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Major Histocompatibility Complex Class II I-E-Independent Transmission of C3H Mouse Mammary Tumor Virus

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C57BL/6 mice are resistant to C3H mouse mammary tumor virus (MMTV)-induced mammary tumorigenesis and lack major histocompatibility complex class II I-E molecules that are essential for presentation of C3H superantigen to T cells. T cells are needed for transmission of milk-borne MMTV from the gut to the mammary gland. In this report, we show that infectious C3H MMTV is produced by C57BL/6 mice that nurse on C3H mothers but that virus production in the mammary gland is delayed compared with that in I-E$^+$ mouse strains.

Mouse mammary tumor virus (MMTV) is a type B retrovirus that causes mammary tumors at a high frequency in susceptible strains of mice (14). Neonatal mice ingest C3H MMTV by nursing on virus-infected mothers, and the ingested virus infects B cells within the Peyer's patches of the small intestine (9). These infected B cells present an MMTV-encoded superantigen (Sag) to T cells (1, 2). Several reports have suggested that C3H MMTV Sag presentation is completely dependent on the presence of major histocompatibility complex class II I-E molecules, whereas other MMTV Sags primarily use I-E molecules but also I-A molecules to some extent (15, 16). Sag appears to be required for virus infection, because mice devoid of Sag-reactive T cells are resistant to infection by C3H MMTV (3, 6, 7). Furthermore, recent evidence has shown that the mammary glands of major histocompatibility complex class II I-E-negative C57BL/6 (B6) mice, a strain resistant to tumorigenesis by C3H MMTV, are not infected by the virus (15). However, when a functional I-E molecule was reconstituted by the I-Eα transgene in B6 mice, the mammary glands of the transgenic animals were infected by MMTV (15).

Most inbred strains of mice contain integrated copies of endogenous MMTVs within their genomes. Endogenous MMTVs are transmitted as Mendelian traits from parents to offspring and often are defective for production of virus particles (11). Recently, it has been found that B6 mice that nursed on C3H/HeN mothers increased expression of RNAs potentially encoding the endogenous MMTV Sag proteins (18), and these RNAs were derived from endogenous *Mtv-17* expressed in gut-associated lymphocytes. These results suggested that there was a limited degree of immune-cell stimulation in class II I-E-negative mice infected by milk-borne MMTV, since endogenous MMTVs have increased expression in stimulated lymphocytes. Although limited compared with that in I-E-positive mice, the stimulation observed in B6 animals may be sufficient to allow infection by exogenous MMTV. Indeed, B6 mice are not completely resistant to C3H MMTV infection, since 6 to 63% of B6 mice that nurse on C3H MMTV-positive mothers (B6fC3H mice) develop mammary tumors, in contrast to <1% of B6 mice that nurse on their natural mothers (8, 12).

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We compared MMTV expression in the mammary glands of B6fC3H mice with expression in mammary glands of two different I-E-positive mouse strains, BALB/cfC3H and C3H/HeN, during their first lactations. RNA was extracted from the lactating mammary glands of these mice at 3 to 5 days postpartum by the single-step guanidinium method (10). RNase protection assays (RPAs) were performed with a riboprobe spanning the polymorphic region of the MMTV long terminal repeat (−455 to −116) as described by Golovkina et al. (5). This probe allows us to distinguish between C3H and endogenous MMTV expression since C3H MMTV provides full-length protection (~340 nucleotides) from RNase digestion, whereas endogenous *Mtv-6* and *Mtv-1* (C3H/HeN), *Mtv-6* (BALB/c), and *Mtv-17* (B6) provide only partial protection (122 and 105 nucleotides for *Mtv-6* and *Mtv-1* or 134 and 122 nucleotides for *Mtv-17*). The level of expression of C3H MMTV in the mammary glands of B6fC3H mothers was very low during the first lactation (Fig. 1). However, expression by the infecting C3H MMTV was 17-fold greater in BALB/cfC3H and 34-fold greater in C3H/HeN mouse mammary glands during the first lactation, as determined by densitometry (Fig. 1; compare lanes 1 to 3). Since the BALB/c and C3H/HeN strains are highly susceptible to tumorigenesis by C3H MMTV but the B6 strain is not, our data support the hypothesis that tumor resistance correlates with the lack of viral replication during early lactation periods.

