Brief Definitive Report

Endogenous Superantigen Expression Is Controlled by Mouse Mammary Tumor Proviral Loci

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Summary

Superantigens are defined by their ability to stimulate T cells based predominantly on their Vβ expression and ability to delete T cells in the thymus when expressed endogenously. We show here that the expression of one endogenous superantigen, Etc-1, is controlled by the expression of the open reading frame region of the 3′ long terminal repeat of the mouse mammary tumor proviral gene, Mtv-9. We show that Mtv-8 controls a superantigen with similar specificity, and that both Mtv-8 and Mtv-9 stimulate some Vβ17$^+$ T cells. A third provirus, Mtv-6, controls a superantigen with specificity for Vβ3. Data presented raise the possibility that endogenous superantigens may compete for class II molecules in a single B cell.

Superantigens (SAGs) are characterized by their ability to stimulate subsets of T cells based primarily on the expression of certain TCR Vβ elements. Endogenous expression of SAGs results in the clonal elimination of these T cells in the thymus as a means of self tolerance (1). We have previously described an endogenous murine superantigen, I-E tolerogenic coligand 1 (Etc-1), that mediates the clonal elimination of Vβ5$^+$ and Vβ11$^+$ T cells in strains of mice that coexpress functional I-E molecules (2, 3). The gene for Etc-1 is very tightly linked to a mouse mammary tumor integrant on chromosome 12, Mtv-9 (2, 3). Given the genetic linkage between other endogenous mouse mammary tumor proviruses (MMTV) and SAGs (2–5), and the demonstration that other SAG genes are encoded by exogenous MMTV (6–8), we transfected Mtv-9 genes into an appropriate recipient cell to determine if this retrovirus controls Etc-1 expression. Here we show that presentation of Etc-1 to T cells is controlled by the open reading frame (ORF) of the Mtv-9 long terminal repeat (LTR). The data also indicate that: (*a*) other endogenous MMTV proviral loci in the BALB/c genome, including Mtv-8 and Mtv-6, control the expression of SAGs that interact at least with Vβ5$^+$ and Vβ3$^+$ T cells, respectively; and (*b*) both Mtv-8 and Mtv-9 control the SAG that interacts with some Vβ17$^+$ T cells. Furthermore, our data raise the possibility that endogenous SAGs may directly compete for binding to class II molecules and presentation to T cells.

Materials and Methods

DNA Constructs. The Mtv-9 envelope (env) + LTR region was isolated by digesting a 3′ EcoRI genomic fragment from the BALB/c Mtv-9 gene with BamHI. This generated a 3.6-kb insert containing the entire coding region for both the env and LTR ORF genes. The BamHI sites are ~300 bp upstream of the translation initiation codon of the env gene and 100 bp downstream of the 3′ Mtv-9 LTR coding regions. The Mtv-8 gene was isolated from a CH12 genomic library which contains the MMTV proviral loci of the B10 background (9). The 3′ Mtv-8 EcoRI fragment was digested with HindIII, giving rise to a 4.3-kb fragment containing the entire coding region for the Mtv-8 env and LTR ORF genes. The HindIII sites are ~460 bp upstream of the env mRNA translation initiation codon and 650 bp downstream of the 3′ Mtv-8 LTR coding region. The Mtv-9 and Mtv-8 env + LTR regions were cloned in both sense and antisense orientations into the BamHI or the HindIII site, respectively, of the β actin expression vector pHβAPr-1-neo (10). A 1.4-kb BglII-BamHI fragment containing the entire Mtv-9 LTR ORF was isolated and cloned in the sense orientation into the BamHI site of pHβAPr-1-neo. The 5′ BglII site is ~35 bp upstream of the first potential ORF AUG site.

Transfection. 2×10^6 A20/2J cells were resuspended in 200 μl of 270 mM sucrose containing 4 μg of linearized plasmid DNA and pulsed at 550 V for 30 μs. Electroporated cells were selected in medium containing 900 μg/ml Geneticin (Gibco Laboratories, Grand Island, NY) and maintained in 500 μg/ml Geneticin.

Stimulation Assay. B cells and T cell hybridomas were cultured for 24 h and supernatants were assayed for IL-2 as described (2).

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Responses to immobilized antibodies were determined by culturing hybridomas in wells coated with RR3-15 (anti-Vβ11) or MR9-4 (anti-Vβ5.1 + Vβ5.2) as described (3).

