The Use of Mammary Tumor Virus (*Mtv*)–negative and Single–*Mtv* Mice to Evaluate the Effects of Endogenous Viral Superantigens on the T Cell Repertoire

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Summary

Most laboratory strains of mice have between two and eight endogenous superantigens. These viral superantigens (vSAGs) are coded by genes in the 3′ long terminal repeats of endogenous mammary tumor viruses (*Mtv*'s). A line of *Mtv*-negative mice and several lines of mice containing single *Mtv*'s were created by inbreeding the F_2 progeny of CBA/CaJ and C58/J mice, which have no *Mtv* integrants in common. This allowed the T cell repertoire of H-2^k mice, unaffected by *Mtv* superantigens, as well as the effects of vSAGs upon that repertoire, to be studied. Although each individual mouse had a different mix of C58/J and CBA/CaJ background genes, the T cell repertoires of different *Mtv*-negative mice were very similar and were reproducible. Since the background genes did not affect the Vβ repertoire, there are no superantigens, other than those encoded by *Mtv*'s, that differ between CBA/CaJ and C58/J. CD4 and CD8 T cells had quite different repertoires in the *Mtv*-negative mice because of the effects of class I and class II major histocompatibility complex molecules on positive and negative selection. vSAG3 was found to delete Vβ5 T cells, while vSAG8 deleted Vβ7 T cells, and vSAG9 deleted Vβ13 T cells in addition to their previously reported specificities. vSAG17 deletes a small proportion of $CD4^+$ T cells bearing Vβ11 and -12. vSAG14 and -30 have little effect on the T cell repertoire and are not expressed in thymocytes and splenocytes. An endogenous superantigen that has a low avidity for a particular Vβ may positively select thymocytes, leading to an increased frequency of peripheral T cells bearing the relevant Vβs. We found evidence that vSAG11 may positively select T cells bearing Vβ8.2. Our data, which analyzed the effects of seven endogenous *Mtv*'s, showed little evidence of positive selection by any other vSAGs on T cells bearing any Vβ tested, despite published reports to the contrary.

Mls gene products can stimulate T cells (1) because of the ability of the superantigens encoded by Mls-like genes to stimulate and/or delete T cells bearing certain Vβ chains in their α/β TCRs (2, 3). The known viral superantigens (vSAGs)[1] in mice are encoded in the 3′ LTR of mammary tumor viruses (MTVs) (4–7). These MTVs exist both as replicating virions, passed from mother to pup in the milk, and as endogenous chromosomal integrants, which are inherited genetically. There are at least 30 known endogenous MTVs, distributed among the various mouse strains and wild mice.

The specificities of these vSAGs have been demonstrated by their ability to stimulate T cells or by their ability to delete T cells bearing certain Vβs in mouse strains containing the superantigen genes compared with strains that do not. The difficulty with this analysis has been that almost all presenting cells and mouse strains contain several *Mtv* integrants, and the effects of particular superantigens cannot be unambiguously separated from those of other superantigens present in the same cell line or mouse. In addition, the effects of the H-2 haplotype on the T cell repertoire and on the vSAGs make interpretation of differences difficult.

To circumvent these problems, a line of mice was created that was free of *Mtv*'s. Although two strains of mice free of *Mtv*'s have been previously described, neither contained a well-known MHC haplotype (8, 9). Simulta-

[1] *Abbreviations used in this paper:* MTV, mammary tumor virus; RT, reverse transcriptase; vSAG, viral superantigen.

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neously, mouse lines carrying the same MHC genes (H-2^k) and single *Mtv* integrants were produced. This was accomplished by crossing two strains of H-2^k–expressing mice that do not have any *Mtv* integrants in common, CBA/CaJ and C58/J, and selectively breeding their progeny. These studies allowed an assessment of the T cell Vβ repertoire in the absence of vSAGs and a search for superantigenlike proteins other than vSAGs that differ between the two strains.

Comparison of the single-*Mtv* mouse lines with the *Mtv*-negative lines allowed the evaluation of the effects of each vSAG on positive and negative selection. In general, only thymocytes that recognize antigen in the context of the MHC molecules found in the thymic epithelia are positively selected for maturation, survival, and export to the periphery (10–13). However, developing thymocytes that recognize self-antigens bound to MHC molecules are eliminated by a process called negative selection (2, 3, 14–18). The "thymic paradox" is that thymocytes must recognize self-MHC to survive but are eliminated if they recognize self-MHC plus self-peptide. The avidity hypothesis explains this paradox by stating that thymocytes with a low but perceptible avidity for self-peptide/MHC are positively selected, while thymocytes with a higher avidity for self-peptide/MHC are deleted (19, 20). Recent experiments using fetal thymus organ culture support this hypothesis (21–23). If the avidity hypothesis is correct, a vSAG expressed in the thymic epithelium that had a very low avidity for a particular TCR Vβ element would allow T cells bearing that Vβ element to be positively selected, and the frequency of peripheral T cells bearing that Vβ element would increase. When we compared the Vβ frequencies of eight single-*Mtv* mice with those of *Mtv*-negative mice, we found evidence of such positive selection by only one vSAG.

Materials and Methods

Mice. C58/J, CBA/CaJ, AKR/J, and C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Crosses between mice were performed in the National Jewish Center animal care facility (Denver, CO).

Southern Blots. Genomic DNA was prepared from mouse tails by proteinase K digestion (0.8 mg/ml in 5% SDS, pH 7.5) and was digested with either NcoI or BstXI, subjected to electrophoresis on 0.75% agarose gels, and transferred to nylon filters (Genescreen; DuPont, Wilmington, DE) using blotting apparatus (VacuGene XL; Pharmacia Biotech, Inc., Piscataway, NJ). The filters were probed with an EcoRI-BamHI fragment of the MTV(C3H) LTR cloned into pTZ18R-orf1 (24). The probe was labeled with ^{32}P by random priming (Random Primers Kit; GIBCO BRL, Gaithersburg, MD), and hybridized at 65°C in 500 mM phosphate buffer, pH 7.2, 7% SDS. The filters received two final washes with 1% SDS, 40 mM phosphate buffer, and were exposed for 1–7 d on x-ray film (XR; Eastman Kodak Co., Rochester, NY).