MMTV infection of the mammary gland is known to be amplified during successive lactation periods (8, 17). Therefore, we used RPAs to determine if C3H MMTV expression was detectable in infected B6 mice at later lactations. To our surprise, the level of infection detectable in the mammary gland of a B6fC3H mouse was approximately equivalent to that of a C3H/HeN animal at the same lactation (Fig. 1, lanes 4 to 7). It has been reported previously that B6 mouse mammary glands are not infected after C3H MMTV infection (15), a result inconsistent with previous reports of C3H MMTV-induced mammary tumors in B6 mice (8, 12). Nevertheless, we confirmed the identity of our B6 mice by the presence of specific endogenous MMTV DNA by using Southern blotting, by the expression of specific endogenous MMTVs by RPAs, by the presence of *Mtv-17*-specific PCR products derived by reverse transcription of RNA from B6 mice, and by the inability of this strain to delete cognate T cells for endogenous MMTVs (18) (Fig. 2 and data not shown).

To ensure that we had not documented a rare occurrence,

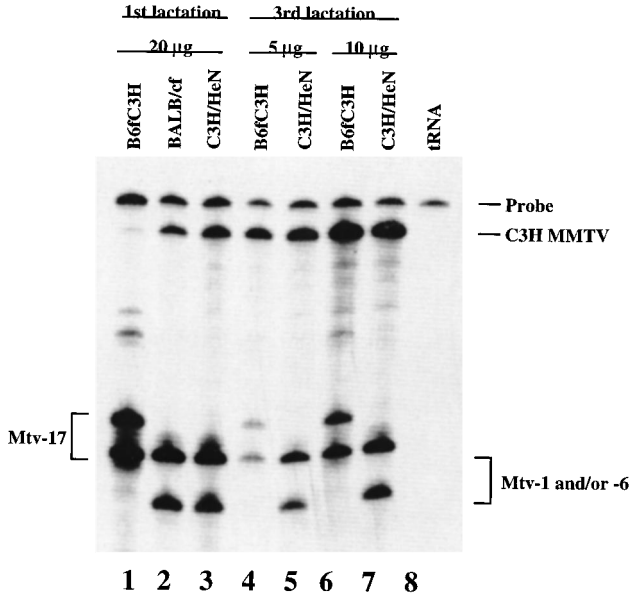

FIG. 1. Mammary glands of B6fC3H mice have low-level C3H MMTV expression during the first lactation period. Total RNA was extracted from B6fC3H, BALB/cfC3H (BALB/cf), and C3H/HeN mouse mammary glands during the first or third lactation period at 3 to 5 days postpartum. Cellular RNA (5 to 20 μg as indicated and the appropriate amount of yeast tRNA to adjust to 50 μg) was hybridized to 6×10^5 cpm of an antisense probe for the polymorphic region of the C3H MMTV long terminal repeat (5). Expression of C3H MMTV is indicated by a single band, whereas the endogenous MMTVs give two bands with higher mobilities. Differences in the sequences of the endogenous viruses allow for different amounts of protection of the riboprobe. Endogenous MMTV expression from *Mtv-17* in B6 mice, *Mtv-6* in BALB/c mice, and *Mtv-1* and/or *Mtv-6* in C3H/HeN mice is observed. Yeast tRNA (50 μg) was used as a negative control. The position of the probe (440 bases) is shown.

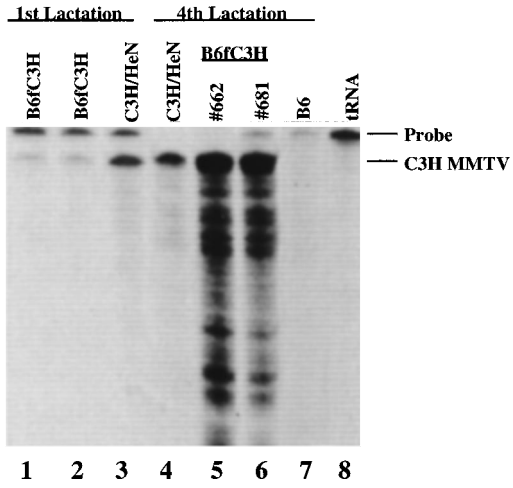

FIG. 2. B6fC3H mice produce low levels of MMTV particles during the first lactation period. Virus particles were extracted from the stomachs of three to five neonatal mice for each sample. Samples were obtained from two B6fC3H female mice and one C3H/HeN mouse during their first lactation period or one C3H/HeN mouse and two B6fC3H mice during their fourth lactation period. RNA was used for RNase protection assays as described for Fig. 1. An identical experiment was performed with uninfected B6 mice that nursed on their own mothers. Yeast tRNA was used as a negative control. The positions of the probe and the fragment indicating C3H MMTV expression are indicated. The fragments that migrate faster than the C3H-specific band presumably are due to degradation of viral RNA extracted from MMTV particles.

two additional B6 mice that nursed on C3H/HeN mothers were tested for the presence of C3H MMTV in their milk at the fourth lactation. Viral RNA was obtained from milk as described by Golovkina et al. (5) and used for RPAs. Although this method is not extremely quantitative, MMTV particles could be detected in the milk of both B6fC3H mice tested as well as in the milk of a C3H/HeN MMTV-infected mother, whereas no virus was detected in B6 mice that nursed on their own virus-free mothers (Fig. 2). RPAs using milk obtained from additional B6fC3H females at their first lactation indicated that a low level of C3H virus was detectable (Fig. 2, lanes 1 and 2).

