# Retroviral Infection of Neonatal Peyer's Patch Lymphocytes: The Mouse Mammary Tumor Virus Model

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## Summary

Mouse mammary tumor virus is known to infect newborn mice via mother's milk. A proposed key step for viral spread to the mammary gland is by the infection of lymphocytes. We show here that although in suckling mice retroviral proteins are found in all epithelial cells of the gut, viral DNA is exclusively detectable in the Peyer's patches. As early as 5 d after birth the infection leads to a superantigen response in the Peyer's patches but not in other lymphoid organs draining the intestine. Viral DNA can be detected before the superantigen response and becomes first evident in the Peyer's patches followed by mesenteric lymph nodes and finally all lymphoid organs.

Mouse mammary tumor virus (MMTV) is a milk-transmitted retrovirus (1). After crossing the gut the virus infects susceptible cells and ultimately the mammary gland (2). During lactation, large quantities of MMTV are secreted into milk, thus transmitting the infection to the offspring.

To date, the absence of in vitro models for MMTV infection has delayed the understanding of the identity of cells supporting viral replication and the early events in the host immune response. Therefore the infectivity in vivo has been established by the late (6 mo–1 yr) appearance of mammary tumors in susceptible strains of mice (3). Most mouse strains contain endogenous MMTV DNA which does not usually produce infectious viruses. MMTV superantigen molecules are expressed by B cells after both infection by exogenous or expression by endogenous viruses (4–8).

Superantigens are a class of molecules that interact with a large percentage of T cells that share expression of particular TCR $V\beta$ elements. They are encoded in an MMTV open reading frame (orf) and it has been shown that a sequence polymorphism at the 3' end of orf correlates with the TCR $V\beta$ specificity (9–13). The superantigen effect is an absolute requirement for the establishment of a productive infection (8, 14).

In a previous study (7, 15) we have characterized MMTV (SW), an infectious virus which stimulates a strong T and B cell superantigen response after systemic inoculation into naive adult mice. The responsive CD4$^+$ T cells in vivo express TCR $V\beta6$. BALB/c mice have three endogenous MMTV proviruses (Mtv-6, −8, and −9) which differ in both superantigen TCR $V\beta$ specificity as well as in the nucleotide sequence from MMTV(SW) orf (4).

Exploiting these differences we were able to analyze the site and kinetics of infection by the exogenous MMTV(SW), and the superantigen response in neonatal mice. In this study we find that MMTV infects the newborn mice through intestinal epithelium. The infection first occurs in the Peyer's patches and stimulates superantigen-responsive T cells as early as 5 d after birth. Finally this study shows that after infection of Peyer's patch lymphocytes, systemic spread of the virus involves first mesenteric lymph nodes and then all lymphoid organs.

## Materials and Methods

*Mice.* BALB/c mice were purchased from Harlan Olac (London, UK). MMTV(SW)-infected mice were obtained from IFFA Credo (L'Arlabesque, France) and bred in our animal facility. BALB.D2 Mls-1 mice were originally obtained from H. Festenstein (London Hospital Medical College, London, England) (16) and maintained in our animal facility.

*Isolation of Neonatal Lymphoid Organs.* Mesenteric lymph nodes were dissociated mechanically and cells were kept in RPMI medium supplemented with 0.1% BSA on ice until use. Peyer's patches of mice at different ages were removed surgically under magnification, lightly minced, and subjected to collagenase digestion (Type IA; Sigma Chemical Co., St. Louis, MO) 5 mg/ml in RPMI for 30 min at room temperature. Digested tissue was dissociated by pipetting until homogenous cell suspensions were obtained. The suspension was added to 10 ml of ice-cold RPMI containing 0.1% BSA in a 15-ml conical tube (Falcon model 2095; Becton Dickinson & Co., Mountain View, CA) and centrifuged at 700 g for 3 min. The pellet was resuspended and recentrifuged. The cells from the supernatants, almost free of endothelial cells, were used for FACS® analysis (Becton Dickinson & Co.).

*FACS® Analysis.* Lymphocytes ($0.2\text{--}10^6$) were stained with anti-V$\beta$6 (44-22-1) (17) or anti-V$\beta$14 (14.2) (18) hybridoma supernatants followed by FITC-conjugated goat anti–rat IgG or anti–rat IgM sera (Caltag Laboratories, San Francisco, CA). As a third layer, PE-conjugated anti-CD4 (GK1.5, Becton Dickinson & Co.) was used. For analysis of B cells, the cells were stained with an FITC-conjugated goat anti–mouse Ig F(ab')$_2$ fragment. The cells were analyzed on a FACScan® (Becton Dickinson & Co.) after exclusion of dead cells using forward and side scatter analysis.

