

# B Cells Are Essential for Murine Mammary Tumor Virus Transmission, but Not for Presentation of Endogenous Superantigens

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## Summary

Murine mammary tumor viruses (MMTVs) are retroviruses that encode superantigens capable of stimulating T cells via superantigen-reactive T cell receptor V $\beta$  chains. MMTVs are transmitted to the suckling offspring through milk. Here we show that B cell-deficient mice foster nursed by virus-secreting mice do not transfer infectious MMTVs to their offspring. No MMTV proviruses could be detected in the spleen and mammary tissue of these mice, and no deletion of MMTV superantigen-reactive T cells occurred. By contrast, T cell deletion and positive selection due to endogenous MMTV superantigens occurred in B cell-deficient mice. We conclude that B cells are essential for the completion of the viral life cycle in vivo, but that endogenous MMTV superantigens can be presented by cell types other than B cells.

Murine mammary tumor viruses (MMTVs)<sup>1</sup> are infectious retroviruses transmitted through milk from the mother to newborns. The virus infects lymphocytes after passing through the gut epithelium of the pups and is then transmitted from the lymphocytes to the mammary tissue, where it replicates and is secreted into the milk (1–3). MMTVs encode superantigens, which stimulate T cells expressing superantigen-reactive TCR V $\beta$  chains. The superantigen is encoded by the MMTV *sag* gene in the U3 region of the 3' LTR (4, 5). MMTV(C3H), carried by the mouse strain C3H/He, encodes a superantigen that stimulates V $\beta$ 14<sup>+</sup> T cells, whereas the superantigen of another MMTV used in this study, MMTV(SW), stimulates V $\beta$ 6<sup>+</sup> T cells (6, 7). MMTV(SW) is of special interest, because its superantigen is virtually identical to Mls-1, the prototype of endogenous superantigens encoded by *Mtv-7* (8, 9).

Endogenous MMTVs are derived from rare infections of germ line cells, leading to Mendelian inheritance of the provirus (10). About 45 different endogenous MMTVs have been identified in inbred and wild mice (1). Most of them no longer produce infectious virus particles (11). On the other hand, most if not all of these proviruses have an open reading frame in the U3 region, which encodes polymorphic superantigens. These endogenous MMTV superantigens lead to deletion of T cells expressing the appropriate TCR V $\beta$  genes by virtue

of self-tolerance induction during thymic development. For instance, Mls-1, the superantigen encoded by *Mtv-7 sag*, leads to deletion of V $\beta$ 6<sup>+</sup>, 7<sup>+</sup>, 8.1<sup>+</sup>, and 9<sup>+</sup> T cells (12–16). In addition, Mls-1 causes positive selection of V $\beta$ 14<sup>+</sup> T cells (17). Superantigens encoded by infectious MMTVs also induce clonal deletion, but the deletion process begins later in the development of the mouse and occurs at a slower rate compared with superantigens encoded by endogenous MMTVs (7, 18, 19). Experimental evidence indicates that superantigen-mediated stimulation of T cells is essential for transmission of infectious MMTV, because mice specifically lacking superantigen-reactive T cells do not transmit the virus (20–22).

Although it has been known for a long time that B cells express endogenous MMTV superantigens (23), their role in viral transmission was only addressed recently. Held et al. (21, 22) demonstrated that MMTVs primarily infect B cells, and that activated B cells show the highest level of MMTV proviral infection. Furthermore, they found that viral infection leads to specific activation and proliferation of B cells. In this study we investigated whether B cells are essential for MMTV infection in mice deficient in B cells because of disruption of one of the membrane exons of the IgM H chain gene (24). We tested these mice for their capacity to transmit infectious MMTVs and delete T cells due to superantigen expression by infectious as well as endogenous MMTVs.

We found neither deletion of T cells reactive to infectious MMTV superantigens nor proviral insertions in spleen and mammary tissue of B cell-deficient mice that had been foster nursed by MMTV-secreting mothers. However, in foster-

<sup>1</sup> Abbreviation used in this paper: MMTV, murine mammary tumor virus.

nursed heterozygous mutant mice, proviruses were detected in these tissues. Furthermore, heterozygous offspring of MMTV-infected B cell-deficient mice showed no detectable signs of viral infection. We conclude that MMTVs, after passing through the gut epithelium, infect B cells, and that infection of T cells is dependent on the presence of B cells. The infection of B cells is a crucial step in the viral life cycle, because no MMTV proviruses were detectable in the mammary tissue of B cell-deficient mice, and no viral transmission to the offspring of these mice occurred.

On the other hand, negative and positive selection of T cells due to superantigens encoded by endogenous MMTVs was as efficient in B cell-deficient as in control mice. These results indicate that presentation of endogenous superantigens can occur by cell types other than B cells.

## Materials and Methods

**Mice.** All mice used in this study were bred and maintained at the animal facility of Tufts University School of Medicine. One male homozygous  $\mu$ MT mouse (24), backcrossed several generations with C57BL/6, was crossed with a female AKR/J mouse (The Jackson Laboratories, Bar Harbor, ME). The F<sub>1</sub> generation was intercrossed, and the F<sub>2</sub> and F<sub>3</sub> generations were typed for endogenous MMTV, MHC class II, and the mutated C $\mu$  gene by use of Southern blot analysis. C3H/HeOu, C57BL/6, and CBA/Ca strains were obtained from The Jackson Laboratories, and the BALB/c SW mice (7) were kindly provided by Dr. H. Acha-Orbea (Ludwig Institute, Lausanne, Switzerland). For the foster-breeding experiments, offspring  $\leq$  3 d were removed from their parents and nursed by C3H/HeOu or BALB/c SW mothers that had given birth within the last 3 d. The litter of the nursing mother was removed with the exception of two or three pups, which served as control animals for successful MMTV transmission.

