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B and T Cells Are Required for Mouse Mammary Tumor Virus Spread Within the Mammary Gland

Tatyana V. Golovkina, Jaquelin P. Dudley, and Susan R. Ross

Mouse mammary tumor virus (MMTV) is an infectious retrovirus transmitted through milk from mother to newborns. MMTV encodes a superantigen (SAg) whose activity is indispensable for the virus life cycle, since a genetically engineered virus with a mutation in the sag gene neither amplified in cells of the immune system of suckling pups nor infected their mammary glands. When wild-type MMTV was injected directly into the mammary glands of uninfected pubescent mice, their lymphoid as well as mammary gland cells became virus infected. To test whether this infection of lymphoid cells was dependent on SAg activity and required for virus spread within the mammary gland, we performed mammary gland injections of wild-type MMTV(C3H) into two strains of transgenic mice that lacked SAg-cognate, Vβ14+ T cells. Neither the MTV-ORF or LEL strains showed infection of their mammary glands. Moreover, no MMTV infection of their peripheral lymphocytes was detected. Similar experiments with mice lacking B cells (μ-chain knockouts) showed no detectable virus spread in the mammary glands or lymphoid tissues. These data suggest that SAg activity and MMTV-infected lymphocytes are required, not only for initial stages of viral infection, but also for virus spread within the mammary gland. Virus spread at late times in infection determines whether MMTV induces mammary tumors. The Journal of Immunology, 1998, 161: 2375–2382.

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Abbreviations used in this paper: MMTV, mouse mammary tumor virus; LTR, long terminal repeat; SAg, superantigen.
SAG EXPRESSION PREVENTS MMTV SPREAD WITHIN THE MAMMARY GLAND

PRO/Cla transgenic mice containing the genetically engineered MMTV provirus with the mutated sag gene were described elsewhere (15). To avoid viral recombination between the Mtv1 endogenous provirus in C3H/HeN mice and the transgene, the HYB PRO/Cla transgene was bred onto the BALB/c background (Mtv6, -8, and -9) for two generations. Transgenic females null for Mtv1 were identified by Southern blot analysis and showed no deletion of Vβ14 T cells (not shown), in contrast to the HYB PRO/Cla females in the C3H/HeN (Mtv1+/−) background (15). The MTV-ORF no. 16 and LEL no. 1 transgenic mice were previously described (7, 16).

Homozygous IGH6/B6 (H-2b) females (µ-chain knockout mice) (22) purchased from The Jackson Laboratory (Bar Harbor, ME) were crossed with C3H/HeN MMTV (H-2b) males (National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD) to generate F1 progeny. The F1 progeny were intercrossed, and F2 generation female mice were typed at the National Cancer Institute, Frederick Cancer Research Facility.

Abs and FACS analysis

Surface expression of IgM and MHC class II was determined by staining PBLs with FITC-labeled anti-mouse IgM and IgG Abs (Jackson ImmunoResearch Laboratories, West Grove, PA) or anti-mouse H-2b Abs (PharMingen, San Diego, CA), respectively. FITC-coupled mAbs against the Vβ14 TCR chain were purchased from PharMingen. Anti-CD4 Abs and FACS analysis were accomplished using the following LTR-specific primers: 5′-TAATGTCTATATTGCTACGCACTTGG-3′ (nt 268–289) and 5′-m.w. DNA (0.25 μl) isolated from spleens, thymi, or Peyer’s patches was amplified by PCR. Amplification of newly integrated copies of the virus preparation (0.15 mg/ml of the protein level) was injected per mouse.

Results

A functional MMTV SAg is required to establish infection in vivo

MMTV can infect only the mammary glands of mice with both functional B cells and SAg-cognate T cells (5, 7, 8). Although these immune system cells are important for MMTV transmission, an absolute requirement for SAg in the MMTV life cycle has not yet been shown. We introduced a transgene, HYB PRO/Cla, with a frameshift mutation at amino acid 113 in the sag gene into C3H/HeN mice. This frameshift caused premature termination of this protein (15). Because this termination occurred upstream of the SAg hypervariable region that determines TCR Vβ specificity, no deletion of Vβ14 T cells should occur in these mice. However, this mutation was corrected in transgenic females by retroviral recombination with the endogenous Mtv1 virus present in C3H/HeN mouse strain, leading to the generation of a Vβ14-deleting, milk-transmitted virus (15). To prevent this recombination, the transgene was crossed into BALB/c mice, which lack Mtv1. The Mtv1− HYB PRO/Cla transgenic animals generated from these crosses expressed the transgene, shed virus in their milk (Fig. 1A, lanes 4 and 5, respectively) (15), and showed no deletion of their SAg-cognate Vβ14 T cells (data not shown).

