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The Neonatal Fc Receptor Is Not Required for Mucosal Infection by Mouse Mammary Tumor Virus

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The milk-borne mouse mammary tumor virus (MMTV) infects newborn mice via the intestine. Infection is initially restricted to Peyer's patches and later spreads to the epithelial cells of the mammary gland. The receptor that mediates uptake and transport of MMTV across the intestinal barrier has not yet been identified. The neonatal Fc receptor (nFcR), which is expressed by enterocytes during the first two weeks of life, is downregulated at weaning, and its disappearance correlates with the onset of intestinal resistance to MMTV. To test whether the nFcR mediates transport and allows infection, we foster nursed on infected MMTV mothers β2 microglobulin-deficient (β2m-deficient) newborn mice that are unable to express the nFcR at the surface of their enterocytes. Exposure of β2m-deficient mice to milk-borne virus resulted in the deletion of peripheral blood T cells reactive to the superantigen encoded by MMTV. Since β2m-deficient newborn mice are susceptible to MMTV infection despite the lack of the nFcR, we conclude that the nFcR is not required for MMTV transport.

The mouse mammary tumor virus (MMTV) is a retrovirus transmitted through milk from mother to offspring (7). Following footpad injection of infectious MMTV, it has been shown that MMTV primarily infects B cells in the draining lymph nodes (12) and, because of the retroviral superantigen (SAg) activity, triggers an intense proliferation of T cells expressing the appropriate T-cell receptor Vβ domain thereby providing T helper function to infected B cells that in turn proliferate (14). These early cognate interactions between B and T cells facilitate the subsequent spread of MMTV to the mammary gland via lymphocytes (5, 14). While systemic infection has been intensively analyzed, little is known about the early steps of mucosal infection. Recently it was documented that when pups were fed by infected mothers, viral DNA was detected only in Peyer's patches (PP) although all intestinal cells were loaded with viral antigens (16). The MMTV SAg triggered an intense T-cell proliferation in the newborn PP which was maximal 10 days after birth. The mechanism whereby MMTV infectious particles cross the tight epithelial barrier of the PP follicle-associated epithelium (FAE) in order to infect the underlying lymphocytes is not yet understood. Previous studies have shown that absorptive enterocytes both in neonatal intestinal villi and in the FAE were able to take up MMTV (6, 11) which accumulated in large vacuoles. In the FAE two cell types could potentially mediate transepithelial transport of MMTV. M cells are known to transport intact pathogenic microorganisms into the underlying lymphoid follicle (for a review, see reference 23). M cells lack the organized apical brush border and its associated glycocalix, which facilitate uptake and transport of antigens and microorganisms (17), and some viruses such as reovirus selectively adhere to M cells (34). Thus, M cells could constitute the portal of entry of MMTV. If M cells are able to mediate transport, it is difficult to understand why infection is restricted to the neonatal period, since M cells are present in both newborns and adults. Enterocytes are known to take up lumenal macromolecules by fluid-phase endocytosis, but usually the internalized material is targeted to the lysosomal compartment and degraded (9). In newborn mice, however, maternal antibodies are efficiently transported across enterocytes via the neonatal Fc receptor (nFcR) (28, 29, 31). Indeed, upon binding to the nFcR, receptor-immunoglobulin G (IgG) complexes are internalized via clathrin-coated pits and shuttled by vesicular transport across the cell, bypassing the lysosome (1). MMTV could take advantage of this transcytotic pathway provided that the viral particles are coated by antibodies and the receptor is expressed on the enterocytes of the FAE. Since nFcR expression is restricted to the neonatal period, the absence of receptors after weaning might explain the resistance to oral infection by MMTV that is observed after 2 weeks of life (2a).

In order to test whether the nFcR mediates uptake of MMTV and allows infection of PP lymphocytes, we foster nursed β2 microglobulin-deficient (β2m-deficient) pups on MMTV (SW)-infected BALB/c mothers. β2m-deficient mice (36) lack the major histocompatibility complex (MHC) class I molecule expression and the CD8-positive T-cell population (35). Furthermore, these mice do not produce functional nFcR (15), since nFcR, which belongs to the MHC class I gene family (31), requires β2 microglobulin for proper assembly and cell surface expression in the neonatal small intestinal enterocytes (15). In this study, we show that FAE enterocytes of wild-type mice but not of β2m-deficient mice express the nFcR during the neonatal period, and we demonstrate that β2m-deficient neonates are susceptible to oral MMTV infection.