*Statistical Analysis.* The statistical analysis and curve fitting were performed by Dr. Guy van Melle (Institut de Médicine sociale et préventive, Université de Lausanne). The control lymph nodes have a constant level of TCR V$\beta$6 expression with a mean of 10.0% and a SD of $\pm$ 0.61%. The 99% $t$-prediction interval for a future observation was 8.4–11.8%. Thus, observations outside this range are significantly different from the control response. The V$\beta$6-response curve of MMTV(SW)-infected mice was calculated using a bootstrap analysis (19).

*PCR Analysis.* Tissues used for the PCR were snap frozen and kept at $-80°C$. Pools of four to six mice were used for each PCR experiment. DNA of the different organs was isolated by overnight digestion at 52°C in 100 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.1 SDS, containing proteinase K (100 $\mu$g/ml). Purified DNA (300 ng) was amplified using PCR with the following oligonucleotides which recognize all the known orf sequences: 5' GATCGGATCCATGCCGCGCCTGCAGCAGA 3' (VJ77) and 5' GTGTCGACCCAAACCAAGTCAGGAAACCAC-TTG 3'(VJ71) yielding a 1,200-bp product. Alternatively, the oligonucleotide pair specific for MMTV(SW) 5' GATCGGATCCAT-GCCGCGCCTGCAGCAGA 3' (VJ77) and 5' GCGACCCCC-ATGAGTATATTTC 3'(VJ83), were used yielding a 766-bp product.

40 cycles of amplification were performed as described (7, 15). In short, 300 ng of DNA were amplified in 2 mM dNTP, 1× PCR buffer (20 mM Tris-HCl, pH 8.3, 50 mM KCl, 2 mM Mg2Cl and 0.01% gelatin), 1 $\mu$M oligonucleotides, and 0.5 U Taq-polymerase (Perkin Elmer Corp., Norwalk, CT) with the cycling steps 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C. Thereafter, the products were separated on 1.5% agarose gels and analyzed by Southern blot analysis with MMTV(GR) LTR probe using standard conditions.

*Histology.* The tissues from 10-d-old mice were quickly excised. After embedding in OCT (Miles Scientific, Naperville, IL) gut epithelium containing a pair of jejunal Peyer's patches was snap frozen in liquid nitrogen–cooled isopenthane. Sections (7 $\mu$m) on silane-treated slides were air-dried, acetone-fixed, treated with 0.1% BSA-containing PBS, pH 7.4, and incubated with rabbit anti-gp52 polyclonal serum obtained from Dr. P. Hainaut (University of Liège, Belgium) (20). After washings, the labeling was revealed with a biotinylated donkey Ig anti–rabbit serum and streptavidin-FITC (Amersham International, Amersham, Bucks, UK). Controls included a rabbit anti–mouse secretory component (SC) serum and the gp52-preadsorbed anti-gp52 rabbit serum. For detection of B cells, an FITC-conjugated goat F(ab')$_2$ fragment specific for mouse Ig was used.

*Confocal Imaging.* Confocal microscopy was performed on a confocal microscope (model MRC-600; Bio Rad Laboratories, Brussels, Belgium) mounted on a Zeiss Axiovert 35M microscope with a 63 × 1.4 NA objective, zoom 2, and 1 s/scan with 7–10 frames per image.

## Results

*Viral Uptake by Epithelia in the Newborn Intestine.* Mucosa-associated lymphoid tissue has been proposed to be a portal of entry of many pathogens (21, 22). In the small intestine these specialized areas of antigen sampling called Peyer's patches are overlaid by the follicle-associated epithelium (FAE). FAE consists of two distinct epithelial cell types, the M cells and the enterocytes. The enterocytes of the newborn ileum including the FAE, differ from their adult counterparts by their capacity to internalize proteins and accumulate them into a large supranuclear vacuole (23–25).

