we analyze the genetic mechanisms responsible for low levels of V β 3.1⁺ T cells in a relatively large subset of normal individuals.

Materials and Methods

Sample Preparation. Mononuclear cells were isolated from peripheral blood or cord blood samples by Ficoll-Hypaque density gradient separation, and used for analysis of TCR V β expression and preparation of RNA (11). For genetic studies in families, neutrophils were also isolated from the pellet during gradient separation of mononuclear cells and used for preparation of genomic DNA.

Immunofluorescence Analysis. Mononuclear cells were stained using biotinylated mAb directed to V β 2, V β 3.1, V β 5.1, V β 5.2/5.3, V β 6.7a, V β 8.1/8.2, V β 12, V β 13.1, V β 13.2, and V β 17 (12–15). Streptavidin-PE (Fisher Biotech, Pittsburgh, PA) was used as a second-step reagent. Samples were also stained with FITC-conjugated mAbs directed to CD4 and CD8 (both from Becton Dickinson & Co., Mountain View, CA). Fluorescence intensity was analyzed with a cytofluorograph (Epics Profile; Coulter Corp., Hialeah, FL), and at least 10⁴ cells were analyzed for each antibody combination.

The specificity of the 8F10 mAb was assigned based on its ability to stain T cell hybridomas transfected with a DNA construct containing VB3.1 as described (15), but not their untransfected parents or hybridoma cells expressing other human V β elements (data not

shown). A similar perfect correlation was seen when a panel of T cell clones was typed for surface expression with the 8F10 mAb and for VB3.1 mRNA expression by PCR (11). Additionally, stimulation of peripheral blood cells with the 8F10 mAb resulted in a selective increase of VB3.1 mRNA expression (13, and Donahue, J. P., P. Marrack, J. Kappler, and B. L. Kotzin, unpublished observations).

Sequencing of TCRBV3S1 Genes. TCRBV3S1 (hereafter called VB3.1) sequences were obtained by amplifying cDNA derived from unstimulated cells using PCR with oligonucleotides specific for the VB3.1 leader (GTGAGAATTCACCATGGGAATCAGGC-TCTCTGTGTC) and TCRBC (CGGGTGGGAACACCTTGTTT-GGATCCTC). Amplified DNA was cloned into pTZ18R (Pharmacia Fine Chemicals, Piscataway, NJ) using EcoRI and BamHI sites, or into the pCR™ cloning vector as specified by the manufacturer (Invitrogen, San Diego, CA). The ligation product was transformed into competent *Escherichia coli* cells (Invitrogen), and colonies containing appropriate inserts were chosen randomly for sequencing. DNA sequencing was performed by the dideoxy method using Sequenase (United States Biochemical Corp., Cleveland, OH). At least five cDNA clones derived from each sample were sequenced.

Identification of Polymorphisms within the TCRB Gene Complex. Previously described VB gene polymorphisms were used primarily to genetically type family members and to determine the inheritance pattern in families. For example, in Families A and B, we utilized polymorphisms in simple sequence repeats (simple sequence length polymorphisms [SSLPs]) contained within the VB6 subfamily of gene segments as described (16). Sequences of the oligonucleotides used for PCR amplification were as described (16). PCR employed a thermal cycler (Cetus/Perkin Elmer; Emeryville, CA). In each reaction, 100 ng of genomic DNA was mixed with primers (0.3 μ M final concentration), 1.0 U of Taq polymerase (Perkin Elmer), and 20 μ M each of dNTPs in a total volume of 50 μ l. Amplification was performed for 35–40 cycles, with 1 min of denaturation at 95°C, 1 min of annealing at 55°C, and 2 min of extension at 72°C. PCR products were separated on a 15% polyacrylamide gel, and visualized with ethidium bromide and UV irradiation.

In Family A, we also utilized PCR and polymorphisms within the TCRBC loci as described (17). Polymorphisms in two regions identified as C β 21 and C β 25 (17) could be identified by changes in BglII and KpnI restriction sites, respectively. Primers utilized for PCR were as described (17) and PCR conditions were identical to those described above. After precipitation of 20 μ l of the PCR product, digestion was carried out with 2 U/ μ g DNA of enzyme at 37°C overnight. Undigested and digested aliquots were separated on a 2% agarose gel and visualized with ethidium bromide and UV irradiation.

