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Initial Stages of Mammary Tumor Virus Infection Are Superantigen Independent

Yelena Pobezinskaya, Alexander V. Chervonsky, and Tatyana V. Golovkina

Exogenous mouse mammary tumor virus (MMTV) is transmitted via the milk from infected mothers to newborn pups. Efficient MMTV transmission is dependent on proliferation of T cells with particular TCR β-chains, which occurs upon recognition of virally encoded superantigen (SAg) bound to MHC class II molecules. It is assumed that infection of these dividing cells favors MMTV amplification. SAg is important for MMTV infection, as mice that lack SAg-cognate T cells due to expression of endogenous Mtv loci or mice that express inappropriate MHC haplotypes unable to present viral SAg efficiently were shown to be resistant to MMTV infection. However, this resistance was not absolute, as these mice developed late onset MMTV-induced mammary tumors. In this study, we show that the success of initial MMTV infection in neonates is independent of SAg function but depends on the developmentally regulated proliferation of target cells. However, SAg was absolutely required for virus spread following completion of this proliferative stage. The Journal of Immunology, 2004, 172: 5582–5587.

Mice mammary tumor virus (MMTV) is a retrovirus that can be transmitted exogenously through milk or endogenously through the germline (1). The primary targets for exogenous MMTV are T and B cells located in Peyer’s patches of the gastrointestinal tract of neonatally infected pups (2, 3). Cells of the mammary glands are the major targets for MMTV in adult mice, as secretion of MMTV into the milk is a critical step in its life cycle. A by-product of multiple rounds of reintegration in the mammary glands is the development of carcinomas.

MMTV has evolved mechanisms to evade and to exploit the immune system. For example, MMTV subverts the innate immune response via Toll-like receptor 4-mediated production of IL-10 by B cells, which suppresses the anti-virus adaptive immune response (4). In another exploitation of the immune system, virally encoded superantigen (SAg) (5), which is presented by MHC class II proteins and is recognized by all T cells expressing particular TCR Vβ domains (6), stimulates a large proportion of T cells to divide, thereby creating an infection-competent pool (7).

In contrast to SAgs encoded by exogenous viruses, SAgs encoded by endogenous MMTVs promote deletion of the Vβ T cell subset via negative selection during differentiation of the immune repertoire of the mouse (8–10). Mice lacking SAg-cognate T cells due to expression of endogenous viral SAg were found to be resistant to exogenous viruses bearing SAgs of the same Vβ specificity (7, 11). It was concluded that SAg function is critical for efficient virus transmission. However, such resistance was not absolute, as mice without SAg-cognate T cells developed MMTV-induced mammary tumors, albeit with much longer latency compared with mice having SAg-cognate T cells (12). Furthermore, viruses without functional SAgs were able to establish infection in vivo, although this infection did not spread beyond cells of the immune system (13). Together, these data suggested that there may be two stages in MMTV replication: SAg independent and SAg dependent.

The objective of our study was to determine which stages in MMTV infection and virus spread require SAg and which do not.

Materials and Methods

Mice

All mice used in this study were bred and maintained at the animal facility of The Jackson Laboratory. C3H/HeN MTV-ORF transgenic mice are originally purchased from Dr. I. Piazzon, Instituto de Investigaciones Hematologicas, Academia National de Medicina de Buenos Aires, Buenos Aires, Argentina) were passed on BALB/cJ mice (BALB/cLA mice) (14).

Abs and FACS analysis

FITC-coupled mAbs against the Vβ14, Vβ2, and Vβ6 TCR chains were purchased from BD Biosciences (San Diego, CA). Anti-CD4 Ab (GK 1.5) coupled to PE was purchased from Immunotech (Carlsbad, CA). A FACSScan (BD Biosciences) flow cytometer and CellQuest software program were used for FACS analysis.

B cell proliferation assay

A total of 5 × 10^6 splenocytes isolated from DBA/2J and BALB/cJ mice of different ages was treated with CFSE (Molecular Probes, Eugene, OR) (15) followed by FACS analysis. Dead cells were gated out with propidium iodide.