Cell Lines. The B cell line A20/2J and the T cell hybridoma 11-40 (Vβ11) have been described (2, 3). Other T cell hybridomas used were: 2B23-18 and 2B23-53 (Vβ17 [11]) and 4HB72(Vβ3 [12]), 18bbm11 (Vβ3 [13]), and KE8(Vβ5.1).

Results and Discussion

To test the hypothesis that Etc-1 is encoded by the Mtv-9 proviral locus, we transfected Mtv-9 genes into A20/2J recipient cells, which express class II I-E^d molecules but do not stimulate the Etc-1-reactive hybridomas, KE8 (Vβ5.1$^+$) and 11-40 (Vβ11$^+$) (2, 3). Neither Mtv-8 nor Mtv-9 appear to be transcribed in this cell line (3). A20/2J cells transfected with the Mtv-9 env + LTR in sense and antisense orientations were first tested for their ability to stimulate the Etc-1-reactive hybridoma, KE8. While none of 27 Mtv-9 antisense transfectants stimulated the hybridoma, 12 of 32 sense transfectants stimulated KE8 to secrete IL-2. Stimulation of KE8 was Vβ specific because the transfectants did not stimulate a panel of four Mls-1^a-reactive hybridomas or a panel of hybridomas expressing Vβ2, Vβ4, and Vβ13 (data not shown). Northern blot analysis of the transfectants demonstrated that novel transcripts not present in parental A20/2J cells were present in the transfectants that stimulated KE8 (data not shown). These transcripts were absent in many, but not all, of the transfectants that failed to stimulate KE8. Surprisingly, none of the transfectants stimulated a second Etc-1-reactive hybridoma, 11-40 (Fig. 1 *A*).

We also transfected A20/2J cells with a Mtv-8 fragment containing the env + 3′ LTR. 2 of 18 Mtv-8 sense transfectants stimulated the KE8 hybridoma but not the 11-40 hybridoma to secrete IL-2 (Fig. 1 *B*). None (0/16) of the Mtv-8 antisense transfectants stimulated either Etc-1-reactive hybridoma. These results suggest that at least some Vβ5$^+$ T cells interact with an Mtv-8-controlled SAG, although we have never detected an obvious influence of the Mtv-8 locus on the clonal elimination of Vβ5$^+$ T cells in H-2^d mice (2, 3). The Mtv-8 locus is highly methylated in most tissues (14), perhaps accounting for its failure to be expressed and delete T cells in some strains of mice.

To clarify whether the env gene or the LTR ORF of the Mtv-9 provirus mediates expression of Etc-1, we prepared a second construct in which the Mtv-9-3′ LTR alone was used for transfection. Since the LTR of Mtv-9 contains a single major ORF that has five putative translation start sites (9),

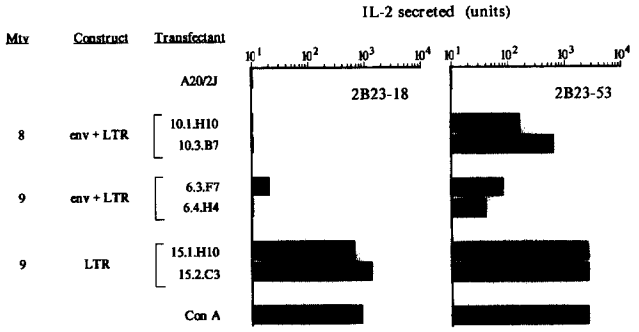

Figure 2. Vβ17$^+$ hybridomas secrete IL-2 in response to A20/2J transfected with Mtv-8 and Mtv-9 DNA. The two responding hybridomas were selected from a panel of seven hybridomas that expressed high levels of CD4 and Vβ17 and produced IL-2 in response to Con A and immobilized KJ23 (anti-Vβ17). The data are representative of three independent experiments.