**Southern Blot Analysis.** DNA was extracted from tail tissue of mice, as described (25), and 10  $\mu$ g DNA was digested with 20 U of PvuII (New England Biolabs, Beverly, MA). Southern blots were carried out as previously described (26), except that denaturation was carried out in 0.2 M NaOH, 0.75 M NaCl. For MMTV typing, blots were hybridized with an MMTV-LTR-specific probe (27); a 2.2-kb band was indicative for *Mtv-7* (9). For typing the disrupted C $\mu$  gene, a BglII-PstI fragment from IgM H chain cDNA (28) was used as probe; an  $\sim$ 3.3-kb band corresponded to the disrupted C $\mu$  gene, and an  $\sim$ 3.2-kb band corresponded to the wild-type C $\mu$  gene. DNA derived from a heterozygous animal was run on all blots as a control. An EcoRI-XhoI fragment, derived from the genomic clone of the I-A $\beta$  gene, was used as a probe for MHC class II typing (29). This probe hybridizes to an  $\sim$ 3.7-kb band for I-A $^b$  and an  $\sim$ 4.4-kb band for I-A $^k$ .

**Antibodies.** The following antibody-producing hybridomas were obtained from the American Type Culture Collection (Rockville, MD): anti-V $\beta$ 6 (44-22-1) and anti-V $\beta$ 8.2 (F23.2). Anti-V $\beta$ 5 (MR9-4) and anti-V $\beta$ 11 (RR3-15) (30, 31) hybridomas were provided by Dr. S. Datta and anti-B220 (RA3-6B2) (32) hybridomas were provided by Dr. H. Wortis (Tufts University, Boston, MA). mAbs were biotinylated after purification from tissue culture supernatant using protein A-Sepharose beads. Biotinylated anti-V $\beta$ 8.1-8.2 (KJ16) and anti-V $\beta$ 14 (14-2) mAb were purchased from PharMingen (San Diego, CA). The anti-CD4 (GK1.5) and anti-CD8 (Tib 105) mAbs were also obtained from the American Type Culture Collection and were used in FITC (Sigma Chemical Co., St. Louis, MO) conjugated form.

**Flow Cytometry.** Single-cell suspensions were prepared from spleen and thymus of 5- and 10-d-old mice, and the spleen cell suspension was treated with Tris-buffered ammonium chloride to lyse red blood cells. Whole blood (0.5 ml) was obtained from individual mice, and PBMC were purified on a gradient (9.9% Ficoll 400; Sigma Chemical Co.), 9.6% sodium metrizoate (Accurate Chemical and Scientific Corp., Westbury, NY)). The cells were washed in HBSS (Gibco BRL, Gaithersburg, MD), incubated on ice for 30 min with the biotinylated anti-V $\beta$  or anti-B220 antibodies, washed again, and incubated for another 30 min with the FITC-conjugated anti-CD4 or anti-CD8 antibodies and with PE-streptavidin (Southern Biotechnology Associates, Inc., Birmingham, AL). Two-color fluorescence cytometry was carried out with a flow cytometer (FACScan<sup>®</sup>; Becton Dickinson & Co., Mountain View, CA).

**MMTV-C3H Provirus Assay.** DNA was prepared from spleen or mammary tissue according to published procedures (25). The MMTV(C3H) LTR sequence was amplified from 5  $\mu$ g DNA with PCR in PCR buffer (Perkin Elmer Corp., Norwalk, CT), 1 mM each dATP, dCTP, dGTP, and dTTP, 1  $\mu$ M each primer, and 2.5 U AmpliTaq DNA polymerase (Perkin-Elmer) in a final volume of 50  $\mu$ l (5' primer: CTCTTACAAGCCGCATCGAT; 3' primer: TAATGTTCTATTAGTCCAGCCACTGT). Amplification was carried out in a DNA thermal cycler (Perkin-Elmer) with the following program: 19 cycles of 1 min at 94°C, 2 min at 55°C, and 2 min at 72°C; 16 cycles of 1 min at 94°C, 2 min at 55°C, and 2–4.5 min at 72°C, finished by 10 min at 72°C and cooling down to 4°C. 20  $\mu$ l reaction mixture was digested with 1 U MunI (Stratagene Inc., La Jolla, CA) for 1 h at 37°C. 500 ng ScaI-digested pSV2cat plasmid was added before the digest; this served as an internal control for a successful restriction enzyme digest. ScaI-cut pSV2cat yields two bands at 1.4 and 0.8 kb after digestion with MunI. 20  $\mu$ l undigested DNA was run next to the digested DNA on a 1% agarose gel (MetaPhor; FMC Corp., Rockland, ME) in 1  $\times$  TBE. Bands were visualized by staining with ethidium bromide. For Southern blotting, the gel was denatured in 0.4 M NaOH, 1.5 M NaCl for 30 min, neutralized for 30 min in 1 M NaCl, 1.5 M Tris-HCl, pH 8.0 (all Sigma Chemical Co.), and soaked for 20 min in 1  $\times$  TAE. DNA was transferred to a nylon membrane (Bio-trans; ICN Biomedicals, Inc., Irvine, CA) in 1  $\times$  TAE with The Panther<sup>™</sup> Semi-Dry Electrobloater (Owl Scientific Inc., Cambridge, MA) for 1 h at 295 mA. The membrane was UV cross-linked and treated as described in Southern blot analysis. A 374-bp RsaI fragment of the MMTV(C3H) LTR (27) was used as probe.

## Results

**Establishment of H-2<sup>k</sup> Mtv-7<sup>+</sup> B Cell-Deficient Mice.**  $\mu$ MT mice are deficient in B cells because of the disruption of one of the membrane exons of the IgM H chain gene (24). They were derived from 129/Sv and were subsequently backcrossed to C57BL/6, two mouse strains that are H-2<sup>b</sup> and *Mtv-7*<sup>-</sup>. Because mice of the H-2<sup>b</sup> MHC haplotype do not express MHC I-E molecules, most MMTV superantigens are not presented. Thus, the  $\mu$ MT mice were crossed with AKR/J mice, which are H-2<sup>k</sup>, an MHC haplotype that presents MMTV superantigens very efficiently (13, 14). In addition, AKR/J mice are *Mtv-7*<sup>+</sup>, allowing us to study the presentation of the endogenous superantigen Mls-1 in B cell-deficient mice. F<sub>2</sub>- and F<sub>3</sub>-generation mice derived from this cross were used for all the studies described in this article. The