Wild-type but not β2m-deficient mice assemble functional nFcR in their FAE enterocytes. Since neonatal MMTV infection occurs exclusively in PP (16) and not in the rest of the small intestine, we first established whether enterocytes of PP FAE were able to express functional nFcR. Some lumenal plasma membrane proteins are down regulated in the FAE enterocytes. For instance, the polymeric Ig receptor which mediates transcytosis of polymeric immunoglobulins from

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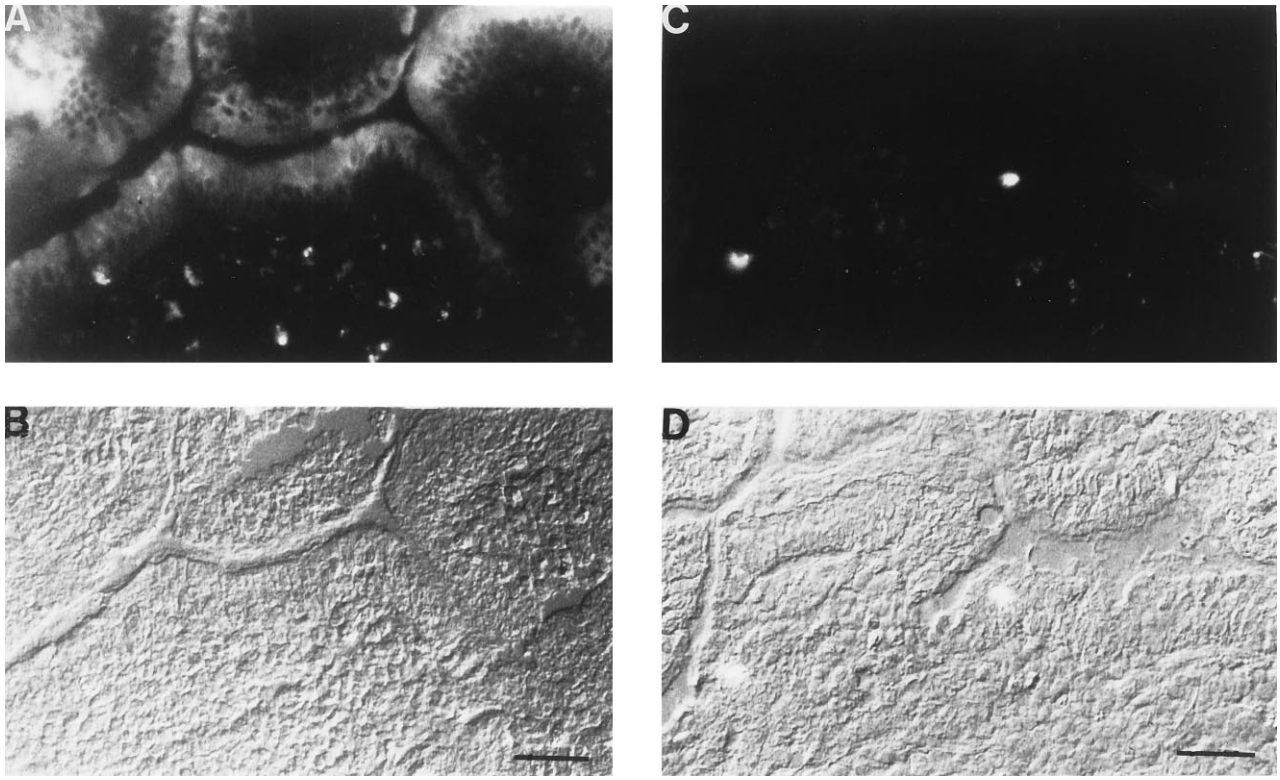

FIG. 1. FITC-labeled IgG2a staining of the nFcR expression on intestinal epithelial cells of β2m-deficient and wild-type newborn mice. Frozen sections (7 μm) of jejunal PP from 10-day-old mice were stained at pH 6 with FITC-labeled IgG2a. (A and B) PP of BALB/c mouse. Staining is present on the epithelial cells of the intestinal villi and also on the FAE. (C and D) PP of β2m-deficient mouse. Only a few cells expressing the gamma FcR were stained in the lymphoid follicle of the β2m-deficient mice, and no staining was observed on the intestinal villi or on the FAE. The gamma FcR-positive cells are also present in the PP of the BALB/c mouse shown in panel A. Bar = 45 μm.

their site of synthesis in the interstitial space into the intestinal lumen is not expressed in FAE enterocytes (24, 33).