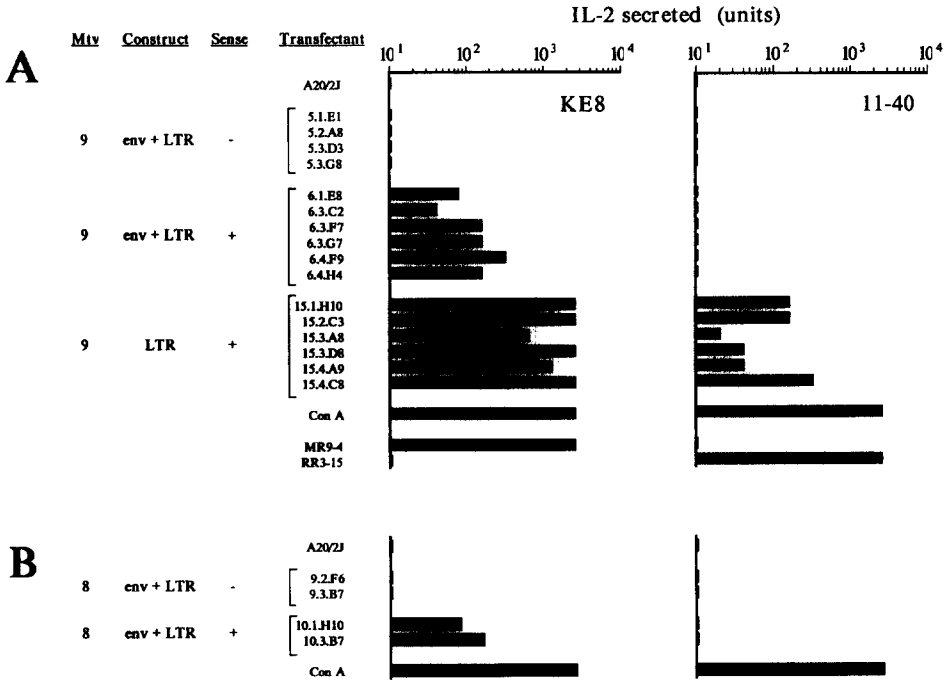

Figure 1. Etc-1-reactive hybridomas, KE8 (Vβ5.1$^+$) and 11-40 (Vβ11$^+$), secrete IL-2 in response to representative panels of A20/2J cells transfected with Mtv-9 (*A*) and Mtv-8 (*B*) DNA. A20/2J transfectants were assayed for the expression of Etc-1 by testing their ability to stimulate Etc-1-reactive hybridomas as described in Materials and Methods. The data are representative of three independent experiments.

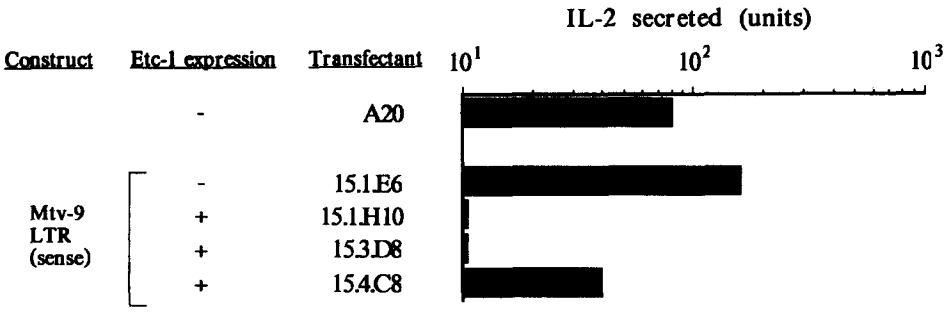

Figure 3. The response of the Vβ3$^+$ hybridoma, 4HB72, to A20/2J is reduced after transfection with Mtv-9 LTR DNA. IL-2 responses were determined as described in Materials and Methods; stimulation of 4HB72 by A20/2J cells and its transfectants was less than maximal, as determined by stimulation with Con A. Responses of Etc-1-reactive T cell hybridomas to these transfectants are shown in Fig. 1. The data are representative of four independent experiments.

we cloned a fragment that was known to encompass all of these sites. 25 of 40 A20/2J transfectants generated with this construct were very strong stimulators of KE8 (Fig. 1 *A*), indicating that Etc-1 expression is specifically controlled by the ORF of the Mtv-9 LTR. Moreover, the 5–10-fold greater stimulation of KE8 by the LTR ORF transfectants suggests that these cells express more of the SAG/I-E complex than cells transfected with the env + LTR. Analysis of the Mtv-9 LTR transfectants revealed that 15 of the 25 transfectants that stimulated KE8 also stimulated 11-40 cells, but only at moderate levels relative to positive controls (Fig. 1 *A*). These data are consistent with the hypothesis that the 11-40 hybridoma has a lower affinity for Etc-1 than KE8.