To determine whether the mutated MMTV produced by the Mtv1− HYB PRO/Cla transgenic females was infectious, these mice were bred and offspring generated. None of the nontransgenic Mtv1− HYB PRO/Cla-nursed offspring acquired virus, since no transgene-specific RNA was detected in either their mammary glands or milk (Fig. 1A, lanes 2 and 1, respectively). In contrast, mice nursed on a Mtv1− HYB PRO/Cla transgenic mice acquired infectious virus (Fig. 1A, lanes 5 and 6), as previously shown (15). These results indicated that a SAg-minus MMTV could not infect mammary gland cells.

The primary targets for MMTV during milk-borne infection are lymphocytes. To determine whether SAg was also required for infection of lymphoid cells, we examined the relative levels of viral DNA present in the lymphoid organs of nontransgenic mice nursed on the Mtv1− HYB PRO/Cla-transgenic mothers using PCR analysis (see Materials and Methods). After 31 cycles of PCR amplification, no exogenous MMTV DNA was detected in DNA isolated from the spleens, thymi, or Peyer’s patches of four different 3-mo-old Mtv1−, HYB PRO/Cla-infected mice (mice 1–4, Fig. 1B, top panel), in contrast to mice nursed on Mtv1−, HYB PRO/Cla-infected mice (E.C., Fig. 1B). However, after 35 cycles, viral DNA was detectable in these organs (Fig. 2B, bottom panel), indicating that there was low level infection of lymphoid cells. Thus, the SAg-mutated virus did not amplify in the lymphoid cells and this was responsible for our failure to detect MMTV infection in the mammary gland.

MMTV spread within the mammary gland requires B cells

Infected lymphoid cells are required for the primary infection of mammary gland cells during milk-borne transmission, but it is not known whether they play a role in MMTV spread within the mammary gland. Although mice naturally acquire virus through the milk, MMTV can also be introduced by injection into the mammary gland of subcutaneous mice. To determine whether MMTV injection into the mammary gland resulted in systemic infection of lymphoid cells, we performed PCR analysis on DNA isolated from spleen and Peyer’s patches of mice infected in this manner, using...
MMTV(C3H) LTR-specific primers. We found that both tissues from the infected mice acquired new proviral copies of MMTV (Fig. 2), indicating that lymphoid cells were infected after virus injection into the mammary gland.

B cells are believed to be the first cells infected during milk-borne MMTV transmission to offspring (3, 8). To determine whether they were needed for MMTV spread within the mammary gland, we injected virus into mice that lacked B cells due to the targeted mutagenesis of their Ig μ-chain gene (strain IGH6/BL6 (22)). This gene disruption was available only in C57BL/6 mice, which are H-2b and do not express the MHC class II I-E molecule. Because of this, most MMTV SAgs, including that encoded by MMTV(C3H), are presented inefficiently and these mice are less susceptible to MMTV(C3H) infection (23). IGH6/BL6 mice were crossed with C3H/HeN mice, which are H-2k, an MHC haplotype that efficiently presents the MMTV(C3H) SAg (24, 25). F1-(IgM1/H-2k1) and F2-generation female mice (IgM1H-2k1, IgM2H-2k2, IgM2H-2k2; see Fig. 3) derived from these crosses received mammary gland injections of MMTV at 3 wk of age. Four weeks after injection, immune response to the virus was assessed by determination of the percentage of peripheral CD41/Vb141 T cells. Both the F1- and F2-generation IgM1H-2k+ mice were infected, since they deleted about 30% of their Vb14+ SAg-reactive T cells (Table I). In contrast, none of the H-2k− or IgM− mice showed deletion (Table I).
IgM