Virus infection

Mice were either fostered by viremic BALB/cLA females or injected as neonates or as adults (3–4 wk old) with 5 × 10^6 splenocytes isolated from infected syngeneic mice. T cells were removed from splenocytes before injection using negative selection with anti-CD4 and anti-CD8 Abs followed by anti-rat IgG Abs coupled to magnetic beads.
RNA isolated from lactating mammary glands or milk (shown for CBA/CaJ mice) of uninfected (MMTV+) or infected (MMTV+) mice injected with syngeneic MMTV(LA)-infected splenocytes as adults was subjected to RNase T1 protection analysis with virus-specific probes. Other MMTVs, partial protection. Endogenous Mtv7 was not detected in the mammary glands, as it is expressed only in lymphoid tissues (17, 35).

**RNase T1 protection assays**

RNase T1 protection assays were performed as previously described (16), using probes specific for BALB2, BALB14, BALBLA (17), and MMTV/C3H (18) viral transcripts. Forty micrograms of total RNA isolated from the lactating mammary glands and 5 μg of RNA isolated from the milk of the mice described in the text were used.

**Isolation of spleen cell subsets and PCR for newly integrated MMTV proviruses**

CD4+ T cells, B220+ B cells, and Mac-1+ cells were purified from spleens of MMTV(LA)-infected BALB/cJ mice by the magnetic bead system using the manufacturer’s protocol (Miltenyi Biotec, Auburn, CA). Cells were purified by positive selection using anti-CD4 Abs (for CD4+ T cells), anti-B220 Abs (for B cells), and anti-CD11b Abs (for CD11b+/Mac-1+ cells). All cells were >95% pure as determined by FACS analysis.

DNA isolated from purified cells was subjected to semiquantitative PCR analysis with primers specific for BALBLA provirus as previously published (17).

**Results and Discussion**

Genetically “resistant” mice are susceptible as neonates to MMTV infection

To elucidate the role of SAg in the different stages of viral infection, we took advantage of MMTV(LA) produced by BALB/cLA mice. MMTV(LA) contains three different exogenous viruses: BALB2, BALB14, and BALBLA, with Vβ2-, Vβ14-, and Vβ6/ Vβ8.1-specific SAg, respectively (14, 17). The sag gene of BALBLA is identical with the sag gene of Mtv7 (17). We reasoned that, unless infection in neonates is SAg independent, Mtv7-positive mice should be resistant to BALBLA, because SAg-cognate T cells used by this virus are either anergized or deleted in these mice at around birth (19, 20). To test this hypothesis, Mtv7-positive (CBA/J, I/LnJ, DBA/2J) and Mtv7-negative (BALB/cJ, CBA/CaJ, C3H/HeN) mice were infected with MMTV(LA) via two different routes: young adult animals (3–4 wk old) were injected with syngeneic MMTV(LA)-infected splenocytes, and newborn mice were foster-nursed on BALB/cLA milk. All animals were then bred, and RNA isolated from their milk was subjected to RNase T1 protection analysis with probes specific for BALB2, BALB14, and BALBLA (17). As expected, adult Mtv7-negative mice (BALB/cJ, CBA/CaJ, and C3H/HeN) became infected with all three viruses, while adult Mtv7-positive mice (DBA/2J, CBA/J, and I/LnJ) were resistant to BALBLA, transmitting BALB2 and BALB14 viruses only (Fig. 1, C3H/HeN and I/LnJ mice not shown). However, surprisingly, newborn mice of both the Mtv7-positive and Mtv7-negative strains were permissive for all three viruses, including BALBLA (Fig. 2A). Thus, newborn mice of the Mtv7-positive strains were susceptible to BALBLA, which encodes SAg of the same Vβ specificity as SAg of Mtv7, whereas adult mice from these strains were resistant.