Tissues from 10-day-old BALB/c or β2m-deficient mice were quickly excised. After being embedded in Tissue-Tek OCT compound (Miles Scientific, Naperville, Ill.), gut epithelium containing a pair of jejunal PP was snap frozen in liquid nitrogen-cooled isopentane. BALB/c mice were purchased from Harlan Olac (London, United Kingdom). MMTV (SW)-infected mice were obtained from IFFA Credo (L'Arlabesque, France). The β2m-deficient mice (129 × C57BL/6)F_1 (36) were crossed with the MRL strain of mice, and in $F_1 \times F_1$ matings the β2m-deficient mice were selected for the presence of H-2k and I-E protein expression. These mice were provided by T. Ohteki. The expression of the I-E MHC class II proteins allows the optimal presentation of SAg encoded by the SW strain of MMTV (2).

Frozen sections (7 to 10 μm) of unfixed jejunal PP were treated with 1% bovine serum albumin (BSA)-containing phosphate buffer (pH 8); this was followed by a treatment with 1% BSA-containing phosphate buffer (pH 6). After these two washes, the sections were incubated with fluorescein isothiocyanate (FITC)-labeled mouse IgG2a (Sigma, Buchs, Switzerland) diluted in 1% BSA-containing phosphate buffer (pH 6). After washes with phosphate buffer (pH 6 or 8), sections were fixed with 2% paraformaldehyde in phosphate buffer (pH 6). All enterocytes in the FAE of wild-type mice bound FITC-IgG2a at pH 6.0 (Fig. 1A and B) but not at pH 8.0 (data not shown). We next examined whether the nFcR was absent in FAE enterocytes of β2m-deficient neonates as previously reported for villi enterocytes (15). No labeling was detected in the FAE (Fig. 1C and D) following incubation of frozen sections with FITC-IgG2a, indicating that FAE enterocytes were unable to assemble a functional nFcR. Therefore, β2m-deficient mice constitute an excellent system for determining the role of the nFcR in MMTV intestinal infection.

We also examined whether MMTV gp52 antigens accumulated in the vacuoles of villi and FAE enterocytes of infected β2m-deficient mice, as reported for wild-type BALB/c mice (6, 16). We stained frozen sections of jejunal villi and PP of β2m-deficient mice with a rabbit anti-gp52 antibody and found the same accumulation of MMTV antigens as in the wild type, indicating that MMTV is not only internalized via the nFcR (data not shown).

Milk of infected mothers contains antibody-coated MMTV particles. Both free antibodies and antigen-antibody complexes are transported by the nFcR (1). Infected adult mice have circulating anti-gp52 antibodies (2, 4, 10) which are efficiently transported into milk during lactation. Therefore, MMTV particles could be coated by IgG antibodies and be transported across the epithelial barrier of the gut by the nFcR. We determined the concentration of MMTV and bound IgG in milk by enzyme-linked immunosorbent assay (ELISA) (Fig. 2) and found that MMTV particles were coated with antibodies but not saturated. MMTV-antibody complexes were detected as follows: Nunc immunoplates I (Nunc, Roskilde, Denmark) were coated with polyclonal sheep anti-gp52 IgG (kindly provided by P. Hainaut, University of Liège, Liège, Belgium) and incubated overnight at 4°C. After washing and saturation of nonspecific sites, serial dilutions of milk were added and incubated for 2 h at 37°C. We used as a standard purified MMTV