2

MMTV

glands. This RNA was subjected to RNase T1 protection analysis were infected, we isolated RNA from their lactating mammary

for the F 2 generation) were infected, since they expressed high

levels of MMTV-specific RNA in their mammary glands (Fig. 5,

lanes A–J). In contrast, no MMTV-specific transcripts were de-

lated in either the IgM

2

lanes P–R

MMTV(C3H)-specific primers under non-quantitative conditions using DNA isolated from spleen (SP) and Peyer’s patches (P.P.) of three different C3H/HeN mice. Endogenous MMTVs contain no MfeI site and are visible after digestion with this enzyme. MMTV was injected into the mammary gland of 3-wk-old mice. Mice were sacrificed 5 wk after injection. Abbreviations: N.C.; negative control, DNA isolated from the spleen of the C3H/HeN MMTV– mouse.

indicating that these mice were not MMTV infected. However, the absence of cognate T cell deletion in the B cell-deficient and H-2k–

(I-E–) mice could also be due to inefficient SAg presentation, because of the lack of B cells or appropriate MHC haplotype. To determine whether the mice were infected, we isolated DNA from the spleens of the injected mice and performed PCR specific for MMTV(C3H) proviral DNA. As shown in Figure 4, only the IgM

2

H-2k+ (lanes A–J) but not the IgM

2

H-2k– (lanes K–N), IgM

2

H-2k– (lane O), or IgM

2

H-2k+ (lanes P–R) mice contained MMTV(C3H) proviruses in their splenocytes. Therefore, lack of deletion of SAg-cognate T cells in IgM– or H-2k– mice directly correlated with the lack of infection.

To determine whether mammary gland cells of these animals were infected, we isolated RNA from their lactating mammary glands. This RNA was subjected to RNase T1 protection analysis with the probe specific for the LTR of the MMTV(C3H) exogenous virus. All the F1 and F2 generation IgM

2

H-2k– (shown only for the F2 generation) were infected, since they expressed high levels of MMTV-specific RNA in their mammary glands (Fig. 5, lanes A–J). In contrast, no MMTV-specific transcripts were detected in either the IgM

2

H-2k– (lanes P–R), IgM

2

H-2k– (lane O), or IgM

2

H-2k+ mice (lanes K–N). After an overnight exposure of the gel in Figure 5, a very faint signal was seen for two of the four IgM

2

H-2k– mice (not shown). As was previously reported, H-2b mice can be infected at a low level (26). One IgM

2

H-2k– mouse also showed no mammary gland expression of MMTV(C3H) (Fig. 5, mouse J); however, this mouse did show lymphocyte infection (Fig. 4, bottom panel, IgM

2

H-2k+, mouse J). Thus, in the absence of B cells, there was no virus spread within the immune system or mammary gland.

Absence of MMTV amplification in the mammary glands of transgenic mice lacking SAg-reactive T cells

One consequence of B cell infection by MMTV is that these cells could function as SAg-presenting APCs that activate cognate T cells. This activation would lead to amplification of virus within the lymphoid compartment. That mice of the wrong MHC haplotype failed to be efficiently infected by mammary gland injection of MMTV indicated that SAg presentation might be a requisite step in the infection of mammary cells. To test directly whether SAg activity and infected lymphoid cells were required for virus spread within the mammary gland, we injected exogenous MMTV(C3H) into the mammary glands of two types of transgenic mice, MTV-ORF and LEL (7, 16). Because the MTV-ORF and LEL transgenic mice both expressed the MMTV(C3H) sag as an endogenous gene, they lack Vβ14+ T cells and thus were resistant to milk-borne MMTV(C3H) infection (17, 27).

Two independent experiments were performed. In the first, LEL transgenic females (n = 7) and their nontransgenic littermates (n = 6) were injected with a high virus dose, whereas in the second

![Figure 3](https://via.placeholder.com/150)

**FIGURE 3.** Establishment of B cell-deficient I-E-positive mice. Homozygous IGH6/BL6 (IgM

2

H-2k–) females were crossed with C3H/HeN MMTV– (IgM

2

H-2k+) males to generate F1 heterozygotes. The F1 mice were intercrossed and the F2 generation female mice were typed for surface IgM and MHC class II H-2k expression. The mammary glands of the four different groups generated were injected with MMTV at 3 to 4 wk of age.

