Expression of a MHC Class II Transgene Determines Both Superantigenicity and Susceptibility to Mammary Tumor Virus Infection

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Summary

Milk-borne mouse mammary tumor virus (MMTV) is a type B retrovirus that induces mammary carcinoma. Infectious MMTV, as well as genomically integrated mouse mammary proviruses, encode superantigens that are recognized by T cells that express appropriate T cell receptor Vβ products. To determine the relationship between the superantigenic property of milk-borne MMTV and its in vivo infectivity, mice which were either positive or negative for expression of a transgene-encoded EαEβ class II major histocompatibility complex (MHC) product were exposed to milk-borne C3H MMTV. Superantigen-mediated deletion of Vβ14-expressing T cells occurred only in Eα transgene-positive mice, indicating that the deletion was EαEβ dependent. When mice were analyzed for viral infection by assaying viral p28 in the milk of recipient females, significant p28 levels were found only in EαEβ transgene-positive mice. Similarly, the presence of C3H MMTV LTR mRNA in mammary glands, as detected by PCR, paralleled p28 levels. These findings indicate that Eα expression or the Eα-dependent T cell response to viral superantigen is causally related to susceptibility to MMTV infection, and that lack of a permissive class II product can protect mice from virus infection.

Materials and Methods

Animals. C57BL/6 (B6) mice were obtained from Frederick Cancer Research Center (Frederick, MD). C3H/OUJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The MHC class II Eα transgenic strains 107 (B6.107) and 36-5 (B6.36) have previously been characterized (12, 13) and were generously provided by David Lo (Scripps Institute, La Jolla, CA) and B. J. Fowlkes (NIH, Bethesda, MD). Inbred transgenic lines had been established by 10 generations of back-crossing to B6 mice and selection for transgene expression; transgenic mice used in experiments had been bred no more than three generations after establishing homozygosity.

Foster Nursing. MMTV-positive C3H/OUJ females were used as foster mothers. Newborn mice of the indicated strains were transferred to C3H/OUJ females for nursing within 3 d of birth.

Flow Cytometric Analysis. When mice were at least 4 mo of age (at which time maximal deletion of Vβ14+ cells had occurred), peripheral blood was analyzed for Tcr Vβ expression. Lymphocytes were isolated from heparinized peripheral blood using Lymphocyte Separating Medium (Organon Teknica, Rockville, MD). Cells were stained using anti-Vβ culture supernatant (14, 15) or control supernatant, followed by FITC-conjugated goat anti-rat...
Ig, biotin-conjugated anti-CD4, and Texas red avidin. Two-parameter flow cytometric analysis was carried out as previously described (16).

Measurement of MMTV p28. Milk for assay of MMTV p28 was obtained from females of the appropriate strains. Pregnancies were induced, and following the birth of offspring, females were treated with 0.25 IU oxytocin i.p. and 5 min later milk was harvested by suction. ELISA was carried out by general procedures previously described (17). ELISA plates were coated with rabbit anti-MMTV p28 serum generously provided by Dr. Pierre Hainaut (University of York, York, England) (18). Plates were then washed and incubated with varying concentrations of milk or with a standard preparation of purified C3H milk virus protein. Plates were again washed and then incubated with biotin-conjugated rabbit anti-p28 antibody, washed, and incubated with avidin-conjugated enzyme followed by substrate. Plates were read spectrophotometrically, and virus protein was calculated with reference to a virus protein standard using software generously provided by Dr. Pierre Henkart (NIH, Bethesda, MD).

Polymerase Chain Reaction (PCR) Analysis of MMTV Expression. For analysis of MMTV LTR-ORF mRNA, total RNA was prepared from surgically removed mammary glands of lactating females as previously described (16, 19). cDNA synthesis was carried out as previously described (20). The cDNA samples were then amplified as previously described (20) in 100 μl PCR reaction mixture consisting of: 5 μl each of 20 μM sense and antisense oligonucleotides in TE, 5 μl of cDNA reaction, 1× PCR reaction buffer, 0.2 mM final concentration of each dNTP, and 2.5 U AmpliTag (Perkin-Elmer Cetus Instrs., Norwalk, CT) per reaction. All reactions were overlaid with mineral oil and subjected to 30 cycles of amplification using a programmed thermal cycler (Perkin-Elmer Cetus Instrs.). Each cycle consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and polymerization at 72°C for 2 min. 20-μl samples were analyzed on 1.2% agarose ethidium bromide gels in 0.5× TBE run at 200 V, stained with ethidium bromide, and visualized under UV light.

Oligonucleotide primers capable of amplifying all known endogenous and milk-borne MMTV LTR ORF molecules were chosen on the basis of highly conserved sequences among characterized ORF molecules (21): 5′ (sense) oligonucleotide: ATGCGGCGCCCTGAGCAGA (Mtv-C5); 3′ (antisense) oligonucleotide: CCAAGTCGAGGAAACCTTGCCTCAGAGCAGA (Mtv-C3). To specifically detect milk-borne C3H MMTV, a 3′ (antisense) oligonucleotide specific for the unique Mtv-C5 ORF carboxy terminus was used in combination with Mtv-C5: TCAGAGTCCACTGACCACT (Mtv-C3-3).