Because B6 mice are known to lose milk-borne C3H MMTV within a few generations (8), we assayed the ability of the B6fC3H mice to transmit C3H MMTV to susceptible (B6fC3H $\times$ BALB/c)F_1 offspring. Because the BALB/c strain is +/+ for the MHC class II I-E molecule (16), the F_1 generation will contain functional I-E molecules that will present MMTV-encoded Sag to cognate T cells and allow the deletion of these stimulated cells. Thus, transmission of C3H MMTV to the F_1 offspring was determined by Sag-mediated deletion of Vβ14$^+$ (C3H MMTV Sag-reactive) T cells. We monitored three successive litters of F_1 mice for Vβ14$^+$ T-cell deletion. Histopaque-purified peripheral blood lymphocytes were stained with labeled CD4 and Vβ14 antibodies by standard techniques and were analyzed by flow cytometry (3). Consistent with the low level of mammary gland infection during the initial lactation period, the first litters of the three B6fC3H females showed sporadic infection of their F_1 offspring as measured by the failure to detect Vβ14$^+$ T-cell deletion after 8 months (Table 1). However, similar experiments showed that all offspring of the second and third litters were infected. The data indicate that B6fC3H mice produce infectious C3H MMTV but that some first-litter offspring receive insufficient virus to allow viral infection. These results explain why B6fC3H mice rapidly lose milk-borne virus (8), particularly if first-litter animals are chosen for breeding.

Since the MMTV infection of mammary tissue in B6fC3H mothers appeared to amplify during successive lactations, the amount of virus received by the offspring should increase with each lactation of the mother. By studying the kinetics of deletion in successive (B6fC3H $\times$ BALB/c)F_1 litters from the same mother, we determined the effect of viral dose on Sag-mediated deletion (Fig. 3). As expected, deletion of C3H MMTV Sag cognate T cells (Vβ14$^+$) was more extensive and occurred earlier in animals receiving the highest viral load (from third-lactation mothers) than in animals receiving the lowest viral load (from first-lactation mothers).

In this report, we have shown that B6 mice are susceptible to infection by C3H MMTV, despite the inability of these mice to present C3H Sag through class II I-E molecules. However, we

TABLE 1. Sporadic infection of first-litter (B6fC3H $\times$ BALB/c)F_1 mice

B6fC3H mother	No. (%) of offspring infected[a]		
	First lactation	Second lactation	Third lactation
662	4/4 (100)	ND[b]	5/5 (100)
681	3/5 (60)	3/3 (100)	5/5 (100)
692	1/5 (20)	ND	5/5 (100)
Total	8/14 (57)	3/3 (100)	15/15 (100)

[a] Mice were considered infected if at least 30% of CD4$^+$ Vβ14$^+$ T cells were deleted at 20 weeks.
[b] ND, not determined.