![Figure 2](https://via.placeholder.com/150)

**FIGURE 2.** Injection of virus directly into the mammary gland results in lymphoid cell infection. PCR followed by MfeI digestion was carried out with MMTV(C3H)-specific primers under non-quantitative conditions using DNA isolated from spleen (SP) and Peyer’s patches (P.P.) of three different C3H/HeN mice. Endogenous MMTVs contain no MfeI site and are visible after digestion with this enzyme. MMTV was injected into the mammary gland of 3-wk-old mice. Mice were sacrificed 5 wk after injection. Abbreviations: N.C.; negative control, DNA isolated from the spleen of the C3H/HeN MMTV– mouse.

Table I. Percentage of Vβ14+/CD4+ T cells in IgM– and H-2k– mice after mammary gland injection of MMTV

<table>
<thead>
<tr>
<th>Mice</th>
<th>Age (in days)</th>
<th>CD4/Vβ14 (%)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1 IgM– H-2k– MTV+</td>
<td>62</td>
<td>5.9 ± 0.51 (n = 7)</td>
</tr>
<tr>
<td>F2 IgM– H-2k+ MTV+</td>
<td>58</td>
<td>6.2 ± 0.70 (n = 20)</td>
</tr>
<tr>
<td>F1 IgM+ H-2k– MTV+</td>
<td>58</td>
<td>8.6 ± 0.30 (n = 5)</td>
</tr>
<tr>
<td>F1 IgM+ H-2k+ MTV+</td>
<td>58</td>
<td>8.7 ± 0.42 (n = 9)</td>
</tr>
<tr>
<td>F1 MTV–</td>
<td>8.1 (n = 1)</td>
<td></td>
</tr>
<tr>
<td>F2 MTV–</td>
<td>8.4 ± 0.20 (n = 3)</td>
<td></td>
</tr>
</tbody>
</table>

a T cells were isolated from the peripheral blood of mice at the ages indicated and analyzed for percentage of CD4+/Vβ14+ as described in Materials and Methods. Values are the mean ± SE.
To determine whether MMTV injection into the mammary gland did result in systemic infection of lymphoid cells, we performed PCR analysis on DNA isolated from spleen and Peyer’s patches of the infected transgenic and nontransgenic mice, using MMTV(C3H) LTR, gag-specific primers. We found that lymphoid tissues from all of the infected nontransgenic mice acquired new proviral copies of MMTV (Fig. 7, LEL n/t and MTV-ORF n/t). In contrast, the lymphoid tissue isolated from the injected transgenic mice showed no evidence of viral infection, except for one LEL transgenic mouse (lane marked LEL p.p.*, Fig. 7). This mouse also developed a mammary tumor. Two methods were used to determine whether the mammary gland cells of the injected transgenic mice were infected. First, we bred these mice and then examined their nontransgenic offspring for the deletion of Vβ14+ T cells. In the first experiment, where a high virus dose was injected, the offspring of seven transgenic females showed deletion of their Vβ14+ T cells (Table II). These results suggested that MMTV transmission was variable due to low level infection. In support of this, when a lower dose of virus was injected, there was no deletion of this subset of T cells in the nontransgenic offspring of either the LEL or the MTV-ORF transgenic mice (Table II). All offspring of nontransgenic mice that received mammary gland injections of virus showed Vβ14+ T cell deletion (Table II).

We also tested directly whether the mammary gland cells of the injected mice were infected. First, we received the lower amount of virus (Fig. 8A), whereas no viral RNA was detected in either the LEL or the MTV-ORF transgenic mice (Table II). This lower viral load had a dramatic affect on MMTV-induced mammary tumorigenesis. Because MMTV integration next to cellular oncogenes is a stochastic event, the higher the viral load, the more rapidly mice develop mammary tumors (13). Both the transgenic and nontransgenic mice from experiment 1 were continuously bred and monitored for mammary gland tumor incidence. The multiparous nontransgenic mice had a 100% mammary gland tumor incidence by the age of 260 days (Fig. 9). At that time, none of the transgenic mice had tumors.