PCR products were transferred to a nylon membrane (Hybond-N+; Amersham, Bucks, UK) by alkali blotting. The membranes were prehybridized for 1 h in QuikHyb solution (Stratagene, La Jolla, CA). They were then hybridized with either 32P-labeled cDNA probe or labeled oligonucleotide Mtv-C5. The cDNA probe was prepared from C3H milk virus LTR-ORF mRNA generously provided by Dr. R. Callahan (NIH) (22) and radiolabeled using the random hexamer priming method. The oligonucleotide probe Mtv-C5 was 32P-5′-end-labeled by Lofstrand Labs. Ltd. (Gaithersburg, MD). Membrane-bound cDNA was hybridized overnight at 42°C with the cDNA probe or at 45°C with Mtv-C3. cDNA-hybridized filters were washed twice with 2× SSC, 0.05% SDS at 45°C for 45 min and then once with 1× SSC, 0.05% SDS at 60°C for 30 min. Mtv-C3 oligonucleotide-hybridized filters were washed at room temperature for 20 min, then once with 1× SSC, 0.05% SDS at 45°C for 20 min. Autoradiography was performed using Phosphor Screens (Molecular Dynamics Inc., Sunnyvale, CA) and Phosphor Screens (Molecular Dynamics Inc.); autoradiography was performed using Phosphor Screens (Molecular Dynamics Inc., Sunnyvale, CA) and exposed screens were scanned on a PhosphorImager (Molecular Dynamics Inc.).

Results

Influence of Eαα Transgene Expression on Vβ14 Deletion. It was previously reported that the ability of the C3H milk virus superantigen to induce Vβ14-specific deletion is MHC dependent, and it was suggested that this dependence is related to expression of an MHC class II Eαα product (2, 6). However, these previous studies did not directly test the role of class II Eαα expression in superantigen-mediated Vβ14 deletion. Consistent with previous findings, it was found in the present study that exposure to milk-borne C3H MMTV resulted in significant deletion of Vβ14+ T cells in B10.A (Eαα positive) but not B10 (Eαα negative) mice (data not shown). To directly examine the relationship of Eαα expression to Vβ14 deletion, B6 mice, which fail to express an Eαα product due to the absence of expressed Eαα, were compared with the Eαα transgenic B6 mouse line B6.107, that expresses Eαα in a normal cellular distribution (12). Newborn B6 or B6.107 mice were exposed to milk-borne MMTV by foster nursing on C3H/HeOuJ females. The resulting adult mice were then analyzed for TCR Vβ expression. Analysis of CD4+ peripheral T cells demonstrated a marked decrease in the proportion of Vβ14+ cells in B6.107 mice which had been exposed to milk-borne MMTV (1.8%), when compared to mice which had not been exposed to virus (9.7%) (Fig. 1). No differences were seen in expression of any other serologically detectable Vβ product (Vβ's 2 through 13) (data not shown). In contrast, exposure of B6 mice (Eαα negative) to milk-borne virus did not result in deletion of Vβ14+ cells (7.1% in virus-exposed mice versus 7.0% in control mice). (B6 × B6.107)F1 mice showed Vβ14 deletion indistinguishable from that of homozygous B6.107 animals, indicating that the effect of transgene insertion and expression is dominant. Another Eαα transgenic line, B6.36, has been found to express Eαα molecules only on thymic epithelial cells (12, 13). Flow cytometric analysis confirmed the expression of Eαα product on peripheral lymphoid cells of B6.107 but not B6 or B6.36 mice (data not shown). To determine whether the restricted expression of Eαα in B6.36 mice is sufficient to support Vβ14-specific deletion, B6.36 mice were also exposed to MMTV. These mice failed to undergo Vβ14 deletion after MMTV exposure, indicating that the expression of Eαα limited to thymic epithelium is insufficient to support deletion. Together, these results demonstrate that appropriate expression of an Eαα class II product is necessary for the superantigen-mediated deletion of Vβ14+ T cells in vivo.

Influence of Eαα Transgene Expression on Susceptibility to MMTV Infection. If susceptibility to MMTV infection were related to the host ability to respond to MMTV superantigen, it might be expected that Eαα expression would have an effect on viral infection that paralleled its effect on Vβ14 deletion. To test this possibility, B6 and Eαα transgenic mice that either had or had not been exposed to milk-borne MMTV were...
Expression of VB14 in MMTV-exposed mice. Thymic epithelium from B6 mice (Ecx negative), Ecx transgenic B6.107 mice (expressing Ecx in normal cellular distribution) and Ecx transgenic B6.36 mice (expressing Ecx only on thymic epithelium) were either nursed by MMTV-negative mothers or were foster-nursed by MMTV-milk-virus-positive C3H/HeOUJ females. When mice were at least 4 mo of age (at which time maximal deletion of VB14+ cells had occurred), they were bled, and peripheral blood was analyzed for the expression of VB14+ cells. Exposure to MMTV did not induce changes in VB6 expression (data not shown).