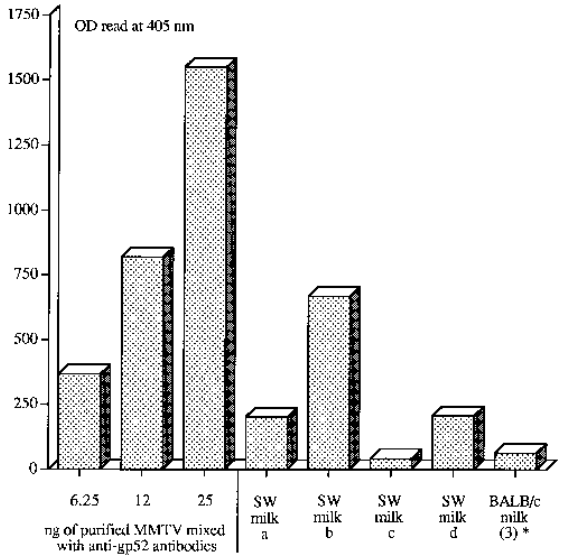

FIG. 2. Detection of MMTV-antibody complexes in milk by ELISA. Plates were coated with polyclonal sheep anti-gp52 IgG; this was followed by the addition of different amounts of MMTV particles coated with either anti-gp52 IgG antibodies as positive control or milk diluted at 1/2 from infected mothers (SW milk a, b, c, or d). Three milk samples (*) aspirated from BALB/c mothers were included as negative controls. The bound MMTV-antibody complexes were detected by the addition of biotin-labeled anti-mouse IgG, which was followed by alkaline phosphatase coupled to avidin and paranitrophenylphosphate. The optical density (OD) was read at 405nm.

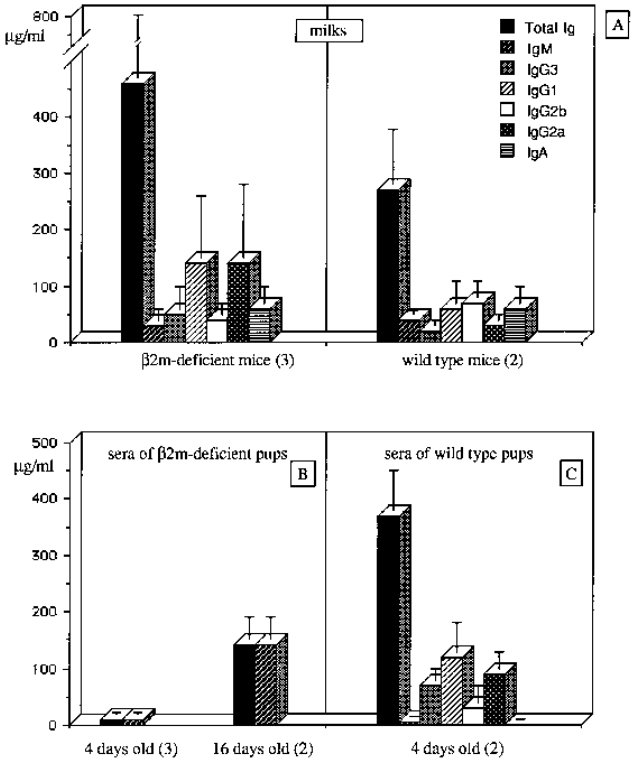

FIG. 3. Milk or serum Ig levels of β2m-deficient mice and BALB/c mice. The milk (A) or serum Ig levels (B and C) are expressed in micrograms per milliliter (means ± standard deviations of individual mouse data). Total Ig levels were calculated for each serum sample by addition of the concentrations of the six major isotypes. The numbers in parentheses indicate the number of individual milk or serum samples which were studied. The serum Ig levels of the β2m-deficient or wild-type mice were measured at 4 or 16 days of age. The serum Ig levels were determined by ELISA.

(purified by ultracentrifugation from the supernatant of cultured GR mammary tumor cells) mixed with an excess of mouse monoclonal IgG antibodies directed against gp52 (1a). The bound MMTV-antibody complexes were detected by the addition of biotin-labeled anti-mouse IgG (Amersham, RPN 1177) for 1 h at 37°C; this was followed by the addition of alkaline phosphatase coupled to avidin (A-2527; Sigma, Switzerland) and paranitrophenylphosphate (Art. 6850; Merck). The optical density was read at 405 nm.

Mouse IgG2a and IgG2b isotypes were reported to bind preferentially to brush border-associated receptors in vitro, while IgG1, IgM, and IgA failed to interact with the nFcR (22). As shown in Fig. 3, wild-type pups acquired maternal antibodies irrespective of their isotype, indicating that the in vitro studies did not correlate with in vivo observations. Therefore, the IgG isotype of the antibodies coating the MMTV particles did not seem to be crucial for the transport of these immune complexes by the nFcR, since all four of the IgG isotypes were transported by the nFcR. Quantification of serum and milk isotypes were carried out by ELISA procedures which have already been reported (18, 19). Since the standards used were serum from BALB/c or C57BL/6 mice, estimated levels of milk Igs could be artificially increased or lowered because of the interference of milk components with the sensitivity of the ELISA test. This, however, would not affect the magnitude of the differences observed between milk Ig levels in BALB/c and β2m-deficient mice.