FIGURE 5. The mammary glands of the B cell-deficient I-E− mice show no amplification of exogenous MMTV. RNA was isolated from the lactating mammary glands of the same mice as in Figure 4, and 40 µg were subjected to RNase T1 protection assay with an MMTV(C3H) LTR-specific probe. All mice were analyzed after their third pregnancy. Abbreviations: p, probe; MMTV(C3H), full protection. Bottom panel. 20 µg of the same RNA were electrophoresed on a 1% formaldehyde gel and stained with ethidium bromide to verify the quality of the RNA. The migration of the 18S and 28S ribosomal bands are indicated. A to R, individual animals, the same as in Figure 4.

FIGURE 4. Direct infection of MMTV into mammary glands does not compensate for lack of I-E or B cell deficiency. Semi-quantitative PCR was performed with DNA isolated from spleens (SP) of the indicated mice followed by MfeI digestion and Southern blot analysis (see Materials and Methods). Mice were analyzed after their third pregnancy. A to R, individual animals.

FIGURE 6. Pedigree of the LEL or MTV-ORF transgenic mice. F1 transgenic and nontransgenic female mice were injected with MMTV at 3 to 4 wk of age. □, Nontransgenic male; ○, nontransgenic female; ◦, transgenic female.
of the transgenic mice developed mammary tumors and only two of seven transgenic mice developed tumors by 300 days (Fig. 9). All the tumors had acquired exogenous MMTV proviruses as detected by Southern blot analysis (not shown).

Discussion

Both T and B cells play an essential role in the transmission of milk-borne MMTV, since the mammary glands of mice lacking either of these lymphoid subsets are not efficiently infected (5, 7, 8, 29). B cells are necessary, as initial targets for MMTV infection at minimum, because they are effective SAg-presenting cells for cognate T lymphocytes (8). Similarly, SAg-reactive T cells are required for the amplification and establishment of MMTV infection, at least within the lymphoid compartment (5, 7). Here, we provide proof that a mutant MMTV lacking a functional SAg gene will infect lymphoid cells, but that insufficient amplification of this lymphoid infection blocks viral transmission to the mammary gland.

Although it is now clear how MMTV initiates infection in vivo, how this virus infects mammary gland cells and spreads within this tissue is poorly understood. Expression of integrated MMTV proviruses in mammary cells is regulated by lactogenic hormones. As a result, pregnancy has a dramatic effect on virus load, which also increases with parity (2). The number of infected cells also increases in multiparous animals, showing that there is virus spread within the mammary gland after the initial infection. Without this spread, tumor induction by MMTV is very inefficient. Because retroviral integration is a stochastic event, increased numbers of integration events will improve the chance of insertion near a given cellular oncogene. For example, MMTV-infected virgin mice have a significantly lower virus load that leads to a decreased mammary tumor incidence and an increased tumor latency (14). In addition, MMTV-induced mammary tumors usually contain large numbers of newly integrated copies of exogenous MMTV, indicating multiple reinfection events (10).

Here we addressed the requirement for lymphoid cells in MMTV infection of and spread within the mammary gland. Not surprisingly, based on experiments by others in which MMTV was injected into peripheral tissue (3), we found that lymphoid cells as well as mammary cells were efficiently infected after the injection of virus directly into the mammary glands of wild-type, pubescent mice. However, when the same mode was used to introduce MMTV into B cell-deficient mice, we found no infection of either lymphoid cells or mammary tissue. Since the B cell-deficient mice had the MHC H-2b haplotype and thus did not express the I-E molecule required for efficient presentation of the MMTV(C3H) SAg, we crossed them to C3H/HeN mice (H-2k), an MHC haplotype in virus spread within the mammary gland. Noting multiple reinfection events (10).

| Table II. Percentage of Vβ14+/CD4+ T cells in the offspring of LEL and MTV-ORF females that received mammary gland injections of MMTV |
|---|---|---|---|
| Expt. | Mice | Age (in days) | CD4+/Vβ14 (%)a |
| 1 | Nontransgenicb | 130 | 4.5 ± 0.89 (n = 6) |
| | LELb | | 1.5 ± 0.2 (n = 7) |
| | Nontransgenic offspring | 52 | 4.0 ± 0.15 (n = 5) |
| | LEL offspring (nontransgenic) | 52 | 4.0 ± 0.13 (n = 5) |
| | LEL offspring (nontransgenic) | 52 | 7.4 ± 0.21 (n = 2) |
| 2 | Nontransgenicb | 152 | 3.9 ± 1.30 (n = 10) |
| | MTV-ORFb | | 1.2 ± 0.2 (n = 6) |
| | LELb | | 1.5 ± 0.2 (n = 4) |
| | Nontransgenic offspring | 52 | 4.0 ± 0.10 (n = 4) |
| | MTV-ORF offspring (nontransgenic) | 52 | 7.5 ± 0.50 (n = 4) |
| | LEL offspring (nontransgenic) | 51 | 7.3 ± 0.40 (n = 4) |
| | C3H/HeN MTV− | | 7.3 ± 0.45 (n = 3) |