Antibody Staining and Flow Cytometry. Nylon wool–purified T cells from lymph nodes were analyzed for the expression of various mouse Vβs with the following mAbs: anti-Vβ2, B20.6 (25); anti-Vβ3, KJ25a (26); anti-Vβ4 (PharMingen, San Diego, CA); anti-Vβ5.1, MR9-4 (27); anti-Vβ5.1 and -Vβ5.2, MR9-4 (27); anti-Vβ6, RR4-7 (28); anti-Vβ7 (PharMingen); anti-Vβ8.2, F23.2 (29); anti-Vβ8.1 and -Vβ8.2, KJ16 (30); anti-Vβ8.1, -Vβ8.2, and -Vβ8.3, F23.1 (29); anti-Vβ9, MR10-2 (31); anti-Vβ10 (PharMingen); anti-Vβ11, RR3-15 (32); anti-Vβ12 (PharMingen); anti-Vβ13 (PharMingen); anti-Vβ14. 14-2 (33); and anti-TCRβ H57-597 (34). Cells were incubated with one of the above-mentioned biotinylated mAbs, washed, and stained with streptavidin-coupled PE and either fluoresceinated anti-CD4 (GK1.5) or anti-CD8 (2.43) as previously described (35). At least 15,000 cells were analyzed on a FACScan® (Becton Dickinson & Co., Mountain View, CA). The frequency of Vβ5.2, Vβ8.1, and Vβ8.3 T cells was calculated by subtraction. The anti-Vβ5 antibody was of variable quality, and the calculated frequencies of Vβ5.2 T cells have a large variance and unusual distribution.

PCR Amplification, Cloning, and Sequencing of vsag30. Genomic murine DNA digested with NcoI or PvuII was separated on agarose gels as described above and purified by GeneClean (BIO 101, Inc., Vista, CA) from the agarose cut out of the appropriate region as determined by Southern blot analysis, as previously described (35). The *vsag* gene was amplified by PCR with primers specific for most of the *vsag*'s. The 5′ sense oligo was GGGAATTCTCGAGATGCCGCGCCTGCAG, and the 3′ antisense oligo was GGGGATCCTCTAGAGGGAACCGCAAGGTTGGG, complementary to a sequence outside the coding region.

The PCR product was cut with EcoRI and BamHI. The 1-kb band was size separated and purified by agarose gel electrophoresis and GeneClean, and then cloned into PTZ18R and sequenced by the chain termination method (36) using Sequenase (United States Biochemical Corp., Cleveland, OH).

Reverse Transcriptase (RT)–PCR. RNA was prepared from splenocytes and thymocytes by lysis and precipitation in 3 M LiCl/6 M urea. The RNA was then resuspended in 10 mM Tris, pH 8.0, 5 mM EDTA, 1% SDS, and was phenol/chloroform, and then chloroform extracted, followed by ethanol precipitation. Residual DNA was digested by incubating for 10 min at 37°C with 5 U of RNase-free DNase I (Boehringer Mannheim Corp., Indianapolis, IN), followed by phenol/chloroform extraction. cDNA was made from 2 μg of total RNA using the superscript RT and random hexamers (GIBCO BRL). *vsag* transcripts were amplified by 35 cycles of PCR (95°C for 1 min, 55°C for 1 min, 72°C for 1 min) using pan-*vsag*–specific primers. The 5′ primer was GGGAATTCTCGAGATGCCGCGCCTGCAG, and the 3′ primer was ACACCAAGGAGGTCTAGC.

Statistics. All results are shown as the average value of all the mice tested ± SEM. The deletion significance levels (P) were calculated using the one-tailed Student's t test. After correcting the nondeleted Vβ frequencies for the deletions (see below), a two-tailed t test was used to determine the significance of any further positive or negative Vβ skewing. A P value <0.003 was required for significance, since 16 tests were performed on each mouse line ($5\% \div 16 = 0.003$).

Frequency Correction for T Cells Bearing Nondeleted Vβs. To normalize the frequency of T cells bearing undeleted Vβs, these frequencies must be multiplied by the ratio of the frequency of T cells bearing these Vβs in *Mtv*-negative mice/the frequency of T cells bearing these Vβs in a single-*Mtv* mouse line. Since Vβ1, -15, and -16 could not be directly measured, two separate methods of normalization were used. The first method included the estimated frequency of Vβ1, -15, and -16 by subtracting the frequencies of measured Vβs from 100%. However, if there was evidence of deletion among Vβ1, -15, or -16, then a second calcu-

lation was used, which normalized only those Vβs that were directly measured and not deleted. For example, in *Mtv8* mice, Vβ5.1, -5.2, -7, and -11 and 12 $CD4^+$ T cells are deleted and account for 6.0% of $CD4^+$ T cells, while they account for 23.9% of $CD4^+$ T cells in *Mtv*-negative mice. By the first method, the correction factor is $(100 - 23.9)/(100 - 6.0) = 0.810$. However, after correcting, it is clear that at least one of the untested Vβ is being deleted. Therefore, these Vβs are not used in the second method, and the correction factor adds the frequencies of T cells bearing the undeleted Vβ2, -3, -4, -6, -8.1, -8.2, -8.3, -9, -10, -13, and -14 = (53.4/73.4) = 0.728.

Results

CBA/CaJ mice contain *Mtv8*, *-9*, and *-14*, while C58/J mice contain *Mtv3*, *-7*, *-17*, and *-30* (37). Since these two strains, both of which are $H\text{-}2^k$, have no *Mtv*'s in common, we were able to create a line of mice that had no *Mtv*'s by crossing the two strains. 422 (CBA/CaJ × C58/J)F_2 animals were tested by Southern blot analysis for the presence of these and any other *Mtv*'s. No other *Mtv*'s were seen, although the probe, the complete 1-kb LTR from MTV-(C3H), should bind to all *Mtv*'s still containing an LTR (37). *Mtv3*, *-7*, *-8*, *-9*, *-14*, and *-17* segregated as expected for a single genetic locus for each virus, with 75% of the F_2 progeny containing one or two copies of the virus (Table 1). However, 93.9% of the F_2 progeny contained the *Mtv30* band, indicating that two separate genetic loci were responsible for the band (Table 1).

Mtv30, like most *Mtv*'s, was originally characterized as a unique band on an EcoRI Southern blot probed with MTV LTR. It was mapped to chromosome 12 in AKR/J mice, but it did not map to chromosome 12 in C57BL/6J or NZB/B1NJ mice (38). Two back-cross experiments were conducted to verify the existence of two distinct integration sites, using CBA/CaJ mice that do not contain *Mtv30*. (CBA/CaJ × AKR/J)F_1 mice were back-crossed to CBA/CaJ to determine if more than one integration site existed for *Mtv30* in AKR/J mice. 50% of CBA/CaJ × (CBA/CaJ × AKR/J)F_1 mice would be expected to be $Mtv30^+$ if there was one integrant, but 75% would be $Mtv30^+$ if there were two integrants. Since only 25% were positive (Table 2), it is likely that only one integration site in AKR/J mice exists. CBA/CaJ × (C57BL/6J × AKR/J)F_1 mice were screened to determine if C57BL/6 and AKR mice contained the same *Mtv30* integration site. If this were the case, then the (C57BL/6J × AKR/J)F_1 parents would be homozygous for the *Mtv30* integration, and their progeny would all contain one copy of *Mtv30*. Since only 16 of 20 progeny were $Mtv30^+$, according to Southern blot analysis (Table 2), the *Mtv30* integrants in C57BL/6J and AKR/J mice are unlinked, and there are two different integration sites for *Mtv*'s that nevertheless have identical DNA band sizes after digestion with EcoRI (38), NcoI, PvuII, BstXI, BsmI, and ScaI (data not shown).

