The Open Reading Frames in the 3' Long Terminal Repeats of Several Mouse Mammary Tumor Virus Integrants Encode Vβ3-specific Superantigens

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Summary

Mice expressing the minor lymphocyte stimulation antigens, Mls-1a, -2a, or -3a, singly on the B10.BR background have been generated. Mls phenotypes correlate with the integration of mouse mammary tumor viruses (MTV) in the mouse genome. The open reading frames within the 3' long terminal repeats of the integrated MTVs 1, 3, 6, and 13 encode Vβ3-specific superantigens. Sequence data for these viral superantigens is presented, indicating that it is the COOH-terminal portion of the viral superantigen that interacts with the T cell receptor Vβ element.

A number of murine alloantigens have been described that, in combination with MHC class II products, stimulate T cells bearing particular Vβ elements (1–8). These alloantigens have been termed superantigens (9). Mice expressing such superantigens delete thymocytes bearing reactive Vβ elements during development in the thymus (10). This clonal elimination of thymocytes maintains self-tolerance by preventing mature self-reactive T cells from reaching the periphery.

The analysis of peripheral expression of T cells bearing particular Vβ elements has recently facilitated the mapping of several of these superantigens to particular chromosomal locations. Woodland et al. (11, 12) found no recombinants between the gene encoding a superantigen (Etc-1), which causes deletion of Vβ11- and Vβ5.2-bearing T cells, and a mouse mammary tumor virus (MTV)1 integrant (Mtv-9) on chromosome 12. In addition, the genes for several other endogenous mouse superantigens map to the same chromosomal regions as MTV integrants (4, 13, 14). In the past, however, some recombinants between the genetic locations of mouse endogenous superantigens and Mtv0s had been reported in the AKXD and BXD recombinant inbred mice. Suspecting that these reported recombinants may have been mistyped, Frankel et al. (15) retyped the collection of AKXD and BXD mice for MTV integrants and, after correcting previous typings, found perfect correlation between the expression of several superantigens and presence of particular MTV integrants in these mice. Thus, these investigators suggested that MTV integrants themselves code for the endogenous mouse superantigens. Independently, Dyson et al. (16) arrived at the same conclusion after mapping several ligands that delete Vβ11+ T cells.

Recently, we have shown that the infectious milk-borne MTV carried by C3H/HeJ mice codes for a superantigen that interacts with T cells bearing Vβ14 and -15 (17, 18). Furthermore, transfection experiments have shown that it is the product of the open reading frame (ORF) within the 3' long terminal repeat (LTR) of the C3H MTV that confers the ability to interact with TCR in a Vβ-specific manner (18). Acha-Orbea et al. (19) have shown, using a transgenic model, that the ORF of the infectious MTV transmitted in the milk of GR mice also causes deletion of Vβ14+ thymocytes. We have suggested the name viral superantigen (vSAG) for these ORF genes.

In this study, we have used mice expressing Mls-1a, -2a, or -3a singly on the B10.BR background to demonstrate conclusively the correlation between the expression of these Mls phenotypes and the presence of Mtv-7, -1, and -6 (vSAG-7, -1, and -6), respectively. Transfection experiments showed that Mtv-1 and Mtv-3 vSAGs encode Vβ3-specific superantigens. The sequences presented here for the MTV vSAGs with Vβ3 specificity (Mtv-1, -3, -6, and -13) strengthen the hypothesis that it is the COOH-terminal region of the vSAG that interacts with the TCR Vβ.

1 Abbreviations used in this paper: LTR, long terminal repeat; MTV, mammary tumor virus; ORF, open reading frame; VSAG, viral superantigens.
Materials and Methods

Mice. Mice were generally purchased from The Jackson Laboratory (Bar Harbor, ME). Mice carrying Mls-1', Mls-2', or Mls-3' on the B10.BR background were bred in our own facility.

Flow Cytometric Analyses. Nylon wool-purified, peripheral blood and lymph node T cells were analyzed for expression of Vβ3 and Vβ6 as outlined previously (9). T cells were stained with biotinylated KJ25 (anti-Vβ3; reference 4) or RR4-7 (anti-Vβ6; reference 20) followed by PE-streptavidin (Tago Inc., Burlingame, CA). Two-color analyses of TCR Vβ expression and CD4 and/or CD8 used directly fluoresceinated GK1.5 (21) and 53.6.71 (22), respectively.