To study the pattern of MMTV uptake in the digestive tract of newborn mouse, the gut tissue from 10-d-old mice breast-fed continuously by MMTV(SW)-infected mothers was analyzed. Gut tissue containing a Peyer's patch was removed and frozen sections were prepared and immunolabeled with an antibody specific for the MMTV envelope protein gp52 (20). In the Peyer's patch, gp52 was seen in the FAE cells, which is consistent with previous observations (27), although it was not possible to identify the labeled cell types. The viral envelope protein accumulated beneath the epithelial lining in contact with Peyer's patch lymphocytes (Fig. 1 *A*). In the adjacent villi, the envelope protein was found in small apical vesicles and in the large vacuoles of the enterocytes (Fig. 1 *B*). This intracellular distribution was similar to that reported by others (26) and suggests a degradative pathway. There was a clear gradient in the villi, with a near complete absence of gp52 in the crypts (Fig. 1 *B*). The labeling using as a control rabbit serum directed against mouse-free SC was restricted to the cell surface of the crypt enterocytes (Fig. 1 *C*). No labeling was detected in the structures positive for gp52 (compare Fig. 1, *B* and *C*). In serial sections incubated with the gp52 preadsorbed anti-gp52 serum, the specific labeling disappeared (Fig. 1 *F*).

*Induction of B Cell Reorganization by MMTV Infection.* In 8–9-d-old mice fed by infected mothers, the size of the patches was on average three times that of control mice. The patches were removed and analyzed by immunohistology. Sections with the largest cross section diameter chosen from MMTV (SW)-infected and noninfected control mice, were immunolabeled with a F(ab')$_2$ fragment specific for mouse Ig. Whereas the B cell distribution in control mice fed by uninfected BALB/c mothers was diffuse with little follicular organization (Fig. 1 *D*), a striking reorganization of B cells was observed in MMTV(SW)-infected mice (Fig. 1 *E*). The proximity of viral material with B cells below the FAE may facilitate retroviral infection at this site. Around day 5 after birth, when the MMTV(SW)-induced T cell response became apparent, control as well as experimental animals showed a drastic increase of the percentage of B cells in the Peyer's patches, from 10 to about 50% (data not shown). Currently we do not know whether this increase is due to maturation of the immune system which starts reacting to environmental antigens or to a programmed increase of B cells.