The Sequence of vSAG30. Genomic DNA from AKR/J, C57BL/6J, and RF/J mice was digested with EcoRI and separated by agarose gel electrophoresis. DNA from the 12–30-kb region, where *Mtv30* is found, was cut out, isolated, and amplified by PCR using pan-vSAG–specific 5′ and 3′ primers. PCR products were cloned into pTZ (35). Clones from all of the mice had the same sequence (Fig. 1), even though AKR/J and C57BL/6J mice have two distinct *Mtv30* proviruses with unlinked integration sites. The amino acid sequence of vSAG30 is most closely related to that of vSAG9 (Fig. 1), from which it differs by 14 residues. Perhaps surprisingly, most of these differences are not clustered at the carboxy-terminal end of the protein, the portion thought to confer Vβ specificity. If expressed, vSAG30 might therefore share the specificities of vSAG9 for T cells bearing Vβ5.1, -5.2, -11, and -12.

Generation of the Mtv-negative and Single-Mtv Mouse Lines. Five of the (CBA/CaJ × C58/J)F_2 mice that contained three or four *Mtv*'s each were selected for further breeding. An *Mtv*-negative mouse line was produced by the pathway illustrated in Fig. 2. Seven other lines were also created, each containing a single *Mtv*. Southern blots from representative mice in each line are shown in Fig. 3. The single-*Mtv* mouse lines are heterozygous for their respective *Mtv* integrants at the moment; however, homozygous lines are being generated. Later, descendants of 129/J class I knock-

Table 1. *Frequency of Restriction Enzyme Fragments Characterizing Various Mtv's in 422 (CBA/CaJ × C58/J)F_2 Mice*

Mtv DNA fragment	Chromosome	Frequency of F_2 mice positive	P (χ^2 test of one gene)	P (χ^2 test of two genes)
3	11	0.755	>0.75	<0.005
7	1	0.782	>0.1	<0.005
8	6	0.749	>0.9	<0.005
9	12	0.766	>0.5	<0.005
14	4	0.746	>0.75	<0.005
17	4	0.771	>0.25	<0.005
30	12	0.939	>0.005*	<0.95

*$\chi^2 = 68.4$.

Table 2. *Frequency of Restriction Enzyme Fragments Characterizing Mtv30 in CBA/CaJ Back-Cross Progeny*

Back-cross	No. *Mtv* 30 positive/total	Frequency
CBA/CaJ×(CBA/CaJ×AKR/J)F_1	4/16	0.25
CBA/CaJ×(C57BL/6J×AKR/J)F_1	16/20	0.80

out mice (β_2m^-) were bred to introduce other *Mtv*'s. (129/J β_2m^- × B10.BR/J)F_2 mice that were I-A^{b-}, I-E^{k+}, β_2m^- (35) were back-crossed to the *Mtv*-negative mice for three generations. This resulted in a line of *Mtv11* mice that were H-$2^{k/k}$, β_2m^+.

Mtv Expression. The level of expression of individual *vsag*'s has been difficult to determine because of the high degree of homology between them. To determine whether the endogenous *vsag*'s are expressed, we prepared cDNA from total RNA prepared from splenocytes and thymocytes from individual mice of each of the single-*Mtv* mouse lines, and from several *Mtv*-negative mice. Any *vsag* genes present in the cDNA were amplified by PCR, using pan-*vsag*–specific 5′ and 3′ primers.

vsag RNA from mice containing *vsag3*, -7, and -*17* was expressed in the spleen and thymus (Fig. 4). *vsag* RNA was found in the spleen, but not in thymocytes of mice containing *vsag8* and -*9*. Even so, however, *vsag8* and -*9* might be expressed in the thymic epithelium or in cell types less frequent in the thymus, such as macrophages, B cells, or dendritic cells. Thymocytes and splenocytes from *Mtv*-negative mice as well as *Mtv14* and *Mtv30* mice did not express detectable *vsag* transcripts.

T Cell Repertoire of Mtv-negative Mice. The native T cell repertoire of H-2^k mice, unaffected by *Mtv* superantigen selection, has not previously been determined. The TCR Vβ repertoire of lymph node T cells from *Mtv*-negative mice was measured by flow cytometry. The Vβ repertoire of CD4 and CD8 T cells was quite different (Fig. 5). For example, 9% of CD8 T cells, but only 2% of CD4 T cells, bore Vβ5.1. Conversely, 8% of CD4 T cells, but only 1.5% of CD8 T cells, bore Vβ12. Generally, Vβ2, -5.1, -5.2, -6, -7, -8.1, -9, -11, -13, and -14 were more frequent on CD8 T cells, while Vβ3, -4, -8.2, -8.3, -10, and -12 were more frequent on CD4 T cells. This disparity implies that class I and class II MHC molecules tend to select T cells bearing different sets of Vβs, because of either negative or positive selection (35).

The existence of a monovariate distribution of Vβ frequencies and small standard deviations from noninbred F_6 to F_{10} *Mtv*-negative mice from several different lines of descent (including several *Mtv*-negative siblings of the single-*Mtv* lines) imply that there are no additional superantigens encoded by background genes that differ between CBA/CaJ and C58.

Mtv14-, Mtv17-, and Mtv30-expressing Mice. T cells from mice containing only *Mtv14, Mtv17,* or *Mtv30* (either or both genes) were analyzed for Vβ frequency and compared with 21 mice containing no *Mtv*'s (Fig. 6 and Table 3). These previously uncharacterized superantigens had only small effects on the T cell repertoire. Only Vβ frequency differences with a P value <0.003 by the Student's t test are discussed.

Mtv14 mice had a slight reduction in the frequency of Vβ2 CD4 but not CD8 T cells by comparison with *Mtv*-negative animals. The sequence of vSAG14 has not been determined but may be similar to the exogenous MTVs previously reported to react with T cells bearing Vβ2 (39–41).

vSAG30 is very similar in amino acid sequence to vSAG9 (Fig. 1) and should therefore delete the same subset of T cells as vSAG9, i.e., those bearing Vβ5.1, -5.2, -11, and -12. However, *vsag30* is not expressed or is only poorly

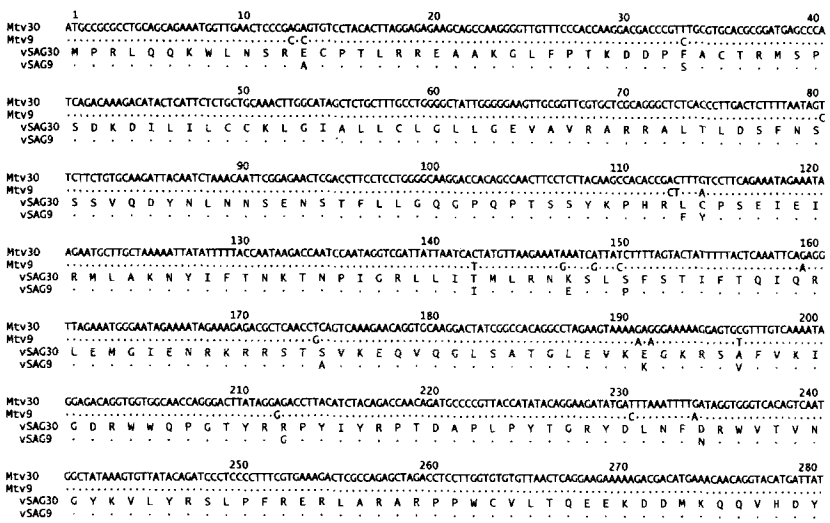

Figure 1. The DNA sequence and deduced amino acid sequence of *vsag30* compared with *vsag9*. These data are available from GenBank under accession number BankIt17116 U37259.