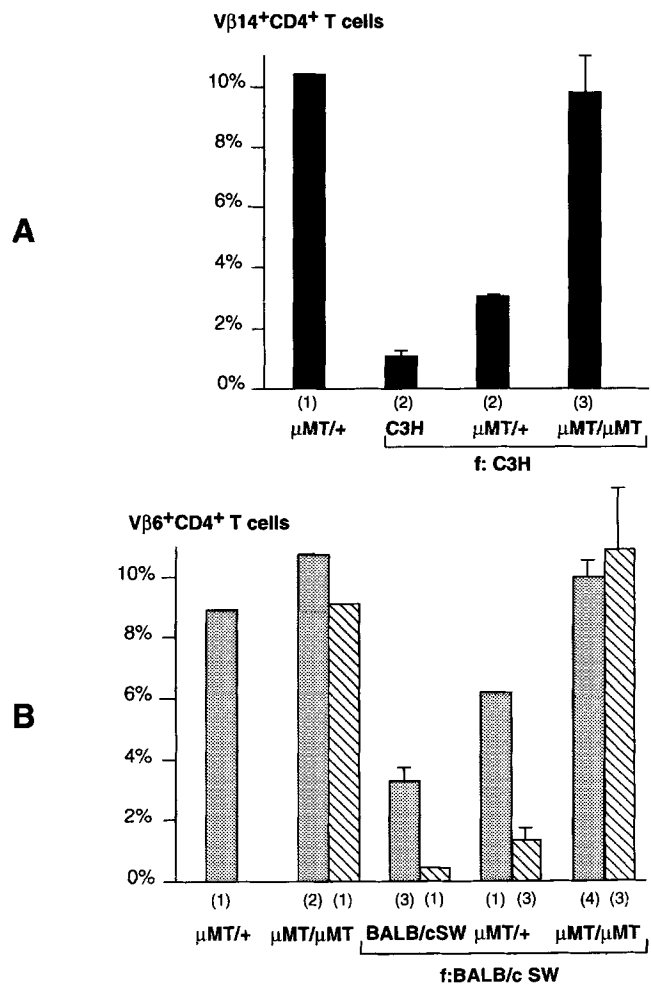
mice were typed for the disrupted  $\mu$  gene, MHC class II, and *Mtv-7* by Southern blotting.

**B Cell-deficient Mice Infected with MMTV(C3H) Do Not Delete  $V\beta 14^+$  CD4<sup>+</sup> T Cells.** It has been suggested that MMTV infection occurs first in B cells and only later in T cells (21, 22). To test, therefore, whether mice deficient in B cells are protected from viral infection, we foster nursed B cell-deficient mice on C3H/HeOu females, which secrete MMTV(C3H). MMTV(C3H) encodes a superantigen leading to the deletion of  $V\beta 14^+$  CD4<sup>+</sup> T cells (4, 5) (i.e., a decrease in  $V\beta 14^+$  T cells is indicative of a successful MMTV [C3H] infection). As shown in Fig. 1 A, no deletion of  $V\beta 14^+$  CD4<sup>+</sup> T cells could be observed in the B cell-deficient mice, whereas heterozygous B cell-mutant and C3H/HeOu mice, which were foster nursed together with the B cell-deficient mice, showed deletion of  $V\beta 14^+$  CD4<sup>+</sup> T cells. The higher percentage of  $V\beta 14^+$  CD4<sup>+</sup> T cells in heterozygous B cell-mutant mice compared with C3H/HeOu mice could be due to the reduced number of B cells in the heterozygous B cell-mutant mice, as well as to strain differences.

We also infected B cell-deficient mice with another virus, MMTV(SW) (7). The MMTV(SW) superantigen is very similar to Mls-1, and, like Mls-1, leads to deletion of  $V\beta 6^+$  T cells. *Mtv-7*<sup>-</sup>, H-2<sup>k</sup> B cell-deficient mice were foster nursed by BALB/c SW mice, which secrete MMTV(SW) in the milk. No deletion of superantigen-reactive  $V\beta 6^+$  T cells was observed in B cell-deficient mice, whereas deletion was already detectable after 6 wk in the heterozygous littermates of the same offspring and the control BALB/c mice, nursed together with the B cell-deficient mice (Fig. 1 B). At 6 mo of age, the infected heterozygous animals had most of their  $V\beta 6^+$  T cells deleted, whereas in the B cell-deficient mice, the amount of  $V\beta 6^+$  T cells was still comparable to the noninfected animals.

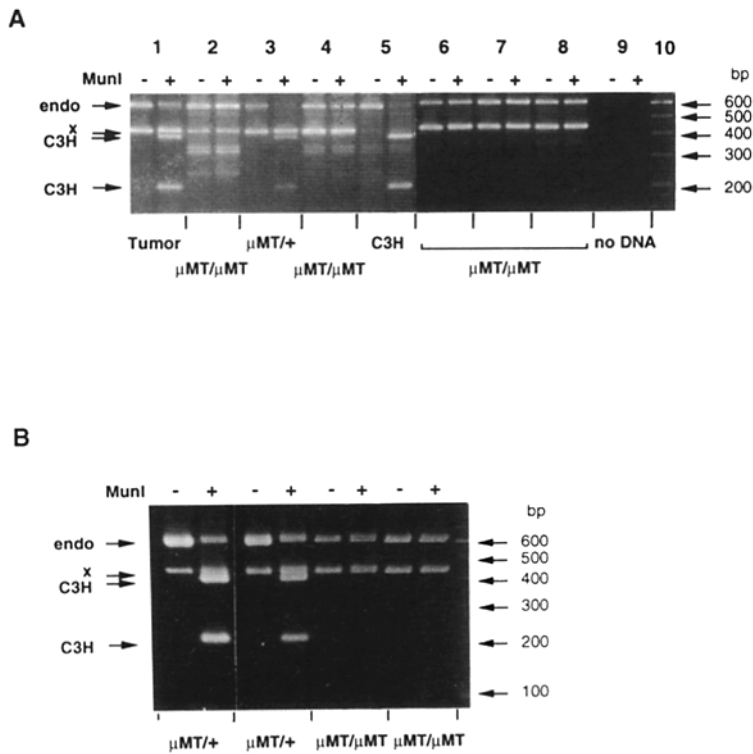
Although these results support the hypothesis that B cell-deficient mice are resistant to MMTV infection, it is conceivable that these mice were actually infected, but the lack of B cells prevented the presentation of the MMTV-encoded superantigens and, thus, prevented the deletion of the appropriate T cells. To address this issue, we determined whether spleen cells and the mammary tissue of these mice were infected with MMTV(C3H), with proviral insertion as a read-out.