In Family B, previously reported polymorphisms in the VB6.1 structural genes were also utilized (8). One of the mutations at amino acid position 62, eliminates a BsiE1 restriction enzyme site. Primers utilized were 5'-GTTCTCAACTTGTGTCTCCG (+ strand) and 5'-GAGATACACGGCTGAGTC (- strand). 25 μ l of amplified product was digested with 2 U/ μ g DNA of BsiE1 (New England Biolabs Inc., Beverly, MA) overnight at 55°C. Undigested and digested products were separated on a 15% polyacrylamide gel and visualized by ethidium bromide staining.

In Family C, typing of maternal TCRB haplotypes utilized Southern analysis and a previously described RFLP defined by a BglII restriction site in the TCRBC2 gene segment (18). In addition, a VB3.1 polymorphism was utilized to determine inheritance of paternal VB gene complexes. To determine the presence of the paternal allele, cDNA from unstimulated PBMC was amplified using

VB3.1 and TCRBC specific primers utilized above for sequencing. This fragment was then reamplified using internal oligonucleotides specific for VB3.1 (GTGAGAATTCACCTGAAAGTAACC-CAGAGCTCG) and TCRBC (TTCTGATGGCTCAAACAC). Fragments were digested with AatII overnight before analysis on a gel.

Results

Variable Percentages of V β 3.1⁺ T Cells in Adult and Fetal Cord Blood. We examined the peripheral blood TCR repertoire in a group of unrelated normal adults to look for possible deletions of specific V β subsets. These studies were accomplished first using a quantitative PCR technique (11) and subsequently using flow cytometry with mAb directed to T cells bearing V β 2, V β 3.1, V β 5.1, V β 5.2, V β 6.7, V β 8.1/8.2, V β 12, V β 13.1, V β 13.2, and V β 17. By PCR analysis and with a mAb directed to V β 3.1 (clone 8F10), we noted a broad range in the percentage of V β 3.1⁺ T cells in the normal population, and discovered several individuals with distinctly low levels (staining data are shown in Fig. 1a). Unlike results with some of the other anti-V β antibodies, low levels of V β 3.1⁺ cells, when present, occurred in both the CD4⁺ and CD8⁺ populations. 7 of 31 unrelated adults studied (or 23%) had low percentages of V β 3.1-bearing T cells, defined as \leq 2.0% of CD4⁺ and CD8⁺ T cells. Most of these individuals were studied multiple times over several months and the percentage of V β 3.1⁺ T cells remained remarkably stable in all cases (data not shown).

We also examined the TCR repertoire of fetal cord blood samples (Fig. 1b). 5 of 25 samples contained low percent-

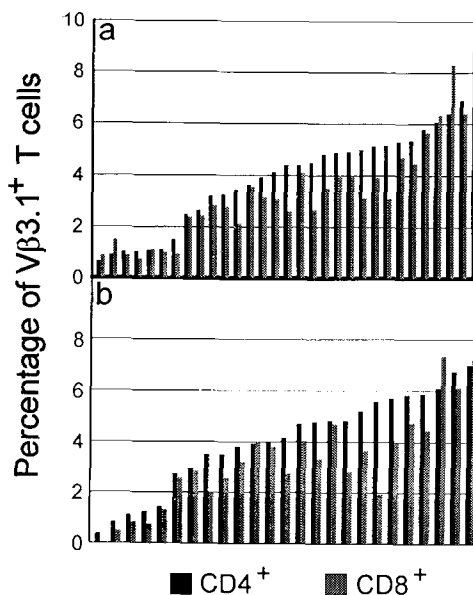


Figure 1. Percentages of T cells expressing V β 3.1 in adult peripheral blood (a) or fetal cord blood (b). Cells from each sample were stained for both V β 3.1 (clone 8F10) and either CD4 or CD8, and the percentages of each population staining positive are shown.

ages of $V\beta 3.1^+$ T cells in both $CD4^+$ and $CD8^+$ subsets. The frequency of low values ($\sim 20\%$) was similar to that observed for adults (Fig. 1 *a*), suggesting that infection after birth does not account for most of the cases with the $V\beta 3.1^{low}$ phenotype.

We carefully examined the density of $V\beta 3.1$ expression per cell, as determined by mean channel of fluorescence intensity, in a large number of different individuals with both low and high percentages of $V\beta 3.1^+$ cells in blood. The density was identical regardless of the percentage of positive cells (data not shown).