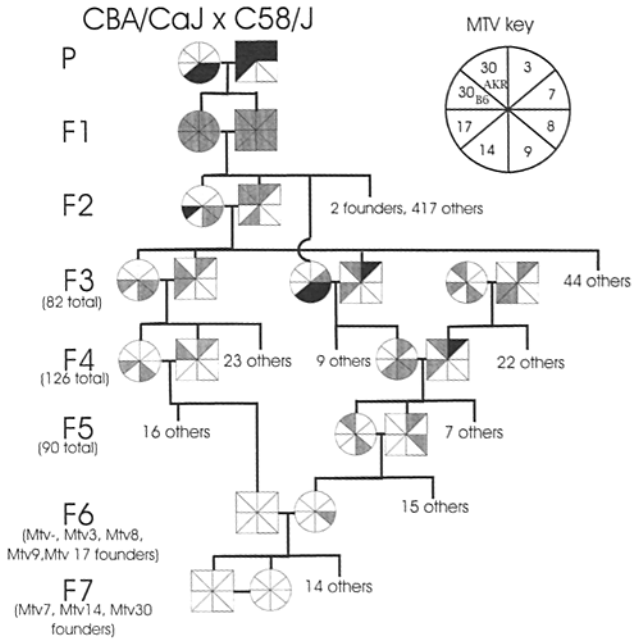

Figure 2. A lineage diagram showing the derivation of the *Mtv*-negative and *Mtv8* mouse lines. Each female or male mouse is divided into eight octants representing the eight *Mtv* proviruses present in the parental strains. The octant is shaded black if the mouse is homozygous for the particular *Mtv*, gray if heterozygous, and white if negative.

expressed (Fig. 4) and had only a slight reduction in the frequency of the Vβs not tested (Vβ1, -15, and -16) in CD4 T cells compared with *Mtv*-negative mice.

Mtv17 mice had significant decreases in Vβ11 and Vβ12 CD4 T cells (Fig. 6 and Table 3). This deletion pattern is similar to that seen in *Mtv8*, *-9*, and *-11* mice, except that T cells bearing Vβ5.1 and -5.2 are unaffected (37). The deletion is variable, not occurring in all of the *Mtv17* mice tested (data not shown). This superantigen protein may be poorly expressed, although we saw *vsag*-containing message in both splenocytes and thymocytes (Fig. 4). The deletions may be dependent on an environmental effect such as in-

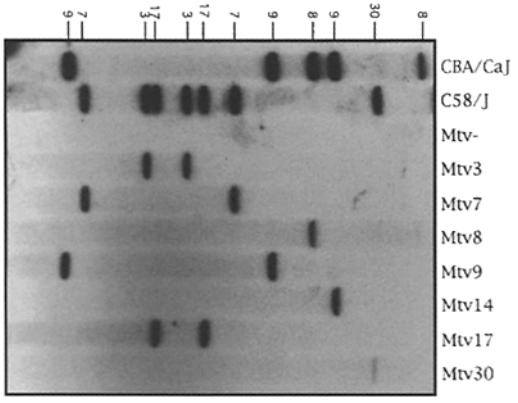

Figure 3. A Southern blot of the various mouse lines. Genomic DNA from the parental strains, the *Mtv*-negative mouse, and the seven single mouse lines containing single-*Mtv* integrants was digested with NcoI and probed with MTV LTR.

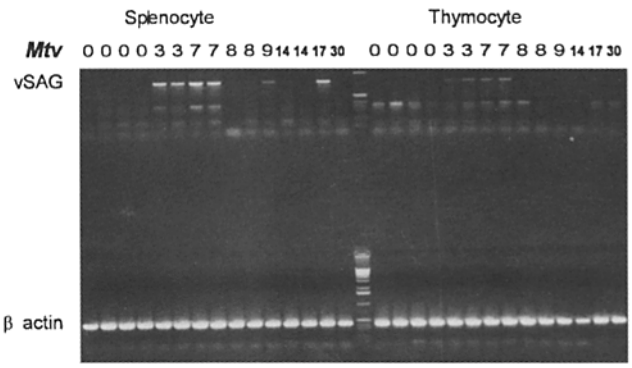

Figure 4. Expression of *vsag* transcripts as shown by RT-PCR. cDNA from spleen and thymus of *Mtv*-negative and single-*Mtv* mice was tested for the presence of *vsag* transcripts by amplifying with primers specific for conserved regions of the 5′ and 3′ ends of the *vsag* gene for 35 cycles. The 790-bp band is the expected size of the amplified *vsag* gene, while the ~400-bp band is an artifact of the PCR that is not present when only 25 cycles are used to amplify the *vsag* gene. As a positive control for the quality of the cDNA, the β-actin gene was used.

fection or stress, leading to *vsag* expression. No effect of *Mtv17* upon T cells bearing Vβ7 was seen, although the reported sequence of *vsag17* (42) is similar to that of Vβ7-deleting superantigen *Mtv23* (35). It is unlikely that *Mtv17* encodes a superantigen that is specific for Vβs that were not tested (Vβ1, Vβ15, or Vβ16), because, if one of the untested Vβs was deleted, the percentage of T cells unaccounted for by the tested Vβs should decrease, and it does not by any significant fraction (Fig. 6).

vSAG3, -7, -8, and -9 Each Delete T Cells Bearing One of Several Vβ Chains. vSAG7, encoded by *Mtv7* (formerly classified as *Mls-1*[a]) has previously been reported to delete T cells bearing Vβ6, -7, -8.1, and -9 (2, 3, 43, 44). The four *Mtv7* mice tested did delete both CD4 and CD8 T

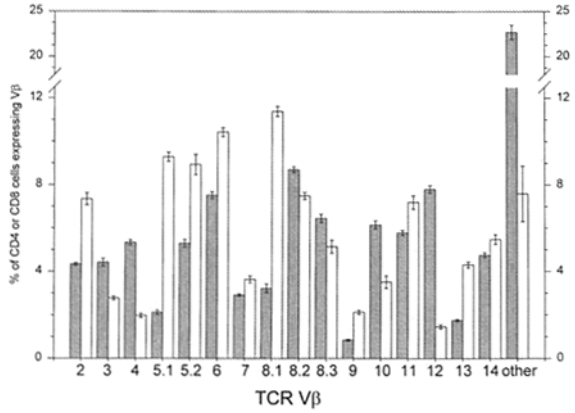

Figure 5. The Vβ T cell repertoire of *Mtv*-negative mice. T cells from lymph nodes of *Mtv*-negative mice were stained with biotinylated antibodies for the various Vβs, as well as CD4 and CD8, and analyzed by FACScan®. The data are presented as the percentage of CD4 or CD8 T cells that are positive for each Vβ. (■), CD4 T cells; (□), CD8 T cells. Each point is the average value of 21 mice ± SEM. Error bars, where not visible, are within the symbol. The frequency of other Vβs (Vβ1, Vβ15, and Vβ16) was calculated for each mouse by subtracting from 100% the frequencies of all the Vβs tested.