Southern Analyses. Liver or tail DNA was digested with PvuII, subjected to electrophoresis on 0.7% agarose gels, and transferred to nitrocellulose as described by Maniatis et al. (23). Filters were hybridized with a MTV LTR probe. The probe was an EcoRI-BamHI fragment of pTZ18R-ORF (18), derived from the milk-borne C3H MTV (kindly provided by Dr. John Majors, Washington University School of Medicine, St. Louis, MO). The probe was labeled by random priming (24). Filters were washed with two final washes with 0.1% SSC, 0.1% SDS at 58°C for 30 min.

PCR Amplification, Cloning, and Sequencing of MTV vSAGs. Oligonucleotide primers specific for C3H MTV vSAG (5' sense, GGGGATCTCGAGAAGCGGGCCCGCTGCA ACTACACTATTACACCTTG) were used to amplify the vSAGs from PvuII-digested genomic DNA from DBA/2 and C58 mice. The DNA was separated on a 0.7% agarose gel and gel pieces corresponding to the 3' LTKs of Mtv-1, -6, and -13 from DBA/2 and Mtv-3 from C58 (Fig. 1) were excised. DNA purified using GeneClean (Bio 101 Inc., La Jolla, CA) was subjected to PCR amplification using a thermocycler (Cetus Corp., Norwalk, CT) using Geneclean (Bio 101 Inc., LaJolla, CA) was subjected to PCR amplification, cloning, and sequencing of MTV vSAGs. Oligonucleotide primers specific for C3H MTV vSAG (5' sense, GGGGATCTCGAGAAGCGGGCCCGCTGCA ACTACACTATTACACCTTG) were used to amplify the vSAGs from PvuII-digested genomic DNA from DBA/2 and C58 mice. The DNA was separated on a 0.7% agarose gel and gel pieces corresponding to the 3' LTKs of Mtv-1, -6, and -13 from DBA/2 and Mtv-3 from C58 (Fig. 1) were excised. DNA purified using GeneClean (Bio 101 Inc., La Jolla, CA) was subjected to PCR amplification using a thermocycler (Cetus Corp., Norwalk, CT) and the following amplification conditions: 95°C melting, 50°C annealing, and 72°C extension, each for 2 rain. The amplified products were restricted with BamHI and EcoRI and cloned into the eukaryotic expression vector pBDWMC52 (26). In case other retroviral products were necessary to ensure high expression of the vSAG products, the vSAG constructs were cotransfected with pUdH, a plasmid containing the 5' sequences of the endogenous C3H provirus Mtv-1 and the 3' sequences of the milk-borne exogenous C3H MTV (27). These plasmids were linearized with XbaI and SalI respectively, and were transfected into the B cell line CH12.1 (28) by electroporation. Transfectants were selected with G418.

Stimulation Assays. Transfectants were screened for their ability to stimulate T cell hybrids expressing Vβ3 (25-49.16 and K25-59.6; reference 4) and Vβ7 (KOX7-6.6; reference 18). 10^6 B cell transfectants were cultured with 10^5 T cell hybrids. Lymphokine production was assayed after 24 h using the indicator cell line HT-2 (29).

Results and Discussion

Endogenous Mouse Superantigens Map to MTV Integration Sites. Previously published analysis of the progeny of a (C3H/HeJ × B10.BR)F1 × B10.BR backcross has shown that C3H/HeJ mice express two Vβ3-specific superantigens, Mls-2' and Mls-3' (30). The progeny that expressed Mls-2' or Mls-3' were further backcrossed on to the B10.BR background. For each generation, the offspring were screened for Vβ3 expression and the mice expressing low levels of Vβ3 + peripheral blood T cells were used as breeders. Expression of Mls-2' causes partial deletion of Vβ3 + T cells, while Mls-3' causes complete deletion of Vβ3 + T cells (Table 1; and reference 30). Fig. 2 shows a Southern blot of genomic DNA from the backcrossed mice hybridized with a probe derived from C3H exogenous MTV LTR. Mice expressing the Mls-2' phenotype carried Mtv-1, while the Mls-3' mice carried Mtv-6. This is a formal demonstration that the Mls-2' phenotype correlates with the presence of Mtv-1, a possibility suggested by Frankel et al. (15).

Concurrently, we have backcrossed the Mls genes of CBA/J on to the B10.BR background. Deletion of Vβ6 + T cells was used as a marker for Mls-1' expression (3) and the absence of Vβ3-bearing cells was taken to indicate the presence of another Mls gene. Mls-1' expression correlates with the

| Source of Mls genes | Percent Vβ3+ lymph node T cells |
|---------------------|-------------------------------|
| B10.BR              | 3.5                           |
| CBA/J               | 0.1                           |
| C3H/HeJ             | 0.1                           |
| B10.BR-Mls-3'(F6)   | CBA/J                         |
| B10.BR-Mls-2'(F8)   | C3H/HeJ                       |
| B10.BR-Mls-3'(F7)   | C3H/HeJ                       |

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endogenous MTV LTR. The higher molecular weight bands for C3H/HeJ and DBA/2, CBA/J carry only one VB3-specific superantigen (31-33). The expression of this superantigen correlates with the presence of Mtv-7 on chromosome 1 (Fig. 2; and reference 15). It has previously been reported that, unlike C3H/HeJ and DBA/2, CBA/J carries only one VB3-specific superantigen (31-33). The expression of this superantigen correlates with the presence of Mtv-6 (Table 1 and Fig. 2), and it is therefore termed Mls-3*.