In β2m-deficient neonates, no detectable Ig was found in the serum 4 days after birth, and the IgM detected 2 weeks after birth probably reflected de novo synthesis (Fig. 3B). We also compared milk Ig isotypes of β2m-deficient and wild-type mice. As shown in Fig. 3A, the milk isotype distribution in wild-type and β2m-deficient mice was not significantly different, indicating that the absence of passively acquired IgG in the serum of neonatal β2m-deficient mice was not the consequence of low milk IgG levels in β2m-deficient maternal mice but was rather due to the absence of the nFcR expression in β2m-deficient neonates. Together these results indicate that antibody-coated MMTV particles should bind to the nFcR and undergo transcytosis across an intact FAE layer.

β2m-deficient newborn mice are infected by MMTV. Since β2m-deficient mice lack a functional nFcR, one would predict that these animals should be resistant to infection if the transepithelial transport of infectious MMTV is mediated by the nFcR. Wild-type and β2m-deficient pups were infected by continuously foster nursing the neonates 3 days after birth with MMTV (SW)-infected BALB/c lactating females. Infection of the pups was monitored 3 and 31 weeks after birth by flow microfluorimetry analysis. Blood lymphocytes were recovered from heparinized blood samples by centrifugation through a Ficoll (Pharmacia, Uppsala, Sweden) cushion. These cells were double labeled with FITC-conjugated anti-T-cell receptor Vβ antibody (44.22.1 rat IgG anti-Vβ6 [25] or 14.2 rat IgM anti-Vβ14 [21]) and phycoerythrin-coupled anti-CD4 (GK1.5; Becton Dickinson and Co., Mountain View, Calif.). In order to confirm β2m deficiency in the knockout mice, we checked for the absence of peripheral blood $CD8^+$ T cells by using an FITC-labeled anti-CD8 (53-6.7; Boehringer Mannheim). Analysis was performed on a Facscan (Becton-Dickinson) with Lysis II software for data evaluation. Dead cells were excluded by forward scatter and side scatter.

$V\beta6^+$ $CD4^+$ T cells which are known to interact with the MMTV (SW) SAg (13) were deleted to the same extent in

