

An Exogenous Mouse Mammary Tumor Virus with Properties of Mls-1^a (*Mtv-7*)

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Summary

The classical minor lymphocyte stimulating (Mls) antigens, which induce a strong primary T cell response in vitro, are closely linked to endogenous copies of mouse mammary tumor viruses (MMTV). Expression of Mls genes leads to clonal deletion of T cell subsets expressing specific T cell receptor (TCR) V β chains. We describe the isolation and characterization of a new exogenous (infectious) MMTV with biological properties similar to the Mls antigen Mls-1^a. In vivo administration of either Mls-1^a-expressing B cells or the infectious MMTV (SW) led to an increase of T cells expressing V β 6 followed by their deletion. Surprisingly, different kinetics of deletion were observed with the exogenous virus depending upon the route of infection. Infection through the mucosa led to a slow deletion of V β 6⁺ T cells, whereas deletion was rapid after subcutaneous infection. Sequence analysis of the open reading frames in the 3' long terminal repeat of both this exogenous MMTV (SW) and of *Mtv-7* (which is closely linked to Mls-1^a) revealed striking similarities, particularly in the COOH terminus, which has been implicated in TCR V β recognition. The identification of an infectious MMTV with the properties of a strong Mls antigen provides a new, powerful tool to study immunity and tolerance in vivo.

The Mls antigens were originally defined on the basis of a very strong proliferative T cell response between MHC-identical mouse strains (1). Several independent Mls loci (*Mls-1*, -2, -3, -4) segregating as single autosomal dominant genes have been defined (for review see reference 2). For each Mls locus, a stimulating (named, e.g., Mls-1^a) and a null (named, e.g., Mls-1^b) allele has been described.

T cell reactivity towards Mls gene products appears to be determined exclusively by the V domain of the TCR β chain (3–8), whereas the variable, junctional, and highly polymorphic CDR3 segments of both the TCR α and β chains determine classical recognition of antigenic peptides associated with MHC molecules (for review see reference 9).

Because of their unique V β specificity, Mls antigens have been key elements in the development of our current understanding of tolerance mechanisms in mice. T cells expressing Mls-reactive TCR V β domains are deleted in an Mls-expressing mouse strain during thymic maturation by a mechanism called negative selection. Thus, carriers of the Mls-1^a gene delete T cells expressing TCR V β 6, 7, 8.1, and 9 from their

peripheral T cell pool as a consequence of self-reactivity (3, 4, 10, 11).

A second tolerance mechanism has been shown to operate for Mls antigens: injection of Mls-1^a-expressing cells into adult Mls-1^b mice leads to a specific unresponsiveness to Mls-1^a with or without subsequent peripheral deletion of the reactive T cell subsets (12–14).

In addition to Mls genes, several other genetic elements were described that delete T cells from the peripheral T cell pool depending on the expression of their TCR V β domains. However, these deletion elements were found to behave differently from Mls-1^a. First, in agreement with earlier proliferation studies (15, 16, and for review see reference 2), I-A as well as I-E can present Mls-1^a for clonal deletion in vivo (3, 4), whereas these other deletion elements strictly require I-E expression for clonal deletion to occur (17–26, and for review see reference 27). Second, a strong proliferative T cell response in vitro can be induced with Mls-1^a-expressing B cells (28–30). Such a proliferative response of T cells expressing the relevant TCR V β domains was not detected (or only very weakly) with these weaker deletion elements. Third, Mls-1^a mice delete V β 6 cells very quickly after birth (31). This is in contrast to the weaker deletion elements, where a much longer amount of time is required to induce

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a near complete deletion (32, 33). Thus, for the rest of this article, these weak deletion elements will be referred to as Mls-like determinants.

It has been shown recently that the gene products encoding most (if not all) Mls and Mls-like determinants in mice are closely linked to endogenous mouse mammary tumor virus (MMTV)² proviral loci (32, 34–37). Until now, >30 different endogenous MMTVs have been mapped and characterized (38). They display ~95% nucleotide sequence homology. Usually, two to eight different MMTV copies are contained in the genome of any inbred laboratory mouse strain. One of these copies, *Mtv-7*, showed close linkage to Mls-1^a (32, 36). Results from transgenic mice as well as transfection experiments showed that this deleting activity is encoded by a 3' open reading frame (orf) within the LTR of MMTV (32, 39). Sequence comparison and transgenic mouse experiments further suggested that the specificity for particular TCR V β domains is localized at the COOH-terminus of these putative MMTV orf molecules (32, 39, and for review see reference 27).