Results for VB14 expression are shown as means of 4 to 12 individual mice per group after subtraction of negative control staining. [ ], Control; [ ] MMTV exposed.

Examined for virus infection. Milk was harvested from female mice and analyzed using an ELISA specific for MMTV p28 gag product. No p28 was detected in control mice of any strain that had not been exposed to milk-borne virus. Virus-exposed Ecx-negative B6 mice had statistically in-

significant levels of p28 (Fig. 2). In contrast, the mean p28 content of virus-exposed B6.107 transgenic mice, which were Ecx positive and deleted VB14+ CD4+ T cells, was 2.03 μg viral protein/ml. Foster-nursed B6.36 mice, in which Ecx expression was confined to thymic epithelium and in which VB14 deletion did not occur, had no detectable p28 in milk. Thus, p28 levels in these strains correlated with the ability to undergo superantigen-mediated deletion of VB14+ cells.

MMTV infection was also analyzed by PCR. cDNA was

Figure 2. Assay of MMTV p28 in milk of virus-exposed or control mice. Female mice of the groups described in Fig. 1 were mated with virus-negative B6 males. Following delivery of offspring, milk was harvested by suction and analyzed for p28. The group designated as B6.107 is a pool of mice homozygous and heterozygous for the Ecx transgene. Results are expressed as ng of viral protein/ml of milk. The number of mice in virus-exposed groups was: B6 n = 11, B6.107 n = 11, B6.36 n = 4.
prepared from the mammary glands of experimental mice and was amplified using oligonucleotide primers that were either specific for the LTR of milk-borne C3H MMTV, or were derived from common sequences expressed by multiple endogenous and milk-borne MMTV LTR's and were therefore capable of amplifying cDNA from all of these genes. Amplified products were analyzed by electrophoresis and hybridization with an MMTV oligonucleotide probe. Virus-exposed Ea-transgenic B6.107 mice were positive for PCR-amplified C3H milk virus product, while transgene-negative B6 mice were negative (Fig. 3 A). Transgene-positive and negative mice, with or without exposure to milk virus, expressed products corresponding to common MMTV sequences shared by endogenous proviruses (Fig. 3 B). The observed products were of a size consistent with that expected for amplified MMTV product (21). The same results were seen after hybridization with a cDNA probe (data not shown). All mice also expressed similar quantities of PCR-amplified β2-microglobulin product (data not shown). PCR analysis thus indicated that expression of the Ea transgene was required for susceptibility to infection by milk-borne MMTV, in agreement with the results of viral p28 analysis.

Discussion

These findings demonstrate that host susceptibility to MMTV infection in vivo is strongly influenced by expression of a class II Ea product. The mechanism underlying this effect is not certain. However, the observation that both the superantigenic effects of MMTV and the susceptibility of mice to viral infection are similarly dependent upon expression of a transgenic Ea product suggests that the T cell response to viral superantigen is causally involved in viral infectivity. The expression of proviral mts genes has been shown to be greatly enhanced in T and B lymphocytes following activation of these cells (9). Thus, it is possible that introduction of infectious MMTV into the host results in infection of cells including lymphoid cells, and that class II EaEβ-positive cells such as B cells express and present the MMTV superantigen to T cells expressing appropriate TCR Vβ products. The resulting activation of T cells and the consequent activation of B cells might results in enhanced viral expression and replication.

From such a model, it would be predicted that any maneuver which interferes with the superantigenic stimulation of T cells by MMTV-expressing cells would result in decreased susceptibility to virus infection and replication. The "knockout" of EaEβ expression in the wild type B6 strain is one such maneuver. In this situation, the failure of class II* cells to express a permissive EaEβ product results in failure of superantigenic stimulation of VB14* T cells and a consequent resistance to viral infection. A different approach was taken in the work of Golovkina et al. (23), who reported that the expression of a transgene encoding the C3H MMTV superantigen resulted in VB14 deletion and in decreased susceptibility to infection by milk-borne MMTV. In this case, the interaction of VB14* T cells with EaEβ* cells expressing an endogenous transgenic superantigen results in the deletion of VB14* T cells and a preempting of their ability to interact with cells bearing the infectious MMTV.

There thus appear to be two distinct mechanisms by which animals can be protected from susceptibility to infection with tumorigenic MMTV retroviruses. One mechanism involves the expression of mts proviruses that lead to deletion of T cells that facilitate MMTV infection. The fact that multiple Vβ-specific endogenous mts superantigens are conserved in the mouse suggests that this may be a biologically relevant mechanism of host protection. A second and distinct mechanism for host protection from MMTV infection is the failure to express class II EaEβ. The fact that many wild as well as inbred mice have conserved genomic defects in class II EaEβ expression is consistent with the biologic relevance of this mechanism as well. Both the expression of endogenous superantigens and the widespread deletion of a permissive class II product are phenomena which have to date been observed only in the mouse, suggesting that they may reflect a unique relationship between host and MMTV retroviruses in this species.

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