a T cells were isolated from the peripheral blood of mice at the ages indicated, stained, and analyzed for the percentages of CD4+/Vβ14+ T cells as described in Materials and Methods. Values are the mean ± SE.

b Transgenic (LEL or MTV-ORF) and nontransgenic littermates were injected with MMTV(C3H) at 3 wk of age.

Figure 7. Injection of MMTV into the mammary gland results in the infection of peripheral lymphoid tissues of nontransgenic but not sag transgenic mice. PCR followed by Southern blot analysis was performed with DNA isolated from the Peyer’s patches (P.P.) and spleens (SP) of the same mice as in Figures 8 and 9 using MMTV(C3H) LTR- and gag-specific primers (see Materials and Methods). Mice were sacrificed either at the time of tumor appearance or after 300 days.
glands of pubescent mice. These data suggest that the SAg stimulation of T cells is required for efficient infection of mammary glands and consequent tumorigenesis. Even when infection of the mammary gland cells was achieved, as in the case of the LEL transgenic mice injected with a high virus dose, the virus spread within this tissue was less efficient than that which occurred in mice that had SAg-cognate T cells.

These results demonstrate that infected lymphoid cells play a critical role in infection of the mammary gland. One possibility is that virus spread between mammary gland cells cannot be achieved in the absence of infected lymphoid cells. This may be because cell-cell contact between lymphocytes and mammary cells is the most efficient way to deliver virus. The architecture of the murine mammary gland may also affect how virus spreads. Mammary gland ducts consist of two main cell types, an inner region of ductal cells and an outer monolayer of myoepithelial cells surrounded by a basement membrane (2). At puberty, the mammary epithelial cells proliferate, resulting in a lengthening and branching of the ductal tree until the whole mammary fat pad is filled with cells. The terminal end bud drives ductal morphogenesis and most DNA synthetic activity is associated with these buds, while the ducts are relatively quiescent. The cells that occupy the terminal buds are most likely the targets for MMTV during puberty, since as with most other retroviruses, it is thought to require cell division for its propagation (30). If infected lymphocytes are present near these terminal buds, they could increase infection level of these dividing cells.

Similarly, lymphocytes may be involved in the virus spread that occurs in multiparous mice. Once puberty is over, the mammary cells do not divide in the mouse until pregnancy. MMTV preferentially buds from the apical surface of the epithelial cells lining the alveolar lumen (31) so that the virus is released into the milk during lactation. Thus, for MMTV to cause a systemic infection of the mammary gland tissue, it would have to be produced by an infected cell that directed its expression toward subepithelial tissues. B or T cells could fulfill this requirement, since both produce cytokines produced by SAg-activated T cells (32). An additional factor that may come into play is that lymphoid cells can circulate within the tissue, thereby possibly coming into contact with mammary cells at multiple locations.

It is also possible that cytokine production by SAg-stimulated T cells affects the mammary gland tissue directly. For example, cytokines produced by T cells could induce expression of gene products necessary for MMTV infection of mammary gland cells or for mammary tumorigenesis. Since MMTV-infected mice gradually delete SAg-cognate T cells after acquisition of milk-borne virus, this mechanism would require the persistence of such T cells in the mammary gland and not in other peripheral sites. Whether this occurs can be tested by examining the Vβ-repertoire of T cells isolated from the mammary gland and their ability to be activated by SAg.

Taken together, we have established that SAg activation of lymphoid cells is needed not only for the initial stages of MMTV infection, but also for virus spread within the mammary gland. These results demonstrate the importance of different cell types during in vivo virus infections.

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References