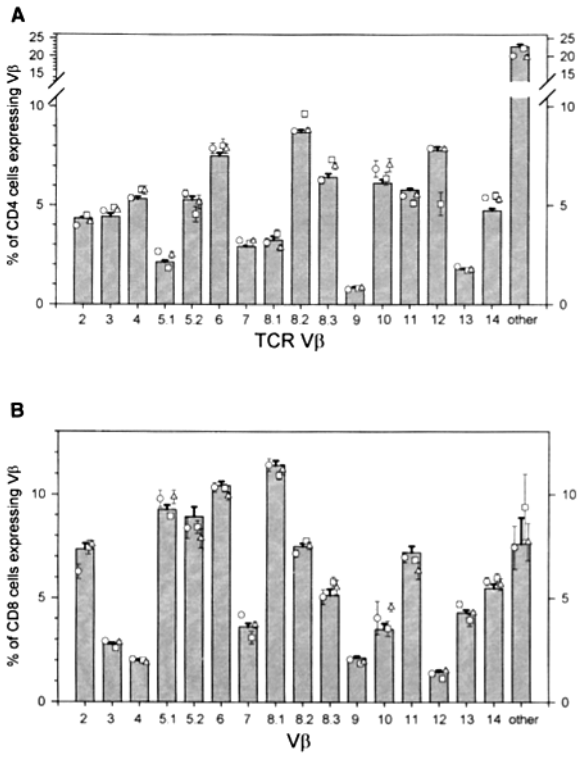

Figure 6. The Vβ T cell repertoire of *Mtv14*, *Mtv17*, and *Mtv30* mice. (*A*) CD4 or (*B*) CD8 lymph node T cells from various mouse lines were analyzed as in Fig. 4. (▨), *Mtv*-negative mice (n = 21); (○), *Mtv14* mice (n = 11); (□), *Mtv17* mice (n = 15); (△), *Mtv30* mice (n = 16).

cells bearing these Vβs and none of the other Vβs tested (Fig. 7 and Table 3). The efficiency of deletion of the various Vβs confirms the hypothesis that vSAG7 has the strongest avidity for Vβ6, followed by Vβ8.1 and Vβ9, and then Vβ7 (45).

vSAG3, a member of the *Mtv1*, *-3*, *-6*, and *-13* family of viral superantigens, has been reported to delete T cells bearing Vβ3, and this is borne out in our mice (Fig. 7 and Table 3). However, vSAG3 also reacts with CD4 and CD8 T cells bearing Vβ5.1 and -5.2, since many of these cells disappeared in *Mtv3* mice. This is a novel finding but not entirely unexpected, as vSAG6, a closely related protein, was previously reported to delete Vβ5-bearing T cells (46). Since the carboxy-terminal 60 amino acids of vSAG1, -3, -6, and -13 are almost identical, we would expect that vSAG1 and vSAG13 would also induce deletion of T cells bearing Vβ5.1 and Vβ5.2. vSAG3 also induced deletion of about one-fourth of the CD4 T cells bearing the Vβs not tested (Vβs1, -15, and -16).

Mtv8, *-9*, and *-11* have related sequences. vSAG8, vSAG9, and vSAG11 all induce deletion of CD4 T cells bearing Vβ5.1, -5.2, -11, and -12, although vSAG9 induces the most complete deletion (Fig. 8 and Table 3). However, the efficiencies of deletion of CD8 T cells differ greatly between the three superantigens, probably because of better expression of vSAG9 rather than sequence differences between the superantigens. vSAG9 deletes most of the CD8 T cells bearing Vβ5.1, -5.2, -11, and -12; vSAG11 deletes Vβ5.2-, Vβ11-, and Vβ12-bearing CD8 T cells; while vSAG8 deletes only Vβ12-bearing CD8 T cells. The specificities of vSAG8, -9, and -11 had some minor differences with those previously reported (47). In the previous paper, vSAG8 and -11 were thought not to delete Vβ5-bearing T cells. This conclusion was probably reached because CD4 and CD8 T cells were not measured separately, and vSAG8 and -11 do not delete CD8 Vβ5 T cells efficiently. Since Vβ5 T cells are overrepresented in the CD8 population compared with the CD4 population, detection of the CD4 deletion might be hindered when total T cells are compared.

Interestingly, vSAG8 also deletes CD4 T cells bearing Vβ7 (Fig. 8 and Table 3), while vSAG9 and -11 do not. This might be because of minor amino acid differences between vSAG8 and -9 (see Discussion). In addition, vSAG9 deletes about one-fourth of CD4 T cells bearing Vβ13.

Positive Selection by Mtv vSAGs. vSAGs are noted for their ability to induce deletion in the thymus of T cells bearing particular Vβ chains. However, they might also participate in positive selection. We investigated this possibility by comparing the frequencies of TCR Vβs in *Mtv*-negative and *Mtv* single-positive mice. In *Mtv* single-positive mice, the frequency of T cells bearing each nondeleted Vβ is, of course, increased. To compare the frequencies of T cells bearing nondeleted Vβs with Vβ frequencies in *Mtv*-negative mice, the frequencies must be normalized with a correction factor that makes the total frequency of T cells bearing nondeleted Vβs equal in both lines of mice (see Materials and Methods).

The corrected Vβ T cell repertoires for mice expressing single *Mtv*'s are shown in Figs. 9 and 10. There is only one significant increase in Vβ frequency compared with *Mtv*-negative mice. *Mtv11* mice have a 10% higher frequency of Vβ8.2 $CD4^+$ T cells (Fig. 10 and Table 3). This could be because of positive selection. The increased frequency of Vβ8.2 CD4 T cells was observed in two separate experiments. There were no other significant increases in Vβ frequency after correction for the deletions.

The corrected frequencies also allowed a more careful study of weak Vβ-specific deletions. If only a small proportion of T cells bearing a particular Vβ are deleted (perhaps because of the α or the D- or Jβ), the remaining T cells bearing that Vβ will increase in frequency because of other deletions, masking the small deletion that takes place. This actually occurs with several vSAGs.