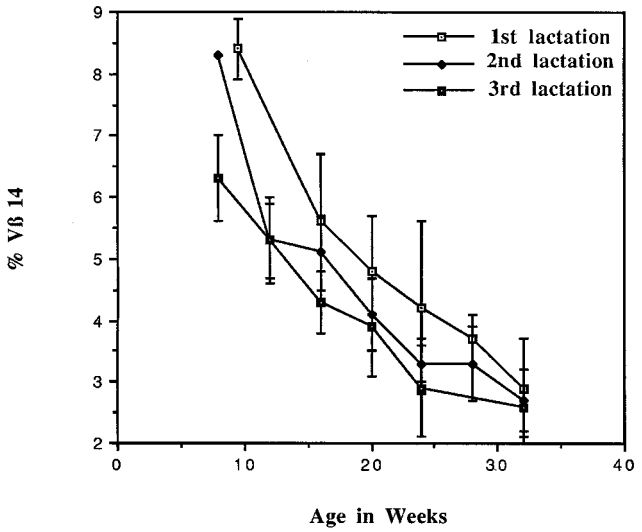

FIG. 3. Deletion kinetics of Vβ14$^+$ T cells in (B6fC3H × BALB/c)F_1 mice. B6fC3H females (I-E −/−) were mated with BALB/c males (I-E +/+) to give F_1 (I-E +/−) offspring. Peripheral blood lymphocytes were obtained every 4 weeks from mice between the ages of 8 and 32 weeks. Lymphocytes were separated with a Histopaque cushion (Sigma Chemical Co., St. Louis, Mo.) and stained with phycoerythrin-conjugated CD4 and fluorescein-conjugated Vβ14 antibodies (PharMingen, San Diego, Calif.). Cells were analyzed by using the Lysis II program and a FACSort cytometer (Becton Dickinson, San Jose, Calif.). Cells were gated for the expression of CD4. Percentages were calculated by dividing the number of CD4$^+$ Vβ14$^+$ cells by the number of CD4$^+$ cells. Each datum point is the average for 3 to 15 mice, and standard deviations are shown. Only mice confirmed to be C3H MMTV infected were included. Mice were grouped according to the litter number of each B6fC3H mother: first litters (1st lactation), second litters (2nd lactation), and third litters (3rd lactation). The average percentage of Vβ14$^+$ CD4$^+$ T cells in (B6 × BALB/c)F_1 mice was 9.2 ± 0.4.

previously found that endogenous *sag* RNA is induced by milk-borne virus of B6 mice, which is consistent with low-level lymphoid-cell proliferation in the intestine and spleen (18). Since C3H mice package endogenous *Mtv-1* RNA (5) and since *Mtv-1* Sag may be presented by class II I-A molecules present in B6 mice (16), lymphoid-cell proliferation in B6fC3H mice may be due to *Mtv-1* Sag. Therefore, we tested for deletion of *Mtv-1* cognate (Vβ3$^+$) T cells in B6fC3H animals. We found that most B6fC3H mice deleted approximately 40% of their CD4$^+$ Vβ3$^+$ T cells (2.6% ± 0.5%), in contrast to B6 mice that nursed on their own mothers (4.1% ± 1.1%). However, B6fC3H mice lacked detectable *Mtv-1* RNA in their mammary glands, implying that any potential for infection by *Mtv-1* is limited. Consistent with this observation, B6 mice that nursed on B6fC3H mothers failed to delete Vβ3$^+$ T cells (4.3% ± 0.5%) but were infected by C3H MMTV, as determined by the presence of viral particles in milk (data not shown). Thus, the Sag activity provided by *Mtv-1* is not required for C3H MMTV infection of B6 mice.

It remains possible that a secondary pathogen provided lymphocyte stimulation in the MMTV-infected B6 mice, thus substituting for the stimulation normally provided by C3H Sag. However, this implies that a normal immune response to other infectious agents would provide an adequate environment for MMTV infection. We believe that this would undermine the necessity for MMTV Sag in wild mice, since such animals probably encounter a multitude of pathogens that trigger an immune response. Nevertheless, all analyzed MMTVs from mice contain functional Sags (16), implying that there is a strong selection for Sag activity.

This study questioned the requirement for Sag in the MMTV life cycle, as did work by Penninger et al. (13). In the latter experiments, CD4 knockout mice were infected by C3H MMTV with delayed kinetics in the absence of detectable Vβ14$^+$ T-cell deletion. However, Golovkina et al. (4) suggested that Sag was required for C3H MMTV transmission since viruses containing a *sag* frameshift reverted at high frequency after a single cycle of MMTV transmission by milk. These seemingly contradictory sets of data might be explained by one or more of the following. First, if Sag expression increases the efficiency of infection, but is not required, *sag*-containing viruses will quickly outnumber those that lack *sag* coding capacity. Second, Sag may be required for lymphoid-cell transmission, but this transmission may not require CD4 or class II I-E molecules. Indeed, we have observed a 30% decrease in the CD4$^+$ Vβ14$^+$ T cells of 11-month-old B6fC3H mice compared with the number in uninfected control animals (data not shown). This implies that C3H MMTV Sag functions, at least partially, in conjunction with I-A molecules or by a class II-independent mechanism. Third, Sag may have a role in the MMTV life cycle other than stimulation of T-cell proliferation. Therefore, selection for several Sag functions during the MMTV life cycle would heavily favor reversion of a *sag* frameshift mutation. Current experiments in our laboratory should provide further insight into Sag function.

The infection of B6 mice was delayed compared with that in mouse strains that are susceptible to C3H MMTV-induced mammary tumors. Therefore, it is possible that Pucillo et al. (15) failed to detect milk-borne MMTV infection because they tested animals during the first lactation period. Nevertheless, our data and those of Pucillo et al. (15) are in agreement that expression of class II I-E increases the efficiency of C3H MMTV infection. Since mammary glands of B6fC3H mice eventually produce C3H MMTV at levels equivalent to those in C3H/HeN animals, our data imply that tumorigenesis may be dependent, at least in part, on MMTV infection of developing mammary tissue.

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