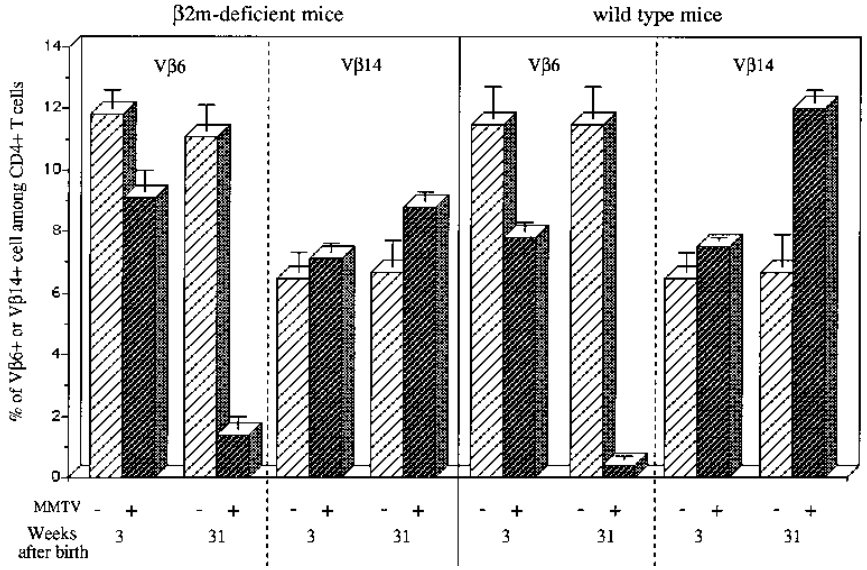

FIG. 4. Clonal deletion of $CD4^+V\beta6^+$ T lymphocytes in β2m-deficient mice nursed by BALB/c mice harboring the infectious SW strain of MMTV. Peripheral blood lymphocytes were isolated as described in the text and double stained with either FITC-labeled anti-Vβ6 and anti-CD4-phycoerythrin or with FITC-labeled anti-Vβ14 and anti-CD4-phycoerythrin. Relative percentages of $V\beta6^+$ and $V\beta14^+$ cells among total $CD4^+$ lymphocytes at 3 and 31 weeks after birth are shown. Five β2m-deficient mice were nursed by an infected BALB/c mother (31 weeks after neonatal infection, these mice showed 2.8% ± 0.7% of $CD8^+$ T cells in the total population of blood lymphocytes). Three β2m-deficient mice were nursed by their own mothers, i.e., retrovirus-negative mothers (at 31 weeks of age, these mice showed 1.4% ± 0.7% of $CD8^+$ T cells in the total population of blood lymphocytes). Three β2m-positive littermates of the β2m-deficient mice were nursed by infected BALB/c mice (36 weeks after birth these mice showed 20.8% ± 2.1% of $CD8^+$ T cells in the total population of blood lymphocytes). −, no infection; +, infection.

wild-type and β2m-deficient mice (Fig. 4). The kinetics of deletion were similar in both groups. As a control, SW-SAg nonreactive T cells, i.e., $V\beta14^+$ $CD4^+$ T cells, were not deleted from the peripheral T-cell population.

We found that neonatal MMTV infection occurred in PP from wild-type as well as from β2m-deficient mice. Indeed, we could detect an intense $V\beta6^+$ $CD4^+$ T-cell proliferation in PP of β2m-deficient newborns after 6 or 9 days of foster nursing on MMTV (SW)-infected BALB/c lactating females (data not shown).

The peripheral deletion of $V\beta6^+$ $CD4^+$ T cells in MMTV-infected β2m-deficient pups indicates that these neonates are as susceptible as wild-type mice to intestinal MMTV infection, and consequently the nFcR is not required for the transepithelial transport of infectious MMTV across the intestinal epithelium.

In our experiments, β2m-deficient pups were foster nursed on MMTV-infected BALB/c females. Since β2 microglobulin can be exchanged among MHC class I molecules, it is possible that cell surface expression of nFcR is triggered or stabilized by the β2 microglobulin present in the milk of MMTV-infected BALB/c mothers. However, this is unlikely, as a recent report (15) indicates that β2m-deficient pups foster nursed by lactating wild-type mothers expressing a distinct IgG2a allotype did not neonatally acquire the maternal IgG2a allotype, clearly demonstrating that milk β2 microglobulin did not rescue the nFcR in the knockout pups.

Our experiments also demonstrate that the deletion of the SAg-reactive T cells is independent of a fully functional $CD8^+$ T-cell population, in agreement with an earlier report (26) in which CD8-deficient mice deleted peripheral SAg-reactive CD4-positive T cells after neonatal MMTV infection.

Other mechanisms for the transepithelial transport of MMTV have now to be envisaged. For example, M cells could represent a possible portal of entry for MMTV (23). Many pathogenic microorganisms including reoviruses (34), poliovirus (30), or human immunodeficiency virus (3) are endocytosed by M cells and delivered into the intraepithelial pocket. Such a mechanism would facilitate infection of B cells which are abundant in the M cell intraepithelial pocket, thus minimizing proteolytic degradation of the virus in the mucosal environment. In addition, endocytosis of MMTV by M cells could be enhanced by maternal milk antibodies, since adherence to and transport by M cells is triggered by the opsonization of the microorganisms (33). If uptake and transport of MMTV is mediated by M cells, one has to explain why infection is restricted to the neonatal period, since M cells are present both in newborns and in adults. The resistance to oral MMTV infection after weaning may also reflect the postnatal maturation of the digestive functions of the gastrointestinal tract with the appearance of acid secretion in the stomach and digestive enzyme secretion both in the stomach and in the gut.

Other retroviruses (human T-cell leukemia or human immunodeficiency virus) are also transmitted by milk, and it has been proposed that infection is mediated by cell-associated virus. Mouse milk contains B and T lymphocytes (8, 20), which are likely to be infected in MMTV-infected mothers. It has been reported that the injection of naive mice with MMTV-infected B or T cells causes disease (32). Whether MMTV-infected B or T cells can cross the FAE barrier, and produce infectious particles in the PP which in turn infect PP lymphocytes, is not known. Retroviral spread from lymphocytes to epithelia has been documented for human immunodeficiency virus (27). These hypotheses are testable, and work is in progress to identify the mechanisms of MMTV entry into PP.

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