Mtv7 and *Mtv*-negative mice have identical frequencies of Vβ8.2 CD4 T cells, and *Mtv7* mice actually have a higher frequency of Vβ8.2 CD8 T cells (Fig. 7). However, after correction for the other deletions caused by vSAG7, the frequency of Vβ8.2-bearing T cells is reduced by 13% in *Mtv7*-expressing mice (Fig. 9 and Table 3). This confirms earlier studies showing that vSAG7 could stimulate some but not most Vβ8.2 T cell hybridomas (2, 48). Similarly, after correction, *Mtv9* and *Mtv11* mice have reduced frequencies of CD8 T cells bearing Vβ13, while *Mtv8* mice have reduced frequencies of CD4 T cells bearing Vβ13.

Table 3. *The Percentage of T Cells Bearing Particular Vβs Deleted or Selected by Single Mtv in H-2^k Mice*

Mtv	Vβ	Percentage of CD4 T cells deleted	*P**	Percentage of CD8 T cells deleted	*P**
Mtv3	Vβ3	95%	$<10^{-16}$	89%	10^{-9}
	Vβ5.1	82%	10^{-9}	95%	$<10^{-16}$
	Vβ5.2	84%	$<10^{-1}$	81%	10^{-10}
	other	24%	0.0007		NS
Mtv7	Vβ6	93%	$<10^{-16}$	96%	10^{-10}
	Vβ7	74%	10^{-10}	66%	10^{-8}
	Vβ8.1	82%	10^{-4}	90%	10^{-5}
	(Vβ8.2)	(13%)	(10^{-5})	(14%)	(0.001)
	Vβ9	82%	10^{-9}	79%	10^{-4}
Mtv8	Vβ5.1	66%	10^{-7}		NS
	Vβ5.2	75%	10^{-4}		NS
	Vβ7	61%	10^{-4}		NS
	Vβ11	59%	0.001		NS
	Vβ12	94%	10^{-13}	65%	0.0003
	(Vβ13)	(25%)	(10^{-5})		NS
	(other)	(34%)	(0.0002)		NS
Mtv9	Vβ5.1	81%	10^{-14}	95%	$<10^{-16}$
	Vβ5.2	98%	$<10^{-16}$	95%	10^{-11}
	Vβ11	94%	$<10^{-16}$	68%	10^{-5}
	Vβ12	98%	10^{-16}	96%	10^{-9}
	Vβ13	25%	0.002	(17%)	(0.0001)
	(other)	(21%)	(0.002)		NS
Mtv11	Vβ5.1	72%	10^{-12}		NS
	Vβ5.2	70%	10^{-9}	58%	10^{-8}
	(Vβ8.2)	(+10%)	(0.0006)		NS
	Vβ11	96%	$<10^{-16}$	80%	10^{-12}
	Vβ12	98%	$<10^{-16}$	88%	10^{-8}
	(Vβ13)		NS	(21%)	(0.003)
Mtv14	Vβ2	9%	0.001		NS
	(Vβ11)	(9%)	(0.003)		NS
	(other)	(15%)	(0.001)		NS
Mtv17	Vβ11	11%	0.002		NS
	Vβ12	35%	0.0003		NS
Mtv20	(Vβ2)	(13%)	(0.001)		NS
	other	13%	0.002		NS

Values in parentheses are significant only after correcting for other deletions. A + before the percentage deleted indicates an increase in the percentage of T cells bearing that Vβ.

*Probability of no deletion as determined by Student's *t* test.

NS, No significant deletion, $P > 0.003$ by Student's *t* test.

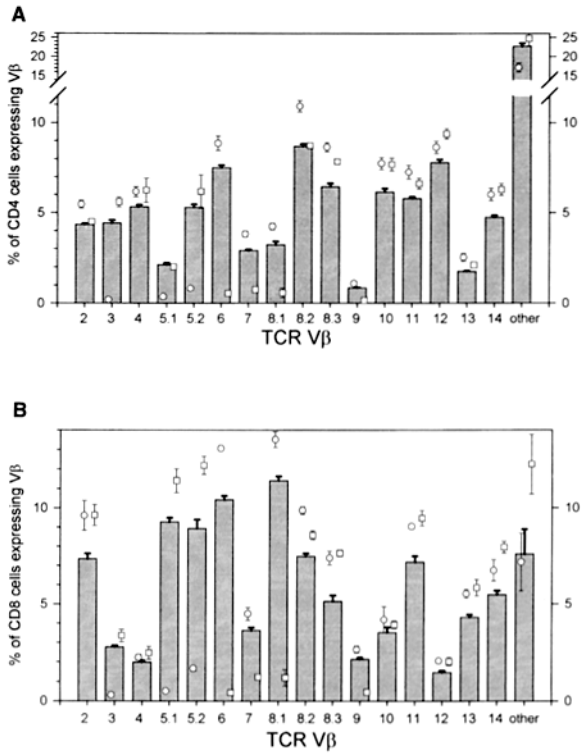

Figure 7. The Vβ T cell repertoire of *Mtv3* and *Mtv7* mice. (*A*) CD4 or (*B*) CD8 lymph node T cells from various mouse lines were analyzed as in Fig. 4. (▨), *Mtv*-negative mice (n = 21); (○), *Mtv3* mice (n = 7); (□), *Mtv7* mice (n = 4).

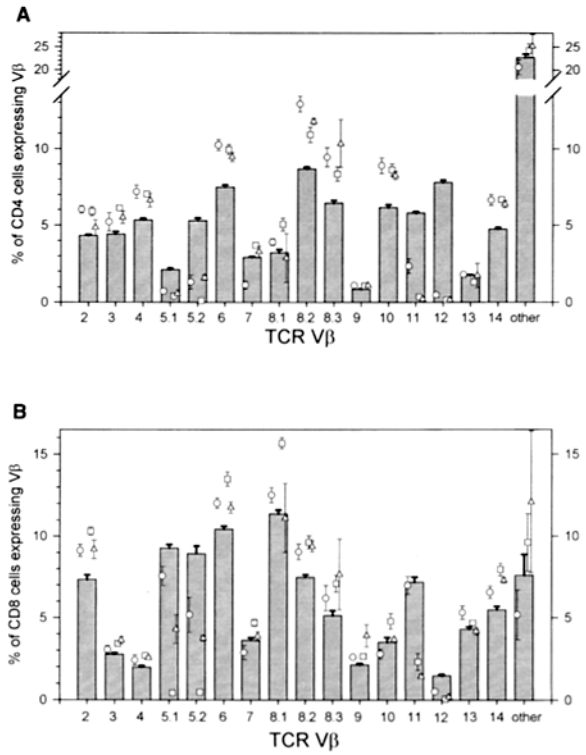

Figure 8. The Vβ T cell repertoire of *Mtv8*, *Mtv9*, and *Mtv11* mice. (*A*) CD4 or (*B*) CD8 lymph node T cells from various mouse lines were analyzed as in Fig. 4. (▨), *Mtv*-negative mice (n = 21); (○), *Mtv8* mice (n = 7); (□), *Mtv9* mice (n = 13); (△), *Mtv11* mice (n = 8).