MMTV also exists as an exogenous infectious virus that is maternally transmitted via milk (40). Our knowledge about the biology and the life cycle of the virus is still incomplete. Uptake of the virus occurs in the gut. There is some evidence that the immune system is involved in transport of the virus to the mammary gland (41). Upon infection of the mammary gland, the virus can complete its life cycle. Integration close to the *int-1*, *-2*, *-3*, and *-4* loci leads to development of mammary tumors and high MMTV titers in milk (42).

With respect to clonal deletion of T cells expressing specific V β chains, the two exogenous MMTVs (GR) and (C3H) have been characterized. MMTV (C3H) was shown to induce a slow deletion of T cells expressing V β 14 after neonatal uptake of infectious particles contained in milk (33). MMTV (GR) has been analyzed using mice containing the entire viral genome as a transgene. In these transgenic mice, MMTV (GR) leads to a slow, I-E-dependent deletion of V β 14⁺ T cells (32). Neither virus is capable of stimulating a strong mixed lymphocyte response. Thus, exogenous MMTVs behave very much like the weak Mls-like structures described above. So far no infectious virus encoding a strong Mls antigen has been described.

In this article we describe the characterization of an exogenous form of Mls-1^a. This infectious MMTV was found in high titers in the milk of several but not all BALB/c mice obtained from IFFA Credo (BALB/c IC), but not in BALB/c mice obtained from Harlan Olac (BALB/c HO). It induces clonal deletion of the same TCR V β -expressing T cells as Mls-1^a. Either fast or slow clonal deletion of the responsive T cells was observed depending on the route of infection. Analysis of the DNA or cDNA sequences of both endogenous *Mtv-7* and of this new exogenous MMTV, respectively, indicates a very high degree of homology in the orf molecules. The most relevant differences to all the other previ-

ously sequenced MMTV orf molecules were found in the COOH-terminal amino acids, which is compatible with the unique V β specificity of Mls-1^a and this new exogenous virus. Since most likely the new MMTV derives from outbred Swiss mice, we propose the designation MMTV (SW).

Materials and Methods

Mice. BALB/c IC mice were purchased from IFFA Credo (L'Arbresle, France), and BALB/c HO mice from Harlan Olac UK Ltd. (Bicester, UK). C3H/OuJ and BALB.D2 (43) mice were bred in our colony.

Antibodies. The following mAbs were used in this study: 14.2 (anti-V β 14) (44); 44.22.1 (anti-V β 6) (45); KJ-16 (anti-V β 8.1-2) (46), F23.1 (anti-V β 8.1, 8.2, 8.3), F23.2 (anti-V β 8.2) (47); KT4.10 (anti-V β 4) (48); MR 10.2 (anti-V β 9) (49); TR310 (anti-V β 7) (11); GK1.5 (anti-CD4) (50), AT 83 (anti-Thy-1) (51).

Milk Collection, Virus Purification, and Virus Titration. Milk was aspirated from lactating BALB/c or C3H/OuJ females after injection of 0.5 IU syntocinon/oxytocine (Sandoz, Basel, Switzerland), pooled, aliquoted, and stored at -70°C.

Freshly sampled milk was diluted 1:20 with water and centrifuged at 600 g for 5 min to skim and remove casein. Milk serum was then centrifuged at 15,000 g for 1 h and the virus pellet was resuspended in water (52).

MMTV gp52 in milk was measured by sandwich ELISA using polyclonal sheep and rabbit anti-gp52 IgG (kindly provided by Dr. P. Hainaut, University of Liège, Liège, Belgium). A biotinylated sheep antibody directed against rabbit IgG and streptavidin peroxidase served as a detection system. MMTV, purified by ultracentrifugation from the supernatant of cultured GR mammary tumor cells, was used as standard. 1 pg of MMTV corresponds to ~10³ viral particles (40).

Injections and Sampling. Mouse milk (20 μ l), which was either MMTV free or contained between 2 \times 10⁸ and 10⁹/ μ l MMTV (C3H) or (SW) particles, was injected into the hind footpad of 6–10-wk-old BALB/c HO mice. After 4 d, the popliteal and inguinal lymph nodes were isolated. Alternatively, mice were tail bled and leukocytes were recovered from heparinized blood samples by centrifugation through a Ficoll cushion. Splenic BALB/D2 Thy-1⁻ cells were prepared by elimination of T cells through complement lysis using the mAb AT83 (anti-Thy-1), and 6 \times 10⁶ cells were injected into the footpad.