Inheritance Pattern for the $V\beta 3.1^{low}$ Phenotype and Contribution of TCR and MHC genes. We searched for families in which the mother and father expressed divergent levels of $V\beta 3.1^+$ T cells. Of the first 13 sets of parents studied, 7 had one spouse with a low level of $V\beta 3.1$ -expressing T cells. In these seven families, the frequency of children with low $V\beta 3.1$ levels ($<2\%$ in both $CD4^+$ and $CD8^+$ subsets) was 12 of 30 compared with 0 of 23 in the other six families ($p < 0.001$ by Fisher's exact test). These results strongly suggest that low levels of $V\beta 3.1^+$ T cells are determined genetically.

We analyzed three of the above families with the largest number of individuals for the contribution of VB or MHC genes to the $V\beta 3.1$ phenotype. The analysis of Family A is shown in Fig. 2 *a*. The mother expressed low levels of $V\beta 3.1^+$ T cells in both $CD4^+$ and $CD8^+$ subsets, whereas the father did not express this phenotype. Six children were analyzed, of which three expressed the $V\beta 3.1^{low}$ phenotype. Other T cell subsets quantitated by monoclonal anti- $V\beta$ antibodies were not strikingly different among family members (only data for $V\beta 8.1/8.2$ are shown). As shown in Fig. 2 *a*, the inheritance of MHC haplotypes in this family was independent of $V\beta 3.1^+$ T cell levels. It is interesting to note that expression of the low phenotype correlated with inheritance of one of the TCRB complexes from the father, who did not express the low phenotype (Fig. 2 *a*). The statistical likelihood of this happening by chance is $p = 0.05$ (by Fisher's exact test). Either of the mother's TCRB complexes appeared to allow for the low phenotype in the children.

This analysis of Family B is shown in Fig. 2 *b*. Again, the mother expressed the low phenotype that was expressed by four of the seven children. $V\beta 3.1$ levels in the other three children were actually higher than those expressed by the father. 5 wk later, repeat studies of three children, including two with the low phenotype, showed identical $V\beta 3.1$ percentages. As shown in Fig. 2 *b*, MHC haplotypes inherited from the mother or father did not correlate with $V\beta 3.1^+$ T cell levels. However, similar to Family A, this family also showed a concordance of the $V\beta 3.1^{low}$ phenotype with one of the paternal TCRB gene complexes ($p = 0.03$ by Fisher's exact test). Again, either TCRB complex from the mother, who expressed the low phenotype, allowed for low levels in her offspring.

One additional family (Family C) with the largest number of available individuals was studied to determine the importance of TCRB or MHC genes (Fig. 2 *c*). The mother (II-2) exhibited a $V\beta 3.1^{low}$ phenotype as did one sister (II-

3). Of the eight children examined, only one child (III-4) was $V\beta 3.1^{low}$. These phenotypes remained stable during the 12-mo period in which the family was studied. For example, percentages of peripheral blood $CD4^+$ T cells expressing $V\beta 3.1$ in samples taken several months apart from the father (II-1) were 4.9, 5.7, and 5.0%. In contrast, values for the mother (II-2) were 1.5, 1.7, and 1.3%, and values for child III-4 were 2.0 and 1.9%.

Inheritance of MHC haplotypes in Family C is shown in Fig. 2 *c*. In several individuals, the same MHC type was associated with disparate $V\beta 3.1$ expression, indicating that a particular MHC type was not sufficient to explain the $V\beta 3.1^{low}$ phenotype.

During the sequencing of VB3.1 genes expressed in Family C, a new VB3.1 allele (designated TCRBV3S1*2[N]) was identified in the father, which differs from previously published sequences (designated TCRBV3S1*1 [19, 20]) by a single nonproductive change (A \rightarrow G) in the third position of the threonine codon at position 84. Although it does not alter the amino acid sequence, this change does generate a unique AatII restriction site, which was useful for identifying the paternal VB complex inherited among family members. Using this AatII polymorphism and a previously characterized genomic TCRBC2 BglII polymorphism (18), we identified two individuals who inherited the same VB complexes but who had different $V\beta 3.1$ phenotypes (III-4 and III-8).