Four Different MTV Integrants Encode VB3-specific Superantigens. Analyses of VB3 expression in RI mice of the AKXD and BXD series suggested that DBA/2 mice carry at least two genes encoding VB3-specific superantigens (4, 14); one mapping to chromosome 4 and a second to chromosome 16. Reanalysis of these data and the MTV integration sites for the RI strains suggested that DBA/2 mice actually carry three VB3-specific superantigens, Mls-2*, Mls-3*, and one other, mapping to the retroviral integrants Mtv-1, Mtv-6, and Mtv-13 on chromosomes 7, 16, and 4, respectively (15). There is, however, evidence for yet another endogenous mouse superantigen that reacts with T cells bearing VB3. Several strains, including C58, NZB, NOD, and CE, have low levels of VB3 expression but do not carry Mtv-1, 6, or 13 (4, 15, 34). Fairchild et al. (35) have recently demonstrated, by analyzing the progeny of a B10.5(9R) x NOD)F1 x B10.BR backcross, that the VB3 deletion phenotype of NOD mice correlates with the presence of Mtv-3 on chromosome 11. To study the relationship between these four VB3-specific MTV integrants, we cloned and sequenced the 3' LTR vSAGs of these viruses as described in Materials and Methods.

The Mtv-1 vSAG sequence from DBA/2 (Fig. 3) differed from the published sequence for Mtv-1 from C3H (36) at two positions; first by a C-G mutation in codon 55 resulting in a Pro-Ala substitution, and second, by the insertion of a single base at the third position of codon 315. The resulting protein encoded by this ORF is therefore three amino acids longer. Since Mtv-1 in C3H mice interacts with VB3+ T cells, its suggests that the terminal three amino acids of the Mtv-1 vSAG in DBA/2 are not required for T cell stimulation or, alternatively, it is possible that the published sequence for C3H Mtv-1 may be wrong.

The Mtv-6 and Mtv-13 vSAGs were amplified from DNA from R1 strains BXD-8 and BXD-1, respectively. Each of these R1 strains carries only one MTV integrant that encodes a VB3-specific superantigen derived from DBA/2 (37). The Mtv-1 and Mtv-6 vSAG genes only differ by eight nucleotides (Fig. 3); however, it is striking that their amino acid sequences are identical. This finding is surprising, since it is known that the products of these genes differ in the efficiency with which they cause T cell deletion. Mice bearing Mtv-6 completely delete VB3+ T cells, while Mtv-1 expression results in only partial deletion of these T cells (4, 14, 15, 30). Perhaps differences elsewhere in the MTV genomes, or the different chromosomal integration sites, cause expression of the various VB3-specific MTV superantigens in different tissues or at different levels.

The Mtv-13 vSAG differs from that of Mtv-1 by seven nucleotides; five of these differences are silent and the other two result in amino acid differences at positions 28 and 45 (Fig. 3). The similarity of the three MTV integrants encoding VB3-specific superantigens derived from DBA/2 mice may be due to the insertion of similar MTVs at distinct sites in the mouse genome, on chromosomes 16, 4, and 7, or alternatively, it may be due to the transfer of the provirus from one chromosomal location to another with subsequent point mutations.