To rule out the possibility that the observed differences in susceptibility between newborn and adult mice were the result of the different routes of infection, newborn mice of the Mtv7-positive strain

**FIGURE 1.** Mice expressing endogenous Mtv7 are resistant to exogenous viruses with the same SAg specificities when infected as adults. RNA isolated from lactating mammary glands or milk (shown for CBA/CaJ mice) of uninfected (MMTV+) or infected (MMTV+) mice injected with syngeneic MMTV(LA)-infected splenocytes as adults was subjected to RNase T1 protection analysis with virus-specific probes. Other MMTVs, partial protection. Endogenous Mtv7 was not detected in the mammary glands, as it is expressed only in lymphoid tissues (17, 35).

**FIGURE 2.** The same mice shown in Fig. 1 can be infected with all viruses as neonates. A, Mice from different strains fostered by BALB/cLA females transmit all MMTV variants. RNA isolated from the milk of BALB/cJ, DBA/2J, CBA/J, and CBA/CaJ mice foster-nursed by BALB/cLA females was subjected to RNase T1 protection analysis with probes specific for BALB2, BALBLA, and BALB14 viruses. DBA/2 MTV+ and CBA MTV+ RNA isolated from the milk of uninfected mice. B, DBA/2J mice are susceptible to all viruses when injected with MMTV(LA)-infected BALB2 and BALB14 splenocytes as neonates. RNA isolated from the milk of BALB/cJ and DBA/2J mice injected with MMTV(LA)-infected BALB/cJ and BALB14 splenocytes, respectively, was subjected to RNase T1 protection analysis with three probes. BALB/cJ, RNA isolated from the milk of uninfected BALB/cJ mice.
DBA/2J and the Mn7-negative strain BALB/cJ (as a control) were injected with MMTV(LA)-infected syngeneic splenocytes. As was the case with Mn7-positive mice fostered by infected mothers, newborn DBA/2J mice injected with MMTV(LA)-infected splenocytes became infected with all three viruses, as did the control mice (Fig. 2B). Thus, lack of SAg-cognate T cells and SAg-mediated T cell activation confers resistance to MMTV in adult but not in neonatal mice.

Susceptibility of neonatal mice to MMTV infection cannot be attributed to concomitant infection with a mixture of viruses

I-E MHC class II molecules are much more efficient than I-A molecules for SAg presentation (21-23). As a result, mice of I-E-negative MHC haplotypes are relatively resistant to MMTV infection and MMTV-induced mammary tumors (24, 25). Some MMTV variants encode I-E-independent SAgs, including BALBLA (26). I-E-negative newborn mice were shown to become infected with an I-E-dependent MMTV variant when infected concomitantly with an I-E-independent MMTV variant (27). Similarly, it was possible that in our experiments BALBLA incapable of inflicting Vβ6+ T cell-deficient mice by itself acquired infectivity upon mixing with BALB2 and BALB14 viruses in I-E-positive mice. To test this possibility we took advantage of two strains of mice with I-E-negative H-2k haplotypes. LP/J and D1.LP/J mice are identical with one another with the exception of the Mn7 locus, which is present in D1.LP/J but not in LP/J mice. As a result, Vβ6+/Vβ8.1+ T cells are absent in D1.LP/J mice due to negative selection, but are present in LP/J mice. Importantly, in mice with I-E-deficiency, deletion or activation of T cells cognate for I-E-dependent SAgs encoded by BALB2 and BALB14 occurs very inefficiently (26). If concomitant infection underlies virus susceptibility in neonatal mice, newborn D1.LP/J mice should be resistant to all three viruses, as BALB2 and BALB14 SAgs are unable to activate cognate T cells, and BALBLA SAg lacks target T cells. Newborn and adult D1.LP/J and LP/J mice were infected with MMTV(LA) via foster nursing or by injection with infected splenocytes, respectively. The newborn mice from both strains became infected with all three viruses, indicating that infection was SAg independent (Fig. 3). At the same time, adult D1.LP/J mice were completely resistant to all these viruses, while adult LP/J mice were susceptible to BALBLA, as its SAg was presented to T cells by I-Aβ (Fig. 3). Therefore, the susceptibility of neonatal mice to MMTV infection cannot be explained by concomitant infection with the three different viruses and does not require SAg presentation. Thus, we concluded that this susceptibility is rather a property of a developmental stage in mice. Table I summarizes our findings on the susceptibility of newborn and adult mice of different strains to MMTV(LA).