**B Cell-deficient Mice Have No Detectable Proviral Insertions in Spleen Cells or Mammary Tissue.** It is difficult to distinguish MMTV(C3H) proviruses from endogenous MMTVs by conventional Southern blot or PCR techniques, because the DNA sequences are closely related (33). Thus, we developed a sensitive and specific assay for MMTV(C3H) proviruses, using a *MunI* restriction enzyme site that is unique for the MMTV(C3H) LTR (position 521 in Brandt-Carlson et al. [33]). Among the sequenced endogenous MMTV LTRs, only the *Mtv-2* LTR contains a *MunI* site; however, *Mtv-2* is not present in mice used in this study. We designed PCR primers homologous to the MMTV(C3H) LTR sequences that should preferentially amplify these sequences, although the primers are not specific enough to prevent amplification of endoge-



**Figure 1.** MMTV-infected B cell-deficient mice do not delete T cells reactive with viral superantigen. (A) Percentage of  $V\beta 14^+$  CD4<sup>+</sup> T cells determined by flow cytometry in peripheral blood of 4-mo-old mice infected with MMTV(C3H). First bar represents results from a noninfected, heterozygous B cell-mutant mouse ( $\mu Mt/+$ ). The remaining bars are the results from MMTV(C3H)-infected animals. C3H; C3H/HeOu mice foster-nursed together with B cell-mutant mice (control mice for successful infection);  $\mu Mt/+$ , heterozygous B cell-mutant mice;  $\mu MT/\mu MT$ , homozygous B cell-deficient mice. Single result for a non-foster-nursed animal was obtained from an age-matched animal in the same FACS experiment. Percentage of  $V\beta 14^+$  T cells in various noninfected  $\mu MT$  mice are also shown in Fig. 7. (B) Percentage of  $V\beta 6^+$  CD4<sup>+</sup> T cells in peripheral blood of 6-wk- (grey bars) and 6-mo- (hatched bars) old mice infected with MMTV (SW). First three bars show representative results from noninfected animals for comparison; percentages of  $V\beta 6^+$  CD4<sup>+</sup> T cells of more uninfected  $\mu MT$  mice are shown in Fig. 4. The remaining bars represent results from animals infected with MMTV (SW). BALB/cSW, offspring foster-nursed together with B cell-mutant mice. Results for each figure were obtained from two independent litters. Numbers in parenthesis represent number of animals tested. Error bars show SD.

nous MMTV LTR sequences. The amplified DNA was digested with *MunI*, yielding two fragments of 400 and 200 bp specific for the MMTV(C3H) LTR. Spleen cells have been shown to contain MMTV proviruses in infected mice (2). Therefore, spleen DNA from C3H/HeOu mice, which carry



**Figure 2.** No MMTV(C3H) proviruses are detected in spleen and mammary tissue of infected B cell-deficient mice. MMTV(C3H) provirus-specific PCR was performed as described in Materials and Methods, and DNA samples were run on an agarose gel and stained with ethidium bromide; Southern blotting was performed with some of the gels, but no additional bands were revealed. Each sample was loaded undigested (*MunI*<sup>-</sup>) and digested with *MunI* (*MunI*<sup>+</sup>) to indicate specificity of the digest. PCR yields a specific band of 600 bp derived from the MMTV(C3H) provirus and endogenous MMTV (*endo*). Another band (*x*) was observed in most samples, however, this band seems to be the result of nonspecific amplification, because an MMTV LTR probe does not hybridize with this band. *MunI* digestion yields the two MMTV(C3H)-specific bands at 200 and 400 bp (*C3H*). (A) Lane 1 shows the results with DNA from a freshly isolated C3H/HeOu mammary tumor as template. Spleen DNA was used as template in lanes 2–8. DNA was obtained from a noninfected B cell-deficient mouse (lane 2), a heterozygous B cell-mutant mouse foster-nursed by C3H/HeOu (lane 3), a C3H/HeOu mouse (lane 5) and four B cell-deficient mice (lanes 4 and 6–8) derived from three different litters. Lane 9 shows no DNA control. Lane 10, 100-bp marker (Gibco BRL). All results shown were obtained in the same PCR experiment. (B) MMTV(C3H) provirus assay performed with mammary tissue DNA. First two samples were derived from heterozygous B-mutant mice, other two samples were derived from B cell-deficient mice. Southern blotting revealed no MMTV(C3H)-specific bands in B cell-deficient mice.

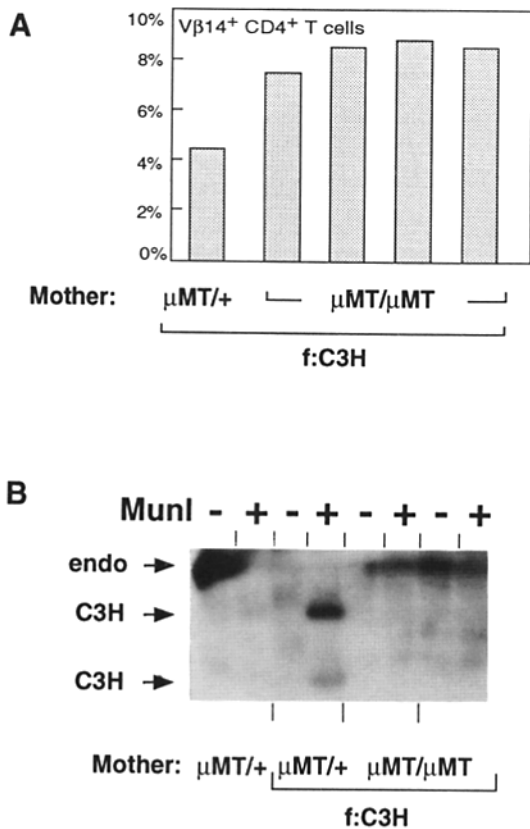
MMTV(C3H), was tested with this assay, and the expected 400- and 200-bp bands were observed after digestion with *MunI* (Fig. 2 A, lane 5). To exclude the possibility that endogenous MMTVs with LTR sequences similar to the MMTV(C3H) LTR cause false positive results, we tested DNAs from various MMTV(C3H)-free B cell-mutant mice containing a variety of endogenous MMTVs. None of the PCR-amplified fragments was cut by *MunI*, indicating that the assay is specific for MMTV(C3H) proviruses (results not shown). By diluting DNA from a mammary tumor (approximately one proviral copy per cell) with DNA from mammary tissue of a noninfected mouse, we estimated that this assay is sensitive enough to detect one infected cell in  $10^4$  cells by ethidium bromide staining and at least one infected cell in  $10^6$  cells by Southern blotting, respectively.