It has been shown that Vβ17$^+$ hybridomas respond to I-E molecules (15), but the SAG(s) involved have not been characterized. To determine whether this activity might be controlled by the Mtv-8 or Mtv-9 loci, we tested the ability of the transfectants to stimulate a panel of seven CD4$^+$/Vβ17$^+$ hybridomas (11). Two of the hybridomas responded strongly to the Mtv-9 LTR transfectants, whereas they did not respond to A20/2J or to A20/2J transfected with antisense constructs of Mtv-9. One of these Vβ17$^+$ hybridomas also responded to the Mtv-8 transfectants that stimulated the KE8 hybridoma (Fig. 2). These data suggest that the Mtv-8 and Mtv-9 genes control SAG(s) that interact with at least some Vβ17$^+$ T cells. The Vβ17$^+$ hybridomas, like the Vβ5$^+$ and Vβ11$^+$ hybridomas, responded more strongly to the LTR ORF transfectants than to the env + LTR transfectants, supporting the idea that the LTR ORF transfectants express higher levels of SAG/I-E on the cell surface.

While Northern analysis revealed that the MMTV 8.7-kb genomic and 3.6-kb env transcripts normally detected in B cells (9) were not present in A20/2J cells, we detected a 1.7-kb transcript (data not shown). The Mtv-6 locus, which is a truncated MMTV provirus containing primarily LTR sequences, gives rise to a 1.7-kb transcript in BALB/c spleen cells (16). Prompted by the reported linkage between the clonal deletion of Vβ3-bearing T cells and the presence of the Mtv-6 locus on chromosome 16 (4), we tested two Vβ3$^+$ hybridomas for reactivity against A20/2J cells. One of the Vβ3$^+$ hybridomas, 4HB72, responded, while the other, 18bbm11, responded only after A20/2J cells were stimulated with LPS, which enhances the expression of Mtv-6 LTR transcripts (data not shown). These results support the association between Mtv-6 and Vβ3$^+$ T cells, and strengthen the conclusion that the LTR ORF is the gene responsible for SAG activity. Together with the results of the Mtv-8 and Mtv-9 transfections, these data demonstrate that all three of the well-characterized MMTV proviruses contained in the BALB/c genome control SAG activity.

Because A20/2J cells express an endogenous SAG, we tested the transfectants to determine if a single B cell clone can express more than one SAG. Surprisingly, the 4HB72 hybridoma did not respond to two transfectants that were capable of presenting the Mtv-9 SAG to the Etc-1-reactive T cell hybridomas (Fig. 3). A third Etc-1-expressing transfectant stimulated less well than the parental A20/2J cells. In contrast, Mtv-9 LTR transfectants that failed to stimulate KE8, such as 15.1.E6 (Fig. 1), retained their ability to stimulate the Vβ3$^+$ hybridoma. The inability of some transfectants to stimulate 4HB72 could not be attributed to reduced I-E expression, since I-E levels in the transfectants were similar to A20/2J, and Northern blot analysis indicated that the levels of Mtv-6 transcripts in all of the transfectants were equivalent to levels in untransfected A20/2J (data not shown). These data suggest that SAGs may compete for binding to class II molecules and that this competition can affect the presentation of these moieties to T cells. Effective competition could result either from quantitative differences in SAG expression, or differences in the affinity of different SAGs for the same class II proteins. Competition between the endogenous Mtv-6 and transfected Mtv-9 or Mtv-8 SAGs may explain why some of the transcript-positive transfectants failed to stimulate Etc-1-reactive hybridomas (Fig. 1).

Our data demonstrate that the LTR of Mtv-9 directly controls the expression of an endogenous SAG responsible for mediating the clonal elimination of both Vβ5$^+$ and Vβ11$^+$ T cells in the thymus. We do not know whether the ORF of the MMTV LTR encodes the SAG or controls the expression of other genes that are the SAGs in murine B cells, although a direct role for the MMTV LTR in encoding SAG activity is the simplest alternative. Regardless, our data are consistent with others (6, 8), who demonstrated that the LTR of exogenous MMTVs control the expression of SAGs that interact with Vβ14$^+$ and Vβ15$^+$ T cells.

The presence of many MMTV proviruses in the murine

genome could have far reaching effects on the immune system by eliminating large fractions of the T cell repertoire. However, not all SAGs are effective in mediating clonal deletion of the relevant T cells. As in the case with Mtv-8, not all potential SAGs may efficiently transcribed in a given strain of mice. We propose a second mechanism that might account for the apparent failure of all possible SAGs to be functionally expressed, through internal competition of different SAGs for binding to MHC class II molecules.

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