VB3.1 Gene Sequences Expressed by Individuals with the Low Phenotype. Expressed VB3.1 gene sequences were analyzed in a number of different individuals to determine if coding region polymorphisms contributed to low levels of $V\beta 3.1^+$ T cells. Table 1 shows that structural VB3.1 gene alterations do not account for differences in $V\beta 3.1$ expression. Table 1 also shows that a relatively large fraction of the cDNA clones from $V\beta 3.1^{low}$ individuals represent nonfunctional rearrangements ($p < 0.005$, compared with sequences from individuals not expressing the low phenotype). It is important to note that the coding region of the nonfunctional clones also matched the TCRBV3S1*1 sequence.

Discussion

We initiated these studies of human TCR repertoire formation by analyzing lymphocytes from normal individuals for $V\beta$ expression using both quantitative PCR and different anti- $V\beta$ mAb. Although considerable variation was found for many $V\beta$ s, only a few, such as $V\beta 3$, were found to be expressed at distinctly low levels in both $CD4^+$ and $CD8^+$ subsets in some individuals. Our results show that $>20\%$ of normal adults express the $V\beta 3.1^{low}$ phenotype. A similar frequency in fetal cord blood samples and a strong inheritance pattern in family studies strongly support the hypothesis that the $V\beta 3.1^{low}$ phenotype is genetically determined in most cases.

In two families studied, the $V\beta 3.1^{low}$ phenotype correlated perfectly with inheritance of one TCRB complex from the parent who was not low. Either allele from the $V\beta 3.1^{low}$ parent was sufficient for expression of the low phenotype.