MTV-ORF transgenic mice are completely protected against exogenous MMTV(C3H) when infected as adults

Previously, we showed that exogenous MMTV lacking a functional sag gene was capable of infecting lymphoid cells in mice that received the virus as neonates (13). In addition, we demonstrated that newborn mice lacking Vβ14+ T cells due to transgenic expression of MMTV(C3H) SAg (MTV-ORF transgenic mice) were apparently resistant to infection by exogenous MMTV(C3H) virus (11). However, these mice developed MMTV-induced mammary tumors after a long latency period and passed infectious virus to their nontransgenic offspring (12). Obviously, MTV-ORF transgenic mice were not completely protected from infection by exogenous MMTV(C3H). We reasoned that both the ability of virus lacking functional SAg to establish initial infection and the susceptibility of MTV-ORF transgenic mice to exogenous MMTV could be due to a SAg-independent stage of virus transmission in neonatal mice. If so, adult MMTV(C3H)-infected MTV-ORF transgenic mice should demonstrate complete resistance to MMTV(C3H). To test this possibility, we injected adult MTV-ORF transgenic and nontransgenic mice with MMTV(C3H)-infected syngeneic splenocytes. Injected mice were bred, and RNA isolated from their milk was analyzed for virus-specific RNA species. In addition, nontransgenic offspring produced by infected transgenic and nontransgenic females were bred and the percentages of SAg-cognate CD4+/Vβ14+ T cells in their peripheral blood were analyzed to determine whether mice were infected (all MMTV-infected mice showed deletion of SAg-cognate T cells).

Table I. Age-dependent susceptibility to MMTV infection in different strains of mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>MHC Haplotype</th>
<th>I-E</th>
<th>Mn7</th>
<th>Infected as Neonates⁴</th>
<th>Infected as Adults⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BALB2</td>
<td>BALB14</td>
</tr>
<tr>
<td>BALB/cJ</td>
<td>H-2d</td>
<td>Yes⁵</td>
<td>No</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CBA/CAJ</td>
<td>H-2k</td>
<td>Yes</td>
<td>No</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C3H/H-2N</td>
<td>H-2k</td>
<td>Yes</td>
<td>No</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DBA/2J</td>
<td>H-2d</td>
<td>Yes</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CBA/J</td>
<td>H-2k</td>
<td>Yes</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LP/J</td>
<td>H-2b</td>
<td>No</td>
<td>No</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D1.LP/J</td>
<td>H-2b</td>
<td>No</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

⁴ RNA isolated from milk or lactating mammary glands of different mice foster-nursed by BALB/cLA or injected with syngeneic infected splenocytes was subjected to RNase T1 protection analysis with probes specific for BALB2, BALB14, and BALBLA viruses.

⁵ Expression of I-E MHC class II molecules and endogenous Mn7 by infected mice is indicated by “Yes” or “No”.

+ or −, presence or absence of viral RNA.
Results showed that adult MTV-ORF transgenic mice were completely resistant to MMTV(C3H), as not only did they fail to show any virus in their milk, they also did not pass infectious virus to their nontransgenic offspring (Fig. 4).

These data again suggest that SAg function is dispensable for MMTV infection in newborn but not in adult mice. However, a question remaining is: why were newborn MTV-ORF transgenic mice relatively resistant to MMTV(C3H), whereas newborn D1.LP/J mice were much more susceptible to MMTV(LA) (Fig. 3)? One possible explanation is that MMTV(C3H) may be produced at rather lower titer, and may therefore require significant amplification via a SAg-dependent mechanism to infect mammary epithelium. In fact, the virus titer produced by MTV(C3H)-infected C3H/HeN mice was much lower than that produced by mice of the same strain infected with MMTV(LA) (Fig. 5). Similarly, in I/LnJ mice, which are capable of producing virus-neutralizing Abs (28), infection with high titer MMTV(LA) progressed to the mammary glands whereas infection with low titer MMTV(C3H) did not progress further than the cells of the immune system before immune response was initiated (29). Together, these data support the idea that a virus transmitted at a lower titer has a greater dependency on SAg.