Using this assay, we tested spleen DNA from B cell-deficient mice that had been foster nursed by C3H/HeOu mice and had been analyzed for their level of  $V\beta 14^+$  T cells. No MMTV(C3H) proviral infections could be detected in the B cell-deficient mice, whereas the heterozygous controls and C3H/HeOu mice clearly showed the presence of MMTV(C3H) proviruses in spleen cells (Fig. 2 A). We also tested DNA from the mammary tissue of these mice for proviral infection, because this tissue shows the highest level of viral infection and is the site of viral transmission to the offspring (2). Again, we could not detect any proviral insertions in homozygous mutant mice, but heterozygous control mice were clearly positive (Fig. 2 B). From these results we conclude that B cells are required for MMTV infection of lymphocytes and mammary tissue.

#### B Cell-deficient Mice Do Not Transmit MMTV to the Offspring

The above results indicate that infection of mammary tissue is inhibited in the absence of B cells. It may be possible, however, that infection of the mammary tissue occurred at a low level, undetectable with the method used but still sufficient for transmission of the virus to the offspring. Thus, we bred female B cell-deficient mice that had been foster nursed on a C3H/HeOu mouse with heterozygous B cell-mutant mice and tested the heterozygous offspring of this cross for signs of viral infection. We used heterozygous offspring because viral infection is not detectable in homozygous B cell-mutant mice, as shown above. We analyzed the level of  $V\beta 14^+$   $CD4^+$  T cells in these mice and found no deletion of these cells. In addition, no proviral insertions were detectable in the mammary tissue of these animals (Fig. 3). As a control, we tested the age-matched offspring of a female heterozygous B cell-mutant mouse that had been foster nursed by a C3H/HeOu mouse and found T cell deletion in the periphery and proviral insertions in the mammary tissue. From these results we conclude that B cells are essential for viral transmission from the gut to the mammary tissue.

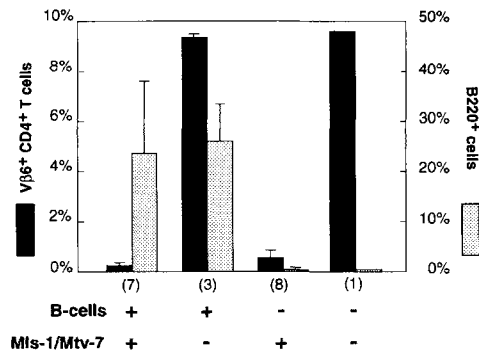
*B Cells Are Not Required for Presentation of Mtv-7 or Mtv-9 Superantigens in vivo.* Having established that B cells are required for MMTV infection of newborn mice, it seemed possible that B cells may also be responsible for presentation of superantigens encoded by endogenous MMTVs, causing deletion of reactive T cells. Thus, we analyzed the TCR  $V\beta$  profile of *Mtv-7*<sup>+</sup> B cell-deficient mice. The lack of B cells, surmised from the Southern blot typing, was confirmed by flow cytometry with a B220-specific mAb and determina-



**Figure 3.** B cell-deficient mice do not transmit MMTV to the offspring. C3H/HeOu foster-nursed female B cell-deficient mice were crossed with heterozygous male B cell-mutant mice and the heterozygous offspring tested for MMTV(C3H) infection. As a control, we used the heterozygous offspring from a C3H/HeOu foster-nursed female heterozygous B cell-mutant mouse bred with a male B cell-deficient mouse. (A) Percentage of Vβ14+ CD4+ T cells in the peripheral blood of 7-wk-old mice, determined by flow cytometry. Left bar represents result from the control breeding, other bars show the results from four individual mice of the test breeding. (B) Southern blot of MMTV(C3H) provirus PCR assay of these mice (for details of assay see Fig. 2 and Materials and Methods). DNA from the mammary tissue of 3-mo-old mice was used as template. First four lanes show results from control mice (a noninfected heterozygous B cell-mutant mouse and the infected heterozygous offspring of the control breeding).

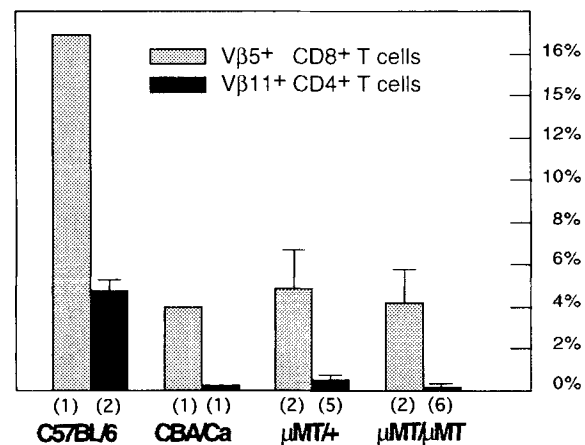
tion of the serum Ig titer. In all cases, homozygous mutant mice had no significant number of B220+ cells ( $\leq 0.4 \pm 0.18\%$ , Fig. 4) and undetectable levels of serum Ig (data not shown), whereas heterozygous mutant mice had intermediate levels compared with control mice. We observed that *Mtv-7*+ B cell-deficient mice deleted Vβ6+ CD4+ T cells as efficiently as the *Mtv-7*+ heterozygous littermates (Fig. 4). The slightly higher percentage of Vβ6+ CD4+ T cells in the B cell-deficient mice is not significant and is probably due to experimental variation seen in noninbred mouse strains.