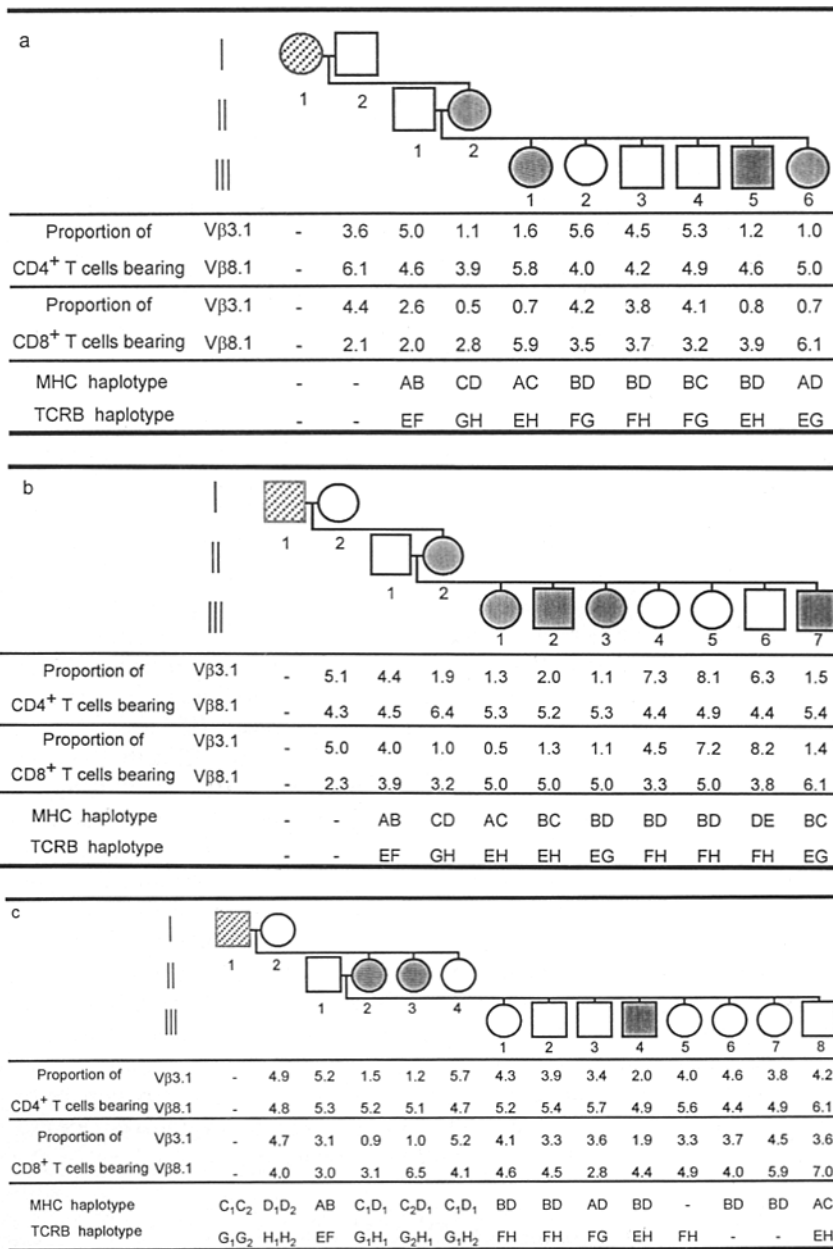


Figure 2. (a) Analysis of Vβ3.1 expression, inheritance of MHC haplotypes, and inheritance of TCRB complexes in Family A. Family members studied, including siblings III-1 to III-6 (ages 24–11), are shown, and individuals with <2.0% Vβ3.1⁺ T cells in both the CD4⁺ and CD8⁺ subsets are indicated by shading. The paternal grandmother, indicated by hatching, could not be located for the study. MHC haplotypes are: A - A2, B35, Cw4, DR4, DR53, DQw3; B - A3, B62, Cw3, DR11, DR52, DQw3; C - A3, B7, Cw7, DR1, DQw1; and D - A24, B7, Cw7, DR11, DR52, DQw3. TCRB haplotypes were revealed by polymorphisms in a VB6.7 SLP (16). In addition, inheritance of the paternal TCRB haplotypes was confirmed using a BglII polymorphism in TCRBC2 (17). Thus, one of the paternal alleles carries a BglII restriction site that cuts the 595-bp PCR fragment into fragments of 465 and 130 bp (designated as allele F). (b) Analysis of Vβ3.1 expression, inheritance of MHC haplotypes, and inheritance of TCRB gene haplotypes in Family B. Family members studied, including siblings III-1 to III-7 (ages 17–3), are shown, and individuals with <2.0% Vβ3.1⁺ T cells in both the CD4⁺ and CD8⁺ subsets are indicated by shading. The maternal grandfather, indicated by hatching, was not studied. MHC haplotypes are: A - A2, B57, Cw6, DR15, DQw1; B - A24, B51, Cw1, DR7, DR53, DQw2; C - A1, B27, Cw1, DR8, DQw4; D - A24, B44, Cw3, DR4, DR53, DQw3; and E - A2, B51, Cw1, DR7, DR53, DQw2. Inheritance of maternal TCRB haplotypes (G and H) was determined by an SLP in the VB6.7 gene (8). Inheritance of paternal TCRB haplotypes was determined by a BsiE1 polymorphism in the VB6.1 gene (8). One paternal allele (F) and maternal allele (H) carries a BsiE1 restriction site that cuts the 300-bp product into two bands of ~220 and ~80 bp. Assignment of genotypes in the children was possible by knowing inheritance of the maternal alleles. (c) Analysis of Vβ3.1 expression, inheritance of MHC haplotypes, and inheritance of TCRB haplotypes in Family C. Family members studied, including siblings III-1 to III-8 (ages 19–3), are shown, and individuals with ≤2.0% Vβ3.1⁺ T cells in both the CD4⁺ and CD8⁺ populations are indicated by shading. The maternal grandfather, who could not be located for study, is indicated by hatching. MHC haplotypes are: A - A2, B8, Cw7, DR15, DQw1; B - A31, B18, DR7, DR53, DQw2; C - A2, B27, Cw1, DR11, DR52, DQw3; and D - A30, B18, Cw5, DR2, DQw1. TCRB inheritance was determined by polymorphism in the VB3.1 gene and a BglII RFLP in the TCRBC2 region. In addition, inheritance of alleles in II-2, II-3, and II-4 was determined with a VB6.7 SLP as described for Families A and B. Maternal (II-2) alleles are designated G and H, and are inherited from the maternal grandfather (G₁, G₂) or grandmother (H₁, H₂). A single nucleotide change from the previously published TCRBV3S1*1 sequence was found in the father but not the mother. The change (A → G in the third position of the threonine codon at amino acid number 84) results in a unique AatII restriction site in this allele (F), which digests the 405-bp PCR product into bands of 266 and 139 bp. A BglII polymorphism in one maternal TCRBC2 allele was identified by the presence of a ~10 kb band (18) and allowed the analysis of inheritance of maternal alleles in the children.

