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 F2 progeny of CBA/CaJ and C58/J mice, which have no Mtv integrants in common. This allowed the T cell repertoire of H-2k 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-

1Abbreviations used in this paper: MTV, mammary tumor virus; RT, reverse transcriptase; vSAG, viral superantigen.
neously, mouse lines carrying the same MHC genes (H-2k) and single Mtv integrants were produced. This was accomplished by crossing two strains of H-2k-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 VB 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 VB element would allow T cells bearing that VB element to be positively selected, and the frequency of peripheral T cells bearing that VB element would increase. When we compared the VB 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 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 (24). The probe was digested with either NcoI or BstXI, subjected to electrophoresis on 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-orfl (24). The probe was labeled with 32p 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 VBs with the following mAbs: anti-VB2, B20.6 (25); anti-VB3, KJ25a (26); anti-VB4 (PharMingen, San Diego, CA); anti-VB5.1, MR-9-4 (27); anti-VB5.1 and -VB5.2, MR-9-4 (27); anti-VB6, RR-4-7 (28); anti-VB7 (PharMingen); anti-VB8.2, F23.2 (29); anti-VB8.1 and -VB8.2, KJ16 (30); anti-VB8.1, -VB8.2, and -VB8.3, F23.1 (29); anti-VB9, MR-10-2 (31); anti-VB10 (PharMingen); anti-VB11, RR-3-15 (32); anti-VB12 (PharMingen); anti-VB13 (PharMingen); anti-VB14. 14-2 (33); and anti-TCRB 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 (OK1.5) 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 VB5.2, VB8.1, and VB8.3 T cells was calculated by subtraction. The anti-VB5 antibody was of variable quality, and the calculated frequencies of VB5.2 T cells have a large variance and unusual distribution.

PCR Amplification, Cloning, and Sequencing of vsag. 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 GGG-AATTTCGAGATGGCGCCCTGACG, and the 3' antisense oligo was GGGGATCCCTCTAGAGGGAACCCGAGGTGGG, complementary to a sequence outside the coding region.

The PCR product was cut with EcoRI1 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 Transcription (RT)-PCR. RNA was prepared from spleenocytes 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 GGG-AATTTCGAGATGGCGCCCTGACG, 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 VB frequencies for the deletions (see below), a two-tailed t test was used to determine the significance of any further positive or negative VB skewing. A P value <0.003 was required for significance, since 16 tests were performed on each mouse line (5% ± 16 = 0.003).

Frequency Correction for T Cells Bearing Nondeleted VBs. To normalize the frequency of T cells bearing undeleted VBs, these frequencies must be multiplied by the ratio of the frequency of T cells bearing these VBs in Mtv-negative mice/the frequency of T cells bearing these VBs in a single-Mtv mouse line. Since VB1, -15, and -16 could not be directly measured, two separate methods of normalization were used. The first method included the estimated frequency of VB1, -15, and -16 by subtracting the frequencies of measured VBs from 100%. However, if there was evidence of deletion among VB1, -15, or -16, then a second calcu-
lation was used, which normalized only those VBs that were directly measured and not deleted. For example, in Mtv8 mice, VB5.1, -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 VB is being deleted. Therefore, these VBs are not used in the second method, and the correction factor adds the frequencies of T cells bearing the undeleted VB2, -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-2k, 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)F2 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 F2 progeny containing one or two copies of the virus (Table 1). However, 93.9% of the F2 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)F1 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)F1 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)F1 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)F1 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/6 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 Scal (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 VB specificity. If expressed, vSAG30 might therefore share the specificities of vSAG9 for T cells bearing VB5.1, -5.2, -11, and -12.

**Generation of the Mtv-negative and Single-Mtv Mouse Lines.** Five of the (CBA/CaJ × C58/J)F2 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)F2 Mice

| Mtv DNA fragment | Chromosome | Frequency of F2 mice positive | P (χ² test of one gene) | P (χ² 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                  |

*χ² = 68.4.
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-2k 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 VBs, because of either negative or positive selection (35).

The existence of a monovariate distribution of VB frequencies and small standard deviations from noninbred F0, to F10, 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, vSAG50 is not expressed or is only poorly
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.

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 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.

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 12 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.

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.

Mtv7 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 Mtv7 mice tested (data not shown). This superantigen protein may be 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 infection or stress, leading to vsag expression. No effect of Mtv7 upon T cells bearing Vβ7 was seen, although the reported sequence of vsag7 (42) is similar to that of Vβ7-deleting superantigen Mtv23 (35). It is unlikely that Mtv7 encodes a superantigen that is specific for Vβ7s 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) 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
cells bearing these VBs and none of the other VBs tested (Fig. 7 and Table 3). The efficiency of deletion of the various VBs confirms the hypothesis that vSAG7 has the strongest avidity for VB6, followed by VB8.1 and VB9, and then VB7 (45).

vSAG3, a member of the Mtv1, -3, -6, and -13 family of viral superantigens, has been reported to delete T cells bearing VB3, and this is borne out in our mice (Fig. 7 and Table 3). However, vSAG3 also reacts with CD4 and CD8 T cells bearing VB5.1 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 VB5-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 VB5.1 and VB5.2. vSAG3 also induced deletion of about one-fourth of the CD4 T cells bearing the VBs not tested (VB5.1, -15, and -16).

Mtv8, -9, and -11 have related sequences. vSAG8, vSAG9, and vSAG11 all induce deletion of CD4 T cells bearing VB5.1, -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 VB5.1, -5.2, -11, and -12; vSAG11 deletes VB5.2, VB11, and VB12-bearing CD8 T cells; while vSAG8 deletes only VB12-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 VB5-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 VB5 T cells efficiently. Since VB5 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 VB7 (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 VB13.