Furthermore, we tested T cell deletion induced by the *Mtv-9* *sag* gene product. This superantigen, also known as Etc-1, deletes Vβ5+ and Vβ11+ T cells in vivo, although the kinetics for deletion are slower than for deletion by Mls-1



**Figure 4.** B cells are not required for Mls-1-induced clonal deletion of Vβ6+ T cells. Percentage of Vβ6+ CD4+ T cells (solid bars, left scale) and percentage of B220+ cells (grey bars, right scale) in the peripheral blood were determined by flow cytometry. The number of mice tested is shown in parentheses. The first two pairs of columns show the results from *Mtv-7*+ and *Mtv-7*- mice, which are heterozygous for the disrupted *Cμ* gene (i.e., they possess B cells), and the second two pairs show the results of B cell-deficient mice. The mice were either H-2<sup>k</sup> or H-2<sup>b/k</sup>, and blood samples were taken between 6 wk and 5 mo of age. Age and MHC haplotype did not significantly influence the level of Vβ6+ T cells. The average of B220+ cells in the B cell-deficient mice was  $0.4 \pm 0.2\%$ .

(34–36). Another difference is the inability of the *Mtv-9* superantigen to stimulate naive T cells in vitro, whereas Mls-1 is a strong stimulator of T cells in vitro (37). Thus, the *Mtv-9* superantigen is considered a weaker superantigen than *Mtv-7*. Fig. 5 shows that deletion of *Mtv-9* superantigen-reactive T cells was not impaired in the *Mtv-9*+ B cell-deficient mice, similar to the result we obtained for Mls-1. C57BL/6 mice were used as negative control animals because they do not

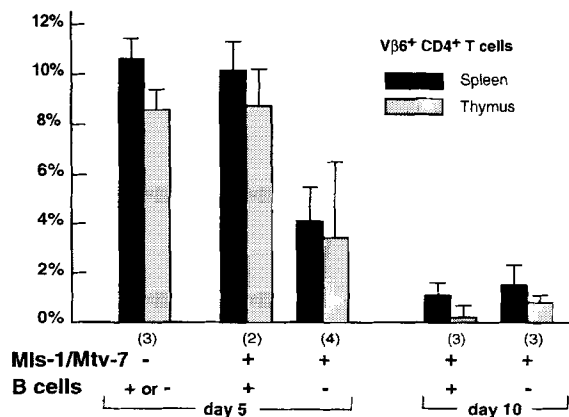


**Figure 5.** B cells are not required for *Mtv-9* superantigen-induced T cell deletion. Percentage of Vβ5+ CD8+ T cells (grey bars) and percentage of Vβ11+ CD4+ T cells (black bars) ( $\pm$ SD) in peripheral blood from mice  $\geq 6$  wk old were determined by flow cytometry. Number of mice tested is shown in parentheses. C57BL/6, CBA/Ca, heterozygous B cell-mutant ( $\mu$ MT/+) and -deficient mice ( $\mu$ MT/ $\mu$ MT) were tested. All these mice are *Mtv-9*+ and H-2<sup>k</sup>, except C57BL/6, which is H-2<sup>b</sup>. The H-2<sup>b</sup> haplotype lacks I-E expression, thus no T cell deletion due to *Mtv-9* superantigen occurs.

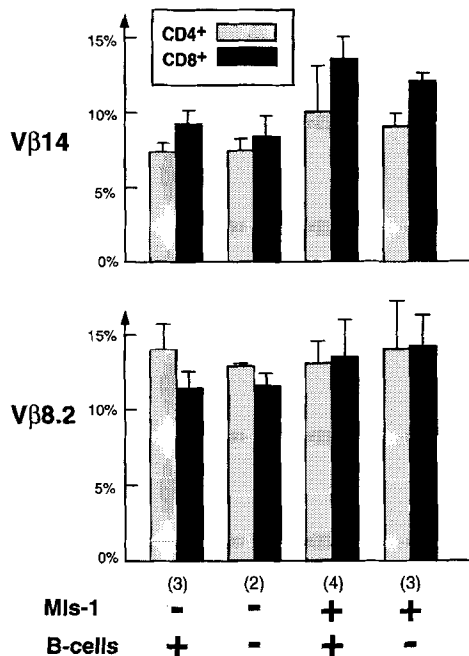
delete *Mtv-9* superantigen-reactive T cells because of the lack of MHC class II I-E molecules (35, 36). These results indicate that the T cell deletion process due to endogenous superantigens in vivo is not influenced by the absence of B cells, regardless of the "antigenic strength" of the particular superantigen.

**T Cell Deletion Kinetics in *Mtv-7*<sup>+</sup> B Cell-Deficient Mice.** Although the absence of B cells had no influence on the deletion of Vβ6<sup>+</sup> T cells in adult *Mtv-7*<sup>+</sup> mice, it was of interest to test whether a difference could be observed during ontogeny. If superantigen presentation is less efficient in B cell-deficient mice than in heterozygous B cell-mutant mice, we would expect slower deletion kinetics in the former mice. Thus, we compared the percentage of Vβ6<sup>+</sup> T cells in the spleen and thymus of 5- and 10-d-old *Mtv-7*<sup>+</sup> hetero- and homozygous B cell-deficient mice. As can be seen in Fig. 6, 5-d-old B cell-deficient mice deleted Vβ6<sup>+</sup> T cells, whereas the heterozygous controls still had high levels of Vβ6<sup>+</sup> T cells. At day 10 the percentage of Vβ6<sup>+</sup> T cells was virtually identical in homo- and heterozygous B cell-deficient mice. We observed in all mice that deletion occurred earlier in the thymus than in the spleen. These results indicate that T cell deletion due to endogenous superantigens is even more efficient in homozygous B-cell deficient mice than in heterozygous control mice, especially at early stages of ontogeny.