The most straightforward explanation for these findings is that the low phenotype is determined in a recessive fashion in these families. If this is the major mechanism for the low phenotype in the human population, then nearly 20% of individuals must be homozygous for the Vβ3.1^{low} allele, and as predicted by the Hardy-Weinberg equilibrium for allele

frequency, nearly 50% of individuals would then be expected to carry such an allele in a heterozygous fashion.

The distribution of Vβ3.1 expression in the population overall suggested that there may be three phenotypes corresponding to the three possible genotypes: Vβ3.1^{low/low}, Vβ3.1^{low/high}, and Vβ3.1^{high/high}. However, although being

Table 1. *Vβ3.1 Sequences Expressed in Peripheral Blood by Different Individuals*

Study group	No. cDNA clones sequenced	No. wild type	No. in-frame
General population			
Vβ3.1 ^{non-low} (n = 3)*	16	16 [†]	15
Vβ3.1 ^{low} (n = 4)*	22	22	13
Family A			
II-2 (Vβ3.1 ^{low})	5	5	4
III-6 (Vβ3.1 ^{low})	5	5	3
Family B			
II-1 (Vβ3.1 ^{non-low})	5	5	5
II-2 (Vβ3.1 ^{low})	10	10	9
II-7 (Vβ3.1 ^{low})	7	7 [§]	4
Family C			
II-1 (Vβ3.1 ^{non-low})	5	5	5
II-2 (Vβ3.1 ^{low})	5	5	3

* A minimum of five sequences were analyzed per individual.

† Nucleotide sequences matched the published TCRBV3S1*1 allele sequence (19, 20).

§ Two sequences carried different single nucleotide changes that were not present in the mother or father, and were felt to be errors introduced during PCR amplification.

|| These sequences contained a single nucleotide difference (see Fig. 2 c), which does not result in a change of the amino acid sequence.

homozygous for the VB3.1^{low} allele appeared to determine low expression in the families studied, heterozygous expression did not necessarily determine a middle range of expression. This is best illustrated in Family B, in which three heterozygous children were identified. Their levels of Vβ3.1⁺ cells, however, were clearly at the high end of the total distribution.

The polymorphism(s) in the VB3.1 allele that causes low expression is currently unknown. However, it appears to be unique compared with other previously described VB gene polymorphisms (7–10). Thus, the VB3.1^{low} allele is not abnormal in its coding region. Functional VB3.1 mRNA and normal VB3.1 gene sequences were expressed in all individuals studied with the low phenotype. In addition, a relatively large number of nonfunctional VB3.1 rearrangements were found in individuals with the low phenotype, and in every case the VB3.1 coding sequence was normal. Finally, it should also be pointed out that despite the lower percentage of Vβ3.1⁺ cells in certain individuals, the amount of TCR protein expressed per cell is normal. We conclude that the VB3.1 allele in these families is defective in its ability to rearrange normally. Although the increased frequency of nonfunctional rearrangements might be expected based on the low percentage of Vβ3.1⁺ T cells alone, this finding may also be secondary to the VB3.1 allele defect itself.

Using techniques that quantitate Vβ mRNA, variability in Vβ3 expression has been observed by others (9, 21, 22).

Vandekerckhove et al. (22) reported that after implantation of fetal liver and thymus from Vβ3-discordant individuals into immunodeficient (SCID) mice, the Vβ3.1^{low} phenotype was determined before negative selection in the thymus and was dependent on the fetal liver rather than thymus donor. These results are consistent with a mechanism whereby the Vβ3.1^{low} phenotype is determined by the TCRB gene complex.

In one individual of a separate family, we found that the Vβ3.1^{low} phenotype was not determined solely by either VB or MHC genes. Although none of the children with disparate phenotypes was identical for both VB and MHC genotypes, it is unlikely that a combination of TCR and MHC genes is required for the low phenotype, since the amino acid coding region sequence of the VB3.1 genes in the family members did not differ. These results suggest a different mechanism for low Vβ3.1⁺ T cells in this individual, including the possible existence of a Vβ3.1-specific superantigen. However, it should be emphasized that the inheritance pattern in this family was unexpected in that only one of eight children expressed the low phenotype. Because our conclusions rest on this one individual, we cannot exclude an infectious exposure after birth that led to the low phenotype. Furthermore, since only one other sibling (III-8) was matched for inheritance of the paternal TCRB haplotype, we also cannot exclude a genetic recombination event that would explain higher levels in this child.

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