*Kinetics of Superantigen Response in Peyer's Patches of Neonatal Mice.* MMTV(SW) encodes the most potent superantigen activity among the known murine retroviruses (15). The responding T cells express TCR V$\beta$6. To establish the time course of MMTV(SW) superantigen–induced T cell activation in Peyer's patch and mesenteric lymph nodes during the neonatal period of suckling mice, we performed FACS®
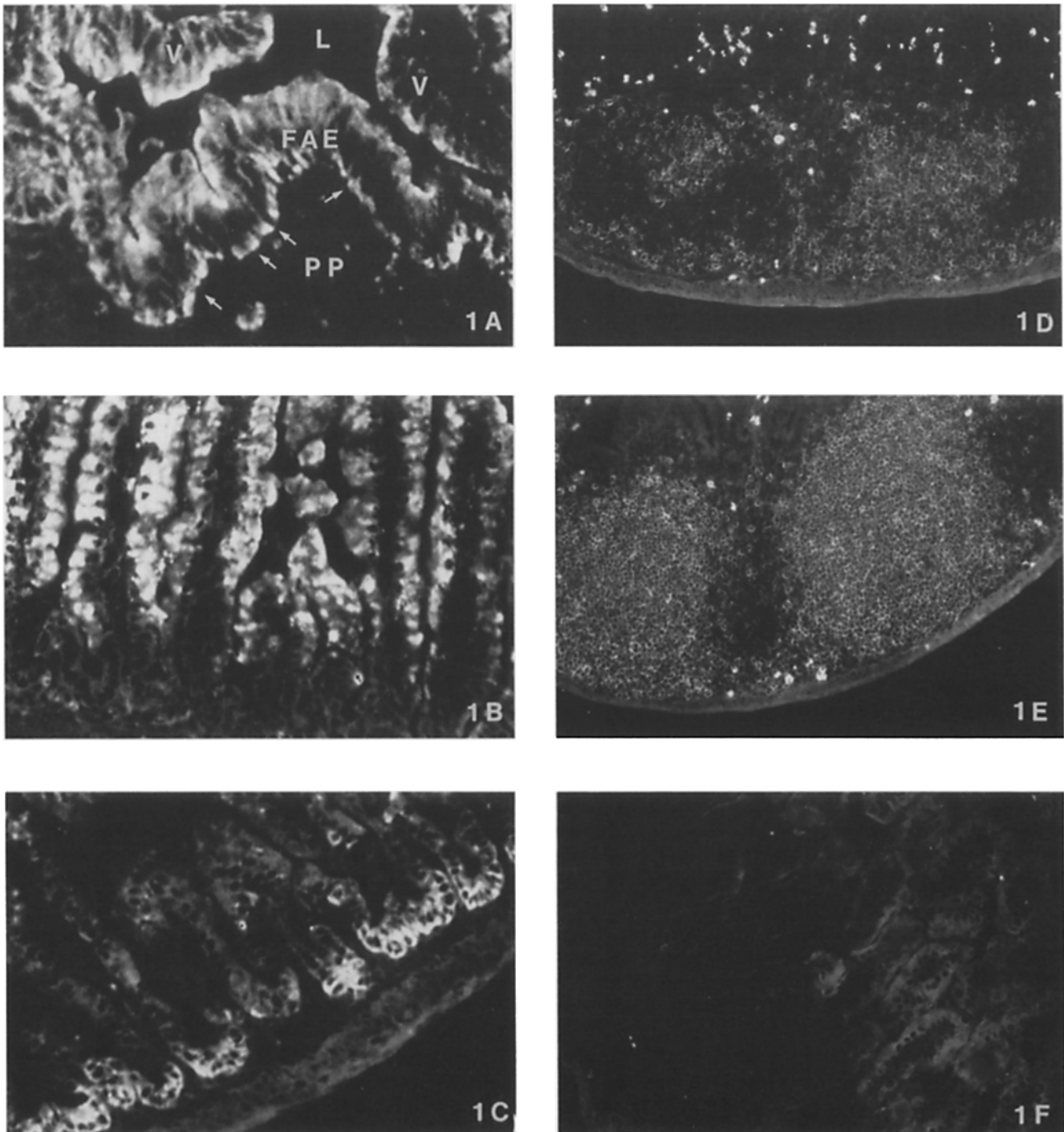
**Figure 1.** Morphology of ileal villi and Peyer's patches of newborn mice fed on MMTV-infected mothers. Frozen sections (7 $\mu$m) of ileal intestinal tissue containing one Peyer's patch from 10-d-old mice were stained with rabbit serum directed against the envelope protein gp52 (*A* and *B*), the same anti-gp52 serum preadsorbed with gp52 (*F*), and anti–mouse SC rabbit serum as a control (*C*) followed by a biotinylated donkey anti-rabbit Ig antibody, or with FITC conjugated goat F(ab')$_2$ fragment specific for mouse Ig (*D* and *E*). (*A–C*) Confocal microscopy; (*D–F*) epifluorescence microscopy. (*A*) Peyer's patch (*PP*). gp52-reactive material is seen beneath the follicle-associated epithelium (*FAE*) (*arrows*) in the dome region of the lymphoid follicle. Adjacent villi (*V*) are labeled and most of the lumen (*L*) is free of reactive material. ×450. (*B*) Ileal villi. gp52-reactive material is detected in the apical and supranuclear region of the enterocytes. Note the absence of labeling in the crypt enterocytes. ×250. (*C*) Ileal villi. Sections were incubated with the rabbit anti-SC serum (*C*) and revealed with the same secondary antibody used for gp52 staining. The labeling is restricted to the enterocytes in the crypt. Note that the structures that are labeled with anti-gp52 antibodies are not stained with the anti-SC antibody. ×250. (*D*) Peyer's patch follicle from control mice. The surface Ig$^+$ B cells form small aggregates in the Peyer's patch. The intensively stained Ig-producing plasma cells are present in the lamina propria of the adjacent villi. ×185. (*E*) Peyer's patch from mice fed on infected mothers. The surface Ig$^+$ B cells are organized in follicles in the Peyer's patch. ×185. (*F*) Ileal villi. Sections were incubated with gp52 preadsorbed anti-gp52 and processed as in *C*. Note the structures labeled with the anti-gp52 antibody (*A* and *B*) are almost unstained. ×130.
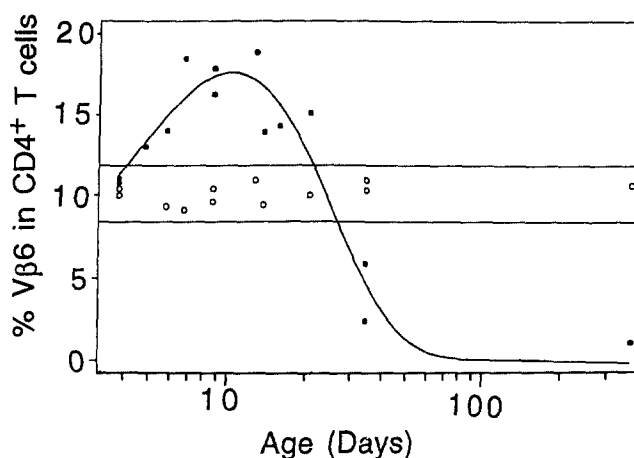
**Figure 2.** Kinetics of the local immune response towards the MMTV (SW) superantigen. Peyer's patches as well as the mesenteric lymph nodes were tested for Vβ6 expression. The mice were fed continuously by MMTV(SW)-secreting mothers. Single cell suspensions were analyzed by FACS® for TCR Vβ6 expression in the CD4+ population. Only the results for Peyer's patches are shown since the other lymph nodes did not show significant increases of the percentage of T cells expressing Vβ6. The percentage of other Vβ such as Vβ14 did not change in these experiments (data not shown). Each symbol represents an experiment with a pool of four to six mice. (O) Results obtained from uninfected BALB/c mice; (●) percentages of Vβ6+ CD4+ T cells of infected mice. The horizontal parallel lines indicate a 99% prediction interval for a future observation (±3 SD). The 99.9th percentile of the first detectable MMTV (SW) immune response is reached by day 5. The 0.1th percentile of falling back to Vβ6 control levels is on day 16. The 99th percentile of falling below control levels occurs on day 48. The x-axis is shown in log scale.