**Positive Selection of Vβ14<sup>+</sup> T Cells in *Mtv-7*<sup>+</sup> Mice Is Not Affected by the Lack of B Cells.** It has recently been reported that the percentage of Vβ14<sup>+</sup> T cells is specifically increased in Mls-1<sup>+</sup> mice (17). In the following experiments, we tested whether the lack of B cells has any influence on this effect. PBL from H-2<sup>k</sup> *Mtv-7*<sup>+/-</sup> hetero- and homozygous B cell-deficient mice were double stained for TCR Vβ14 or Vβ8.2 chains (control) and for CD4 and CD8, respectively (Fig. 7). Similar to the results of Liao and Raulet (17), we detected a significant increase in Vβ14<sup>+</sup> CD8<sup>+</sup> T cells in *Mtv-7*<sup>+</sup> mice compared with *Mtv-7*<sup>-</sup> mice (~50%), whereas



**Figure 6.** Time course of Vβ6<sup>+</sup> CD4<sup>+</sup> T cell deletion in B cell-deficient mice. Spleen (solid bars) and thymus (grey bars) were removed from 5- and 10-d-old mice, and the percentage of Vβ6<sup>+</sup> CD4<sup>+</sup> T cells was determined by flow cytometry. The number of mice tested is shown in parentheses. Day 5 animals were H-2<sup>b</sup> and day 10 animals were H-2<sup>b/k</sup>.



**Figure 7.** Positive selection of Vβ14<sup>+</sup> CD8<sup>+</sup> T cells in *Mtv-7*<sup>+</sup> mice. Blood from 2-3 mo-old H-2<sup>k</sup> mice was analyzed for Vβ14<sup>+</sup> (top) and Vβ8.2<sup>+</sup> (bottom) T cells by flow cytometry. CD4<sup>+</sup> T cells are represented by grey bars, CD8<sup>+</sup> T cells by black bars. The number of animals tested is indicated in parentheses. Results are from (left to right) *Mtv-7*<sup>-</sup> heterozygous B cell-mutant mice, *Mtv-7*<sup>-</sup> homozygous, *Mtv-7*<sup>+</sup> heterozygous, and *Mtv-7*<sup>+</sup> homozygous B cell-mutant mice. Error bars indicate SD. Increase of Vβ14<sup>+</sup> CD8<sup>+</sup> T cells was 46.5% for μMT/+ and 35.9% for μMT/μMT mice between the Mls-1<sup>-</sup> and Mls-1<sup>+</sup> genotypes ( $p < 0.01$  and  $p < 0.05$ , Student's *t* test). The respective increases for Vβ8.2<sup>+</sup> CD8<sup>+</sup> T cells were 18.3 and 19.8% ( $p < 0.2$ ). The increase in Vβ8.2<sup>+</sup> CD8<sup>+</sup> T cells is about twofold lower than the increase of Vβ14<sup>+</sup> CD8<sup>+</sup> T cells and is due to the deletion of Mls-reactive T cells.

the percentage of the control Vβ8.2 CD8<sup>+</sup> T cells increased only slightly (~20%) because of the deletion of the Mls-1-reactive Vβ6<sup>+</sup>, Vβ7<sup>+</sup>, Vβ8.1<sup>+</sup>, and Vβ9<sup>+</sup> T cells. We could not detect a significant difference in the increase of Vβ14<sup>+</sup> T cells in the homo- versus the heterozygous B cell-deficient mice. This result indicates that B cells are not essential for the increase of Vβ14<sup>+</sup> T cells in *Mtv-7*<sup>+</sup> mice.

## Discussion

**Role of B Cells in the Transmission of Infectious MMTV.** MMTV infects T and B cells in the mouse (3, 38). Although the role of T cells in infection has been well characterized (20-22), the importance of B cells was only surmised recently (21, 22). In this study we investigated whether mice deficient in B cells (24) can be infected with MMTV. For this purpose, B cell-deficient mice were foster nursed on MMTV-secreting mothers. No new viral DNA in the spleen and mammary tissue and no T cell deletion due to MMTV superantigens were detectable in these mice. Furthermore, heterozygous offspring of these mice showed no detectable signs of viral infection. Thus, we conclude that B cells are essential for

an early step in the infection and/or spread of virus in mice exposed to MMTV in milk. This indicates that infection of T cells is dependent on the presence of B cells, or else we should have been able to detect MMTV proviruses in the spleens of B cell-deficient mice, which contain mainly T cells.

The role of T cells for the transmission of MMTV has been established by several groups. First, Tsubura et al. (3) discovered that nude mice exposed to MMTV in milk did not transmit the virus to the mammary tissue, and they concluded that T cells, but not B cells, carry MMTV in the blood. Later, Golovkina et al. (20) showed that T cells specifically reactive with the MMTV superantigen are required for viral transmission. Namely, they demonstrated that MMTV(C3H) *sag* transgenic mice, which deleted MMTV(C3H) superantigen-reactive V $\beta$ 14<sup>+</sup> T cells, did not transmit MMTV(C3H) to their mammary tissue after exposure to this virus as newborns. Similar observations were made by Held et al. (21), who showed that reconstitution of nude mice with superantigen-reactive T cells restored susceptibility to infection. Furthermore, they found that transgenic mice expressing exclusively V $\beta$ 8.2<sup>+</sup> TCRs, which are not reactive with the superantigen of MMTV(SW), did not transmit this virus when exposed to it in milk (22). This group also established that both T and B cells can transfer MMTV in lymphocyte transfer experiments (38).

Although our data cannot rule out that T cells transfer the virus to the mammary tissue, we think that this is unlikely for the following reasons. First, Held et al. (21, 22) have shown that predominantly B cells are infected, and that the infection of T cells occurs only at a later time. Second, we have shown that T cells cannot be infected in the absence of B cells. Thus, it is conceivable that the virus is transferred from B cells to T cells through cell-to-cell contact, involving superantigen presentation by B cells and recognition by T cells. However, this leads eventually to clonal deletion or anergy of the reactive T cells (39). Thus, the cells that are supposed to infect the mammary tissue are either removed from the periphery or are inactivated after infection with the virus.

It is more likely, therefore, that B cells transmit MMTV to the mammary tissue, whereas the infected T cells serve as a storage pool for the virus. This is consistent with the observation that MMTV can infect all lymphocyte subsets in a nonmutant mouse (38). Superantigen-reactive T cells are required for the stimulation of the B cells, leading to the expansion of infected B cells (21, 22). Furthermore, this stimulation may induce expression of viral or endogenous genes necessary for the infection of the mammary tissue. The observations of Tsubura and coworkers (3) can easily be explained with this model. In their test system, B cells did not transmit the virus, because nude mice were used as recipients. Thus, no T cells were present to stimulate the infected B cells, which seems to be a requirement for the spread of the virus. On the other hand, T cells were able to infect the nude mice, because the virus transferred to B cells, and the injected T cells could stimulate B cells, thus leading to viral transmission to the mammary tissue.