FIGURE 4. C3H/HeN MTV-ORF transgenic mice are completely resistant to MMTV(C3H) when infected as adults. A, MTV(C3H)-infected MTV-ORF transgenic mice do not produce detectable amounts of the virus into milk. RNA isolated from the milk of nontransgenic and transgenic C3H/HeN MTV-ORF mice infected with MTV(C3H)-infected splenocytes as adults was subjected to RNase T1 protection analysis with probes specific for MMTV(C3H), MMTV(C3H), full length protection. The same RNA samples were separated on 1% agarose gels to test RNA integrity. 18S and 28S, ribosomal RNAs. B, MTV(C3H)-infected MTV-ORF transgenic mice (shown in A) do not transmit virus to their nontransgenic offspring. T cells were isolated from the peripheral blood, stained with Abs against CD4 and Vβ14, and analyzed by FACS. n, Number of mice tested. Values are means ± SD.

FIGURE 5. MMTV(LA) viruses are produced at much higher titers than are MMTV(C3H) viruses. C3H/HeN mice were fostered by MTV(C3H)-infected (right) or MMTV(LA)-infected (left) BALB/cJ mice. Infected mice were bred, and RNA isolated from their milk was subjected to RNase protection analysis with virus-specific probes. 1–3, Individual mice; MTV′, milk RNA from uninfected mouse.

Transition from susceptible to resistant phenotype occurs at approximately day 17 postpartum

To determine the time point when mice lacking SAg-cognate Vβ6/Vβ8.1+ T cells become resistant to BALB/cJ, we foster-nursed several groups of DBA/2J mice on BALB/cLA mothers for different 3-day periods between 15 and 20 days postpartum and then returned them to their uninfected mothers. The foster-nursed mice were subsequently bred and their milk was tested for the presence of MMTV. It became clear that the DBA/2J mice were permissible for all three viruses up until day 17 postpartum but became resistant to BALB/cLA after day 17 (Table II). These studies suggest that developmental changes determine whether the host is susceptible or resistant to retroviruses. Most retroviruses require the target cells to actively cycle to allow integration of the reverse-transcribed retroviral genome into the host chromosome (30). Thus, we hypothesized that MMTV might use the neonatal period as an opportunity to invade the host even without functional SAg.

It has been recognized that the thymocytes and splenocytes of neonatal mice proliferate in vitro (31–33). Thus, we sought to determine whether the ability of these cells from newborn mice to proliferate spontaneously is developmentally regulated. To that end, splenocytes from DBA/2J mice of different ages were labeled with CFSE, cultured in vitro for 4 days, and analyzed by FACS. In contrast to cultured adult splenocytes, cells from newborn mice

Table II. Neonatal mice are susceptible to all MMTVs until day 17 postpartum

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Foster between Days</th>
<th>Mice (n)</th>
<th>BALB2</th>
<th>BALB14</th>
<th>BALB/cLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBA/2J</td>
<td>15–18</td>
<td>8</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>16–19</td>
<td>5</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>17–20</td>
<td>4</td>
<td>++</td>
<td>++</td>
<td>−−</td>
<td></td>
</tr>
<tr>
<td>21–24</td>
<td>5</td>
<td>++</td>
<td>++</td>
<td>−−</td>
<td></td>
</tr>
<tr>
<td>25–28</td>
<td>1</td>
<td>++</td>
<td>++</td>
<td>++/−</td>
<td></td>
</tr>
<tr>
<td>BALB/cJ</td>
<td>1–3…17–19</td>
<td>8</td>
<td>++</td>
<td>++</td>
<td></td>
</tr>
</tbody>
</table>

*DBA/2J mice and control BALB/cJ mice were foster-nursed by BALB/cLA females for different 3-day periods and then returned to uninfected mothers. The foster-nursed mice were bred, and RNA isolated from their milk was subjected to RNase T1 protection analysis with probes specific for BALB2, BALB/cLA, and BALB/cJ.