shown), we never observed any increase of Vβ6+ T cells but found that they started decreasing after 3–4 wk. The percentage of T cells expressing TCR Vβ14 did not change. Similar experiments using MMTV(C3H) did not result in a significant increase of the responding Vβ14+ T cells in the same investigated tissues (data not shown), in agreement with the above cited study on peripheral injection of MMTV(C3H) into adult naive mice (15).

*PCR Analysis of the Route of Infection and Systemic Spread.* To detect viral DNA, we used oligonucleotides specific for MMTV(SW) as well as oligonucleotides able to amplify endogenous as well as exogenous viruses (15). Using a semiquantitative PCR strategy with MMTV(SW)-specific primers, we detected neither amplification of MMTV(SW) RNA nor amplification products from BALB/c DNA. At different time points we removed Peyer's patches, small intestine mucosa freed from Peyer's patches, mesenteric lymph nodes, spleen, peripheral lymph nodes, thymus, and mammary glands, and analyzed the levels of endogenous Mtv or MMTV(SW) DNA. As can be seen in Fig. 3, MMTV(SW) DNA was exclusively detectable in Peyer's patches of infected mice at day 4 and 9 after birth. After 14 d, a signal became apparent in the mesenteric lymph nodes and later all the tested lymphoid organs were positive for MMTV(SW) DNA. At early time points no MMTV(SW) DNA was detected in the gut epithelial tissue when Peyer's patches were removed, despite the high degree of MMTV internalization by the epithelium of the entire small intestine.

analysis. Shortly after birth, the percentages of Vβ6+ CD4+ T cells, and as control Vβ14+CD4+ T cells were determined. In these experiments, the pups were fed by infected mothers from birth. As early as 5 d after birth, a superantigen response was detectable in the Peyer's patches' Vβ6+ CD4+ T cells but in no other lymphoid tissues. This response was reproducible and was maximal between day 6 and 9 after birth (see Fig. 2). After this activation, we observed a gradual reduction of the responsive Vβ6-bearing T cells, approaching a near complete deletion in adult mice in all the lymphoid organs. In mesenteric lymph nodes (data not

## Discussion

Mucosal surfaces which contact the environment are continuously threatened by invasive pathogens. Some microorganisms interact with M cells in the FAE for their uptake (22, 25). Whether MMTV exploits M cells to gain access to lymphoid follicles has not been established. Absorptive cells both in intestinal villi and in the FAE have been shown to take up MMTV (26, 27) and thus represent potential sites for viral entry. These cells are known to take up lumenal macromolecules by fluid phase endocytosis during the first 2 wk of life and direct them into the lysosomal degradative pathway.
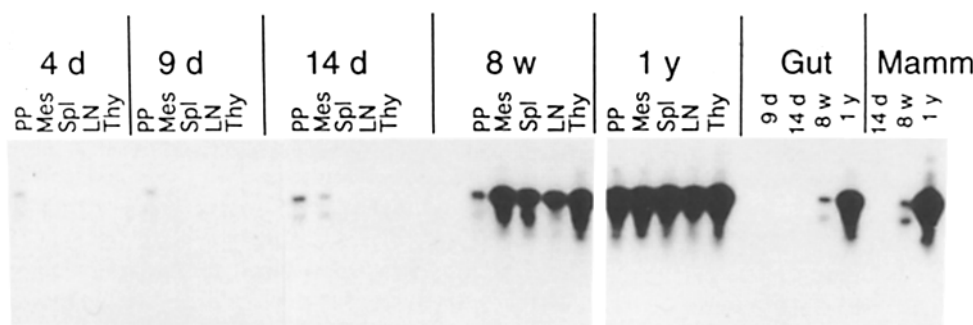