The observation that IL-4 increases functional expression of superantigens encoded by endogenous MMTV fits well

with this model (37, 40, 41). IL-4, also known as B cell stimulating factor 1, is secreted by activated T cells and stimulates B cells. It is reasonable to assume that infectious MMTV superantigen expression increases because of IL-4 stimulation, leading to even stronger T cell activation. On the other hand, we have analyzed MMTV transmission in mice deficient in IL-4 because of a gene-targeted disruption of the IL-4 gene (42), and we could not detect any difference between IL-4-deficient mice and heterozygous littermates in their ability to transmit MMTV or the degree of T cell deletion due to infectious or endogenous MMTV superantigens (data not shown).

*Role of B Cells for Presentation of Endogenous Superantigens.* We also examined the role of B cells for the presentation of superantigens encoded by endogenous MMTVs. As a model we used the superantigen Mls-1, encoded by *Mtv-7 sag*. Although it seems clear that in vitro presentation of Mls-1 to T cells is restricted to B cells (23, 43, 44), it is still controversial whether B cells present Mls-1 in the thymus, thus leading to clonal deletion of reactive T cells. Several cell types have been suggested to induce Mls-1-driven T cell deletion in vivo. Inaba et al. (45) have proposed that CD5<sup>+</sup> thymic B cells, but not splenic B cells, cause T cell deletion, whereas Mazda et al. (46) detected V $\beta$ 6<sup>+</sup> T cell deletion in thymic organ cultures only if Mls-1-expressing B cells were injected in combination with dendritic cells. Faro et al. (47) assumed that B cells exclusively present Mls-1 in the thymus. On the other hand, Webb and Sprent (48) showed in transfer experiments that CD8<sup>+</sup> T cells from Mls-1<sup>+</sup> mice are the most efficient cell type for induction of V $\beta$ 6<sup>+</sup> T cell deletion in vivo. In agreement with this finding, Gollob and Palmer (37, 49) observed V $\beta$ 6<sup>+</sup> T cell deletion in Mls-1<sup>+</sup>  $\mu$ -suppressed mice, which have no functional B cells.

Our results support the findings of the latter two groups, because we could find no requirement for B cells for V $\beta$ 6<sup>+</sup> T cell deletion by Mls-1 in vivo. This does not exclude the possibility that B cells are capable of inducing T cell deletion, but other cell types must be able to present endogenous MMTV superantigens with similar if not even higher efficiency than B cells, because no difference in the degree and the time course of T cell deletion was detectable between B cell-deficient and heterozygous control mice. We are currently investigating which cell types actually present Mls-1 to the V $\beta$ 6<sup>+</sup> T cells in the B cell-deficient mice. Considering the results of Webb and Sprent (48), CD8<sup>+</sup> T cells are the most likely candidates.

Although we obtained the same results as Gollob and Palmer (37, 49) regarding the deletion of Mls-1-reactive T cells in  $\mu$ -suppressed mice, our results differ from theirs for the deletion of *Mtv-9* superantigen-reactive T cells. No deletion of V $\beta$ 5<sup>+</sup> CD8<sup>+</sup> and V $\beta$ 11<sup>+</sup> CD4<sup>+</sup> T cells was observed in  $\mu$ -suppressed mice; however, in B cell-deficient mice, these T cells were deleted to the same degree as in normal mice. We have no explanation for this discrepancy, especially considering that the number of B220<sup>+</sup> cells was lower in the B cell-deficient than in the  $\mu$ -suppressed mice.

Still, it could be argued that a very small and practically undetectable number of B cells mature in the B cell-deficient

mice despite the lack of surface IgM. These B cells would be sufficient to delete the Mls-1-reactive T cells, considering that as few as  $10^5$  B cells can induce Mls-1-driven T cell deletion (38). We think that this is not the case, because we would expect to see a delay in the deletion kinetics or a decrease in the efficiency of the deletion, both of which we did not observe. Furthermore, our results with MMTV-infected B cell-deficient mice suggest that B cells do not reach the periphery. Considering that MMTV-infected B cells are expanded by superantigen-specific T cell stimulation, an initially small number of B cells would be sufficient to allow the virus to spread in the B cells and reach the mammary tissue. However, we detected no viral transmission to the offspring of B cell-deficient mice.

Liao and Raulet (17) have recently demonstrated an increase in  $V\beta 14^+$   $CD8^+$  T cells in Mls-1<sup>+</sup> H-2<sup>k</sup> mice compared with Mls-1<sup>-</sup> mice. This increase was dependent on the H-2 haplotype and was greater than the increase of T cells expressing other  $V\beta$ s, like  $V\beta 8.2$ , which is caused by the deletion of Mls-1-reactive T cells. Thus, they proposed that Mls-1 positively selects  $V\beta 14^+$   $CD8^+$  T cells. We confirmed these results and observed that this increase also occurred in the absence of B cells. This is in accordance with the general assumption that thymic epithelial cells, rather than B cells, govern positive selection. On the other hand, this would indicate that the thymic epithelium expresses Mls-1.

Although this has been suggested before, to our knowledge no direct experimental evidence exists that thymic epithelium expresses Mls-1. In fact, two recent reports demonstrated that thymic epithelium does not express endogenous MMTV superantigens (50, 51).

In this study, we have dissected the role of B lymphocytes in MMTV transmission and superantigen expression in vivo with B cell-deficient mice. With this experimental system, we have unambiguously established that B cells are essential for completion of the retroviral life cycle, because no spread of MMTV occurred after infection in the absence of B cells. Because the transmission of MMTV has already been shown to depend on the presence of MMTV superantigen-reactive T cells, we propose that B cells are necessary for infection of T cells, which in turn activate the infected B cells, a chain reaction elicited by the MMTV superantigen presented by the infected B cells. The superantigen presented by B cells is the likely candidate for an interaction molecule. On the other hand, we have proven that B cells are not essential for the presentation of superantigens encoded by endogenous MMTVs, because both negative and positive selection of T cells due to endogenous superantigen expression occur as efficiently in B cell-deficient as in control mice. Thus, these superantigens can be presented to T cells by cell types other than B cells.

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