The sequence of Mtv-3 vSAG from C58 differs from the Mtv-1 vSAG by 10 amino acids (Fig. 3). All these residues, except Thr at position 317, have been found in other MTV vSAGs. Similar relationships between other MTV vSAGs can be observed in other published sequences (38-41) (Fig. 4). On the whole, amino acids found substituted in one vSAG can also be found in other ORFs, suggesting the occurrence of multiple recombination events; possibly by recombination between milk-borne infectious viruses, or alternatively, by gene conversion between the integrated proviruses. Comparisons of the published sequences of vSAGs from the exogenous C3H MTV (39) and integrated MTVs 1, 8, 9, and 11 (36, 40, 41), led us to speculate that the 30 or so amino acid residues at the COOH-terminal of the vSAGs were responsible for the VB specificity (18). This was supported by the demonstration by Acha-Orbex et al. (19) that transgenic mice expressing a variant of the C3H MTV did not delete VB14+ T cells, whereas the wild-type virus, which differs from the variant at amino acids 288-315 of the ORF, clearly caused the deletion of VB14-bearing T cells.
**Figure 3.** Sequences of V\ø3-specific MTV viral superantigens. Dashes designate spaces to allow alignment with the sequences of the other MTV vSAGs as shown in Fig. 4. These sequence data have been included in the EMBL Data Libraries under accession numbers X63024 (MTV1), X63025 (MTV3), X63026 (MTV6), and X63027 (MTV13).
The finding that all the MTV vSAGs that stimulate Vβ3* cells have similar sequences, which differ significantly from the other MTV vSAG sequences at the 3' end, strengthens the hypothesis that the 3' end of the MTV ORF encodes the Vβ-binding portion of the vSAGs (Fig. 4). This hypothesis is currently being further tested by analyzing T cell responses to chimeric vSAGs.

**Fig. 4.** Comparison of amino acid sequences for MTV viral superantigens. The consensus sequence was derived from the published MTV vSAG sequences (36, 38-41).

| Consensus | 1 | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 | 110 |
|-----------|---|----|----|----|----|----|----|----|----|----|-----|-----|
| **MTV-C3H** | 14,15 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| **MTV-GR** | 14 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| **MTV-1 (C3H)** | 3 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| **MTV-1, MTV-6** | 3 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| **MTV-3** | 3 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| **MTV-13** | 3 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| **MTV-9** | 11 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| **MTV-9** | 5,11 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| **MTV-C3H** | 14,15 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| **MTV-GR** | 14 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| **MTV-1 (C3H)** | 3 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| **MTV-1, MTV-6** | 3 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| **MTV-3** | 3 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| **MTV-13** | 3 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| **MTV-9** | 11 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| **MTV-9** | 5,11 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| **Consensus** | 120 | 130 | 140 | 150 | 160 | 170 | 180 | 190 | 200 | 210 | 220 | 230 | 240 | 250 | 260 | 270 | 280 | 290 | 300 | 310 | 320 |
| **MTV-C3H** | 14,15 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| **MTV-GR** | 14 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| **MTV-1 (C3H)** | 3 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| **MTV-1, MTV-6** | 3 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| **MTV-3** | 3 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| **MTV-13** | 3 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| **MTV-9** | 11 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |
| **MTV-9** | 5,11 | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... | .... |

The finding that all the MTV vSAGs that stimulate Vβ3* cells have similar sequences, which differ significantly from the other MTV vSAG sequences at the 3' end, strengthens the hypothesis that the 3' end of the MTV ORF encodes the Vβ-binding portion of the vSAGs (Fig. 4). This hypothesis is currently being further tested by analyzing T cell responses to chimeric vSAGs.

B cell transfectants expressing the Mtv-1 and Mtv-3 vSAGs were generated to test whether the products of these genes stimulate Vβ3-bearing T cells. CH12.1, a B cell lymphoma derived from B10.A (28), was used as the recipient cell line. Since B10 congenic mice have high levels of Vβ3 expression (4, 34), we inferred that this B cell would not carry any Vβ3-reactive MTV integrants that might confound the results. Transfectants expressing Mtv-1 vSAG or Mtv-3 vSAG stimulated Vβ3+ T cell hybrids, K25-49.16 (Table 2) and K25-59.6, while transfectants expressing the hybrid vSAG C3H construct (pUVH) alone did not stimulate these T cells (data not shown). Thus, in conclusion, these experiments confirm that the vSAGs of these endogenous MTV integrants are superantigens, as has been previously demonstrated for the infectious MTVs.
Table 2. Stimulation of Vβ3+ T Cell Hybrids by Transfectants Expressing Mtv-1 ORF or Mtv-3 ORF

| IL-2 production by T cell hybrids | K25-49.16 (Vβ3) | KOX7-6.6 (Vβ7) |
|----------------------------------|------------------|------------------|
| CH12.1 transfectants             | K25-49.16        | KOX7-6.6         |
| CMTV1-25                         | 80               | -                |
| CMTV1-35                         | 160              | -                |
| CMTV1-40                         | 320              | -                |
| CMTV1-43                         | 160              | -                |
| CMTV3-4                          | 640              | -                |
| CMTV3-8                          | 320              | -                |
| CMTV3-27                         | 320              | -                |
| CMTV3-4.4*                       | 160              | -                |
| CMTV3-4.5*                       | 640              | -                |
| CMTV3-4.7*                       | 640              | -                |

* <10 U of IL-2/ml.
+ These transfectants are clones of CMTV3-4.

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