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

The corrected VB T cell repertoires for mice expressing single Mtv's are shown in Figs. 9 and 10. There is only one significant increase in VB frequency compared with Mtv-negative mice. Mtv11 mice have a 10% higher frequency of VB8.2 CD4 T cells (Fig. 10 and Table 3). This could be because of positive selection. The increased frequency of VB8.2 CD4 T cells was observed in two separate experiments. There were no other significant increases in VB frequency after correction for the deletions.

The corrected frequencies also allowed a more careful study of weak VB-specific deletions. If only a small proportion of T cells bearing a particular VB are deleted (perhaps because of the α or the D- or β), the remaining T cells bearing that VB 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 VB8.2 CD4 T cells, and Mtv7 mice actually have a higher frequency of VB8.2 CD8 T cells (Fig. 7). However, after correction for the other deletions caused by vSAG7, the frequency of VB8.2-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 VB8.2 T cell hybridomas (2, 48). Similarly, after correction, Mtv9 and Mtv11 mice have reduced frequencies of CD8 T cells bearing VB13, while Mtv8 mice have reduced frequencies of CD4 T cells bearing VB13.
Table 3. *The Percentage of T Cells Bearing Particular Vβs Deleted or Selected by Single Mtv in H-2k Mice*

| Mtv  | Vβ   | Percentage of CD4 T cells deleted | P*  | Percentage of CD8 T cells deleted | P*  |
|------|------|----------------------------------|-----|----------------------------------|-----|
|      |      |        | 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^{-3}) | (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}) | (17%) | (0.0001) |
|      | (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^{-15}  | 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^{-15} | 88%    | 10^{-8} |
| (Vβ13) | NS     | (21%) | (0.003) | NS     |     |
| 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.
These results and the previous observation that vSAG9 deletes CD4 T cells bearing VB13 indicate that vSAG8, -9, and -11 have a low avidity for VB13 that results in a partial deletion of those T cells. Mtv8 and Mtv9 mice also have slightly reduced frequencies of “other” CD4 T cells (VB1, -15, and -16), suggesting that one of these VBs is also deleted. Mtv14 mice also have reduced numbers of VB11 and other CD4 T cells after correction, while Mtv30 mice have a slight reduction in VB2-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, Ncol, BstXI, Scal, or BsmI, even though Scal 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 Mtv family, vSAG8, -9, and -11. Thus, we would expect it to have the specificity characteristic of this family for T cells bearing VB5, -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 Mtv family, which includes Mtv8, -9, and -11, has a previously reported specificity for T cells bearing VB5, -11, and -12. Mtv17 also belongs in this family by virtue of its specificity for T cells bearing VB7 and at least one other VB among VB1, -15, and -16. This is probably VB16, as it is quite homologous to VB11 and -12. Mtv17 also belongs in this family by virtue of its specificity for T cells bearing VB11 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 VB7 (35). Although vSAG23 only stimulated T cell hybrids bearing VB7 (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. (I), Mtv-negative mice (c.f. = 1.00); (O), Mtv3 mice (c.f. = 0.80 CD4, 0.81 CD8); (Z), Mtv7 mice (c.f. = 0.87 CD4, 0.75 CD8); (Δ), Mtv14 mice (c.f. = 0.96 CD4, 1.00 CD8); (V), 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. (I), Mtv-negative mice (c.f. = 1.00); (O), Mtv8 mice (c.f. = 0.73 CD4, 0.91 CD8); (Z), Mtv9 mice (c.f. = 0.74 CD4, 0.76 CD8); (Δ), 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-1 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 to 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 stericly 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 VBs in the mouse used in these experiments with a wide range of similarities to one another. For example, the amino acid residues of VB12 are 68% identical to those of VB11, 23% identical to VB2, and interchangeably identical to several other VBs. We expected that at least one of these VBs would have a low enough avidity for the vSAGs which recognize VB12 well to be positively selected. We found that VB8.2 CD4 T cells seemed to be positively selected by vSAG11. vSAG8 also led to an increase in VB8.2 CD4 T cells, that was not significant after correction (P = 0.11). Therefore, we feel that vSAG8 may also slightly positively select VB8.2 T cells, but that this selection is masked by our inability to correct for VB1 and VB15, since no antibody against these VBs is yet available. VB8.2 is the VB most homologous to VB7 (62% amino acid homology). Since vSAG8 has a high enough avidity for VB7 to induce deletion, it may have a lower avidity for VB8.2. Similarly, vSAG11, which does not delete VB7, may have an even lower avidity for VB8.2, an avidity in the right range for positive selection to occur.

Our results indicate that vSAG7 does not positively select CD4 VB14 T cells (P = 0.08) or CD8 VB14 T cells (P = 0.16), an observation which contradicts previous findings that vSAG7 positively selected VB14-bearing T cells in several strains of mice, including some that shared the MHC haplotype (H-2k) 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 VBs, only one increase of VB usage was seen that could be due to positive selection, and the affected VB 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 VBs 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 VBs that bind with sufficient avidity to be positively selected may subsequently be negatively selected. This seems unlikely because of the large number of homologous VBs 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 VB might be influenced by Dβ, Jβ, or Va. In this case, the vSAG might positively select some T cells bearing a particular VB while having a high enough avidity to delete other T cells bearing the same VB. In such a case, there could be an increase, decrease, or no change in the VB frequency. Again, it seems unlikely that the frequencies of all VBs would remain unchanged.

Some aspect of positive selection may preclude most vSAGs from selecting. An interesting possibility is that the vSAG-binding site on VB 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 VBs but not the high-avidity interaction with the deleted VBs. 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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