† + , high virus titer, comparable with that produced by BALB/cJ mice.

‡ + , low virus titer (=1/5 of that produced by BALB/c mice).

§ − , no detectable virus.

∥ ++/− , low virus titer (=1/10 of that produced by BALB/c mice).
MMTV provirus was found in both Mac-1/H11001/H11002 and non-T cells and in Mac-1/CD4-infected mice. DNA isolated from positively selected, as well as non-T and non-B Mac-1/H11001 cells analyzed by FACS. were costained with anti-Mac-1 and anti-Gr-1 Abs and from 3-day-old and adult 3- to 4-wk-old DBA/2J mice were infected with BALB/cJ mice of different ages were stained with CFSE and anti-Mac-1 or anti-Gr-1 Abs, cultured in vitro for 4 days, and analyzed by FACS. In contrast to cultured adult splenocytes, cells from newborn mice showed proliferation as indicated by the dilution of CFSE label and appearance of additional peaks. B and C, Spontaneously proliferating splenocytes express Mac-1 and Gr-1 markers. B, Splenocytes from DBA/2J mice of different ages were stained with CFSE and anti-Mac-1 or anti-Gr-1 Abs, cultured in vitro for 4 days, and analyzed by FACS. C, Splenocytes from 3-day-old and adult 3- to 4-wk-old DBA/2J mice were costained with anti-Mac-1 and anti-Gr-1 Abs and analyzed by FACS. D, MMTV infects T and B cells as well as non-T and non-B Mac-1+ cells in neonatally infected mice. DNA isolated from positively selected CD4+ T cells, B cells, and Mac-1+ cells taken from 5- to 7-day-old MMTV(LA)-infected BALB/cJ mice was subjected to semiquantitative PCR with primers specific for BALB/c provirus (upper panel) or primers specific for all Mtv proviruses (lower panel). PCR products were separated on a 1% agarose gel containing ethidium bromide. One of four representative experiments is shown.

![FIGURE 6. A. Splenocytes from newborn mice proliferate spontaneously in vitro. Splenocytes from DBA/2J mice of different ages were labeled with CFSE, cultured in vitro for 4 days, and analyzed by FACS. In contrast to cultured adult splenocytes, cells from newborn mice showed proliferation as indicated by the dilution of CFSE label and appearance of additional peaks (Fig. 6A). Notably, the number of proliferating cells decreased significantly after day 17 postpartum, which correlated well with the observed resistance to MMTV infection (Fig. 6A and Table II). Susceptibility of 16-day-old DBA/2J mice to infection with BALB/c cannot be explained by residual amounts of SAg-cognate T cells, as these cells have been completely deleted by day 10 (19). The majority of proliferating cells had a Mac-1+/Gr-1+ phenotype (Fig. 6, B and C), and MMTV provirus was found in both Mac-1+/B220−/CD4− non-B and non-T cells and in Mac-1−/B220+ B or Mac-1−/CD4+ T cells isolated from spleens (Fig. 6D).

Thus, it appears that during the early stages of infection MMTV is capable of infecting multiple cell types in both a SAg-independent and a SAg-dependent manner, because MMTV is infecting cells that are proliferating naturally as a result of their developmental program. In contrast, SAg-dependent activation of T cells with consequent activation of B cell proliferation via the CD40 receptor (34) is necessary for MMTV amplification during the later stages of infection. Furthermore, because MMTV has to fight the ongoing anti-virus immune response (4), it is also probable that SAg-induced deletion of cognate T cells helps to eliminate MMTV-reactive lymphocytes. Although this hypothesis requires experimental confirmation, one point is clear: the survival strategy of this retrovirus is much more complex than has been indicated by previous research since the discovery of viral SAGs more than a decade ago.

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References