**Figure 3.** Kinetics of appearance of reverse transcribed and integrated DNA of MMTV(SW) in different tissues. Using a semiquantitative PCR assay, we amplified DNA with oligonucleotides detecting either MMTV(SW) orf specifically or all the endogenous and exogenous orf using primers detecting all MMTV (7, 15). The latter PCR amplification experiments indicated that the quantities of genomic DNA taken from different organs at the indicated time points were comparable

(data not shown). At different time points after birth, when the babies had received MMTV(SW)-infected milk from their mothers continuously, Peyer's patches (PP), mesenteric lymph nodes (Mes), spleen (Spl), thymus (Thy), and peripheral lymph nodes (LN) were removed and DNA isolated. Small intestines after removal of Peyer's patches (Gut) as well as mammary glands (Mamm) were tested using the same approach.

Whether MMTV escapes lysosomal degradation in neonatal ileal or FAE enterocytes and enters the transcytotic pathway to be released in the interstitial space remains to be established. Whatever the mechanism of entry, it appears that MMTV has adopted a strategy to infect mice during the neonatal period. Our study clearly indicates that early infection is restricted to Peyer's patch lymphocytes, in which viral DNA can already be detected 4 d after birth. This could reflect unique transepithelial transport properties of the FAE cells, or alternatively, the presence of cells in the underlying tissue susceptible to infection.

MMTV infection during the first 2 wk after birth has profound effects on the host immune system. Presentation of MMTV orf superantigen by B lymphocytes is required to establish a productive infection (7, 8). Thereafter, the virus is transported by lymphocytes and finally infects its target organ, the mammary gland, where the viral life cycle is completed. Superantigen stimulation has so far only been studied in the peripheral lymphoid system of adult mice. Since the neonatal immune system is not mature yet, it was important to show whether in the case of physiologically infected neonates the superantigen exerts its effects in the Peyer's patches in the same way as for adult mice.

As depicted in Fig. 1 of our study, we observe similar immunolabeling patterns in the entire gut with MMTV(SW) as compared to published studies using MMTV(C3H). However, PCR technology enables us to identify which cells carry viral DNA after infection. Despite the presence of MMTV particles in the whole intestinal epithelium, MMTV DNA was found in the first 9 d exclusively in Peyer's patch lymphocytes. A parallel FACS® analysis of Peyer's patch and of different lymphoid organs draining the digestive tract showed that a specific superantigen response is restricted to Peyer's

patches. This response was already detectable 5 d after birth. The other lymphoid organs did not show a superantigen-induced T cell stimulation even at later time points. Viral DNA, however, was found at later stages first in the mesenteric lymph nodes and thereafter in all the lymphoid organs, as well as in the mammary glands. The reasons for the lack of a superantigen stimulation in mesenteric and other lymphoid organs draining the digestive tract are not clear. Several hypotheses might explain our findings. (a) The lack of activation might be due to the fact that uptake of the virus and then infection of B cells can only happen in the dome area whereas other gut epithelial cells might degrade viral particles; (b) the microenvironment characteristic for Peyer's patches may be required for the superantigen response in the neonatal period; and (c) the immune system in the patches might mature before the peripheral lymphoid organs. An absence of a superantigen stimulation in nondraining lymphoid organs is also observed after footpad injection of MMTV in adult mice.

MMTV-induced deletion is the most sensitive readout for infection (15). The injection of serum from MMTV-infected mice does not provoke superantigen-induced deletion (28). This confirms the absence of detectable viremia. The sequential appearance of MMTV(SW) DNA first in the mesenteric lymph nodes and then in the peripheral lymphoid organs, the gut mucosa, and the mammary glands most likely indicates the migration of infected lymphocytes.

The results we described here allow us to detect MMTV infection as early as 4 d after birth. This is in contrast to the classical in vivo infectivity model, where it takes over 6 mo to observe tumor development (3), or 1–3 mo to detect deletion or superantigen-reactive T cells (15, 29).

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