These results and the previous observation that vSAG9 deletes CD4 T cells bearing Vβ13 indicate that vSAG8, -9, and -11 have a low avidity for Vβ13 that results in a partial deletion of those T cells. *Mtv8* and *Mtv9* mice also have slightly reduced frequencies of "other" CD4 T cells (Vβ1, -15, and -16), suggesting that one of these Vβs is also deleted. *Mtv14* mice also have reduced numbers of Vβ11 and other CD4 T cells after correction, while *Mtv30* mice have a slight reduction in Vβ2-bearing $CD4^+$ T cells.

Discussion

Mtv30 Duplication and Sequence. We have shown that there are two unlinked *Mtv30* bands in C58/J mice and that the *Mtv*'s known as 30 are at different chromosomal locations in AKR/J and C57BL/6J mice. Therefore, it is probable that C58/J mice contain both the integrant found in AKR/J and the integrant in C57BL/6J. Since the two integrants are unlinked, it should be possible to separate them on a Southern blot using an appropriate restriction enzyme. However, we could not separate the two *Mtv30* integrants using six enzymes: EcoRI, PvuII, NcoI, BstXI, ScaI, or BsmI, even though ScaI and BsmI cut within the *Mtv30* LTR (data not shown). This could be explained if the predecessor virus of *Mtv30* integrated in a region of a chromosome that was subsequently duplicated. If the flanking regions were also duplicated, the size of the bands containing the LTR would always be the same.

The sequence of vSAG30 is very similar to that of other members of the *Mls*^{f} family, vSAG8, -9, and -11. Thus, we would expect it to have the specificity characteristic of this family for T cells bearing Vβ5, -11, and -12. However, vSAG30 does not induce any deletions, probably because of poor or no expression, which could result from a defect in the promoter. Another hypothesis is that the whole chromosomal region where *Mtv30* integrated has been shut off, e.g., by excess methylation or centromeric proximity. If it is a region of chromosomal duplication, this might prevent deleterious effects of increased gene dosage to the mouse.

Specificity of vSAG8, -9, and -11. The *Mls*^{f} family, which includes *Mtv8*, *-9*, and *-11*, has a previously reported specificity for T cells bearing Vβ5.1, -5.2, -11, -12, and -17a. Our results show that they also have a weak reactivity with T cells bearing Vβ13 and at least one other Vβ among Vβ1, -15, and -16. This is probably Vβ16, as it is quite homologous to Vβ11 and -12. *Mtv17* also belongs in this family by virtue of its specificity for T cells bearing Vβ11, and -12. However, the published sequence of vSAG17 is slightly different from vSAG8, -9, and -11 (42, 49) and homologous to vSAG23, which is specific for Vβ7 (35). Although vSAG23 only stimulated T cell hybrids bearing Vβ7 (35), it is possible that it induces the deletion of T cells

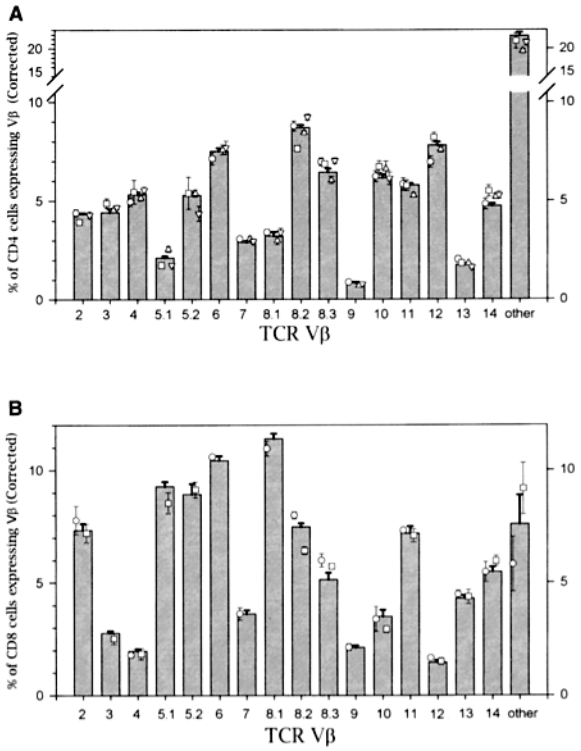

Figure 9. The nondeleted Vβ T cell repertoire of *Mtv3*, *Mtv7*, *Mtv14*, and *Mtv17* mice, corrected for the vSAG deletions. The Vβ T cell repertoire of (*A*) CD4 or (*B*) CD8 T cells from the various single-*Mtv* mouse lines in Figs. 6 and 7 was corrected for the Vβ-specific deletions induced by each superantigen to allow comparison of the nondeleted Vβ percentages with those of *Mtv*-negative mice. Vβs deleted in each mouse line and *Mtv*'s with correction factor (c.f.) = 1.00 are not shown. The c.f. was the percentage of Vβs not deleted by a particular vSAG in *Mtv*-negative mice/the percentage of those same Vβs in the single-*Mtv* mouse. (▨), *Mtv*-negative mice (c.f. = 1.00); (○), *Mtv3* mice (c.f. = 0.80 CD4, 0.81 CD8); (□), *Mtv7* mice (c.f. = 0.87 CD4, 0.75 CD8); (△), *Mtv14* mice (c.f. = 0.96 CD4, 1.00 CD8); (▽), *Mtv17* mice (c.f. = 0.96 CD4, 1.00 CD8). All data are presented as average × c.f. ± SEM × c.f.

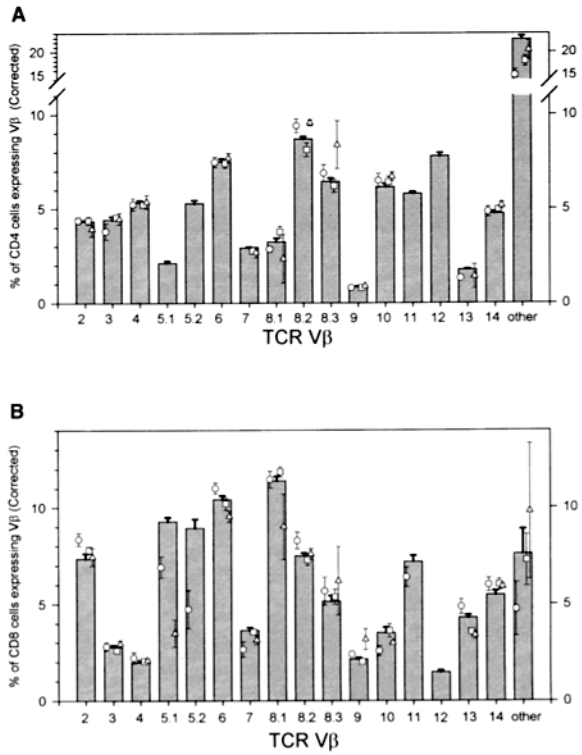

Figure 10. The nondeleted Vβ T cell repertoire of *Mtv8*, *Mtv9*, and *Mtv11* mice, corrected for the vSAG deletions. The Vβ T cell repertoire of (*A*) CD4 or (*B*) CD8 T cells from the various single-*Mtv* mouse lines in Fig. 8 was corrected for the Vβ-specific deletions as in Fig. 9. (▨), *Mtv*-negative mice (c.f. = 1.00); (○), *Mtv8* mice (c.f. = 0.73 CD4, 0.91 CD8); (□), *Mtv9* mice (c.f. = 0.74 CD4, 0.76 CD8); (△), *Mtv11* mice (c.f. = 0.81 CD4, 0.81 CD8). All data are presented as average × c.f. ± SEM × c.f.

bearing Vβ11 and Vβ12. This deletion would normally not be noticed, because all strains containing *Mtv23* also contain *Mtv8*.

Why vSAG8 Deletes Vβ7-bearing T Cells. vSAG8 deletes T cells bearing Vβ7, while superantigens encoded by other members of the *Mls*^{a} family, *Mtv9* and *Mtv11*, do not. The only sequence difference in the carboxy-terminal polymorphic region of these three superantigens is that vSAG8 has an isoleucine rather than a methionine at position 273. vSAGs contain a second polymorphic region at residues 174–198, which was shown not be involved in vSAG7 and vSAG1 specificities for Vβ8.1 and Vβ3, respectively (50). It is noteworthy, however, that the Vβ7-reacting vSAGs: vSAG8, vSAG23, vSAG(M12), and vSAG(SHN), all contain a glutamic acid at residue 176, an arginine at residue 183, and a leucine at residue 197 (35, 51). It is our hypothesis that the first polymorphic region between residues 174 and 198 contributes to specificity for Vβ7, while the specificity for other Vβs may be determined solely by the carboxy-terminal residues. The first polymorphic region may have a purely negative contribution to binding, with certain residues sterically hindering the association with Vβ7. vSAG7 and other members of the *Mls-1* family also react with Vβ7. However, since their sequence at both polymorphic regions is completely different from those of the vSAGs discussed here, they probably bind Vβ7 in a different way, such that their Vβ7 specificity is inseparable from that for Vβ6, -8.1, and -9.

Positive Selection by vSAGs. Positive selection can be defined as the process that selects for survival T cells that bear an avidity for self-MHC and foreign peptide. However, the thymocytes actually selected are only exposed to self-MHC/self-peptide during the event. The avidity hypothesis states that T cells with a low avidity for self-MHC/self-peptide are positively selected, and that these T cells will cross-react with a high avidity for self-MHC and some foreign peptide. If the avidity hypothesis of positive selection is correct, vSAGs might cause the overselection of T cells bearing Vβs with which the vSAGs could react weakly. While there have been reports of possible positive selection by vSAGs (52), others have not found any evidence of it (53–56). However, any positive selection present in these systems could have been obscured by positive selective effects that are ubiquitous to all *Mtv* vSAGs or by the deletions also occurring in most mice.

We could examine the frequency of 16 different Vβs in the mice used in these experiments with a wide range of similarities to one another. For example, the amino acid residues of Vβ12 are 68% identical to those of Vβ11, 23% identical to Vβ2, and intermediately identical to several other Vβs. We expected that at least one of these Vβs would have a low enough avidity for the vSAGs which recognize Vβ12 well to be positively selected. We found that Vβ8.2 CD4 T cells seemed to be positively selected by vSAG11. vSAG8 also led to an increase in Vβ8.2 CD4 T cells, that was not significant after correction ($P = 0.11$). Therefore, we feel that vSAG8 may also slightly positively select Vβ8.2 T cells, but that this selection is masked by our inability to correct for Vβ1 and Vβ15, since no antibody against these Vβs is yet available. Vβ8.2 is the Vβ most homologous to Vβ7 (62% amino acid homology). Since vSAG8 has a high enough avidity for Vβ7 to induce deletion, it may have a lower avidity for Vβ8.2. Similarly, vSAG11, which does not delete Vβ7, may have an even lower avidity for Vβ8.2, an avidity in the right range for positive selection to occur.

Our results indicate that vSAG7 does not positively select CD4 Vβ14 T cells ($P = 0.08$) or CD8 Vβ14 T cells ($P = 0.16$), an observation which contradicts previous findings that vSAG7 positively selected Vβ14-bearing T cells in several strains of mice, including some that shared the MHC haplotype (H-2^k) used in our experiments (52). The most probable explanation for the disparity is that the increased selection was due to strain-specific effects. Another possible explanation for the difference is that several vSAGs somehow cooperate to produce specificities that are not present in the individual vSAGs.

Although eight vSAGs were investigated for effects on 15 different Vβs, only one increase of Vβ usage was seen that could be due to positive selection, and the affected Vβ frequency rose by only 10%, after correcting for the other deletions. Several theories can be put forward to explain the paucity of observed positive selection by the vSAGs. The most interesting would be that thymocytes require another signal in addition to low-avidity binding of their TCR to be positively selected. For example, if a conformational change in the TCR is required for activation (57), this change may preclude positive selection. If vSAGs always induce the conformational change, they would be incapable of inducing positive selection.

A simpler explanation is that few of the other Vβs are homologous at the vSAG-binding site, even though there is much overall sequence homology. Since the vSAG-binding site is not yet known, this cannot be disproved. Similarly, most Vβs that bind with sufficient avidity to be positively selected may subsequently be negatively selected. This seems unlikely because of the large number of homologous Vβs and vSAGs, some of which should have interactions of low enough avidity to avoid deletion in the thymus. However, a low-avidity interaction between vSAG and Vβ might be influenced by Dβ, Jβ, or Vα. In this case, the vSAG might positively select some T cells bearing a particular Vβ while having a high enough avidity to delete other T cells bearing the same Vβ. In such a case, there could be an increase, decrease, or no change in the Vβ frequency. Again, it seems unlikely that the frequencies of all Vβs would remain unchanged.

Some aspect of positive selection may preclude most vSAGs from selecting. An interesting possibility is that the vSAG-binding site on Vβ TCR is occupied by another molecule during positive selection. This hypothetical molecule, possibly a cellular SAG structurally related to superantigens, could be an avidity enhancer molecule present only in positively selecting cells (58) or present in all TCR–MHC interactions (59). The binding of this molecule would block only low-avidity interactions of the vSAG to nonoptimal Vβs but not the high-avidity interaction with the deleted Vβs. Finally, if a particular cell type such as cortical epithelial cells were responsible for positive selection in the thymus in vivo, the positively selecting cell might not express the vSAGs.

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