FACS[®] Staining. Lymph node cells or thymocytes (10⁶) were stained with anti-TCR V β -specific monoclonal hybridoma supernatants followed by fluoresceinated anti-rat or anti-mouse IgM or IgG antisera. PE-coupled anti-CD4 (GK1.5) (Becton Dickinson & Co., Mountain View, CA) was used in the second dimension. Streptavidin-PE Texas red (Tandem; Southern Biotechnology Associates, Birmingham, AL) was used to develop the anti-CD8 (Lyt-2)-biotin labeling. Leukocytes were recovered from heparinized blood samples by centrifugation through a Ficoll cushion. These cells were stained in one step with a mixture of FITC-labeled anti-TCR V β antibody and PE-coupled anti-CD4.

Dead cells were gated by forward scatter and side scatter analysis. Background staining values obtained with the second stage reagents alone were subtracted. Analysis was performed on a FACScan[®] (Becton Dickinson & Co.) cell analyzer using logarithmic scale for data evaluation.

Southern Blot Analysis. High-molecular weight genomic tail DNA was isolated using standard protocols (53). DNA (10 μ g) was completely digested by the restriction enzymes EcoRI (Phar-

² Abbreviations used in this paper: MMTV, mouse mammary tumor virus; orf, open reading frame.

macia, Uppsala, Sweden) or PvuII (Pharmacia), and the DNA fragments were separated on 0.8% agarose gels using standard conditions (53). The DNA was transferred on to nylon membranes (GeneScreen PLUS™; DuPont Co., Wilmington, DE) by vacuum blotting. The baked membranes were prehybridized for a least 2 h in 5× SSC, 100 μg/ml salmon sperm DNA, 1% SDS, 5× Denhardt's solution. The DNA probe was radiolabeled using the random hexamer priming method to ~5 × 10⁸ cpm/μg (54) and then hybridized with membrane bound DNA for 20 h at 65°C. The filters were washed in 2× SSC, 0.1% SDS at room temperature then twice with 0.2× SSC, 0.1% SDS at 65°C. Autoradiography was for 48 h using two intensifying screens and Kodak XAR film.

Reverse Transcription. Reverse transcription was used to prepare viral cDNA. Briefly, partially purified MMTV from milk (~10⁹ particles) was added to a reaction mixture containing: 1× PCR buffer (see below), supplemented with 10 mM DTT, 300 U RNasin (Pharmacia), 0.1% NP-40, and 25 U AMV reverse transcriptase (Boehringer Mannheim Biochemicals, Mannheim, Germany). The reaction was carried out at 42°C for 18 h (55).

PCR, Cloning, and Sequencing. The cDNA products of the reverse transcriptase reaction were amplified with the two oligonucleotides spanning the MMTV orf region: 5' oligonucleotide: GATCGTCGACATGCCGCGCCTGCAGCAGA; 3' oligonucleotide: GTGTGACACCAACCAAGTCAGGAAACCACTTG.

The oligonucleotides were chosen on the basis of high degrees of conservation between the previously sequenced orf molecules. The conditions for PCR were 1 min at 55°C, 1 min at 72°C, 1 min at 93°C for 30 cycles in 1× PCR buffer containing 20 mM TRIS-HCl, pH 8.4, 50 mM KCl, 0.2 mM dNTP, 2 mM MgCl₂, 0.01% gelatin, 2 U Taq polymerase (AmpliQ™, Perkin Elmer Corp., Emeryville, CA) using an LEP amplifier (SCIENTIFIC PREM™; Lep Scientific Ltd. Milton, Keynes, UK). The PCR products were size fractionated in 1% agarose gels and cloned into the pGEM3Zf(+) vector (Promega Biotech, Madison, WI) after Sall digestion and purification using standard techniques (53). The endogenous orf copies were amplified as described above. The BALB/D2 high-molecular weight DNA was size fractionated in agarose gels after complete EcoRI digestion, and the size ranges containing *Mtv-6* (16.7 kb), *Mtv-7* (11.7 kb), and *Mtv-9* (10.0 kb) were electroeluted (Biotrap; Schleicher & Schuell, Inc., Keene, NH) and treated as the PCR products described above. Recombinant plasmids were isolated and used as templates for dideoxy sequencing as described (Sequenase Version 2.0; U.S. Biochemical Corp., Indianapolis, IN).

Results

Age-dependent Deletion of V_β6 T Cells in Certain BALB/c Mice. In BALB/c mice, V_β6 cells make up ~10% of peripheral CD4⁺ T cells (4). Upon repeated testing of peripheral blood from a large number of BALB/c mice obtained from IFFA-Credo (BALB/c IC), we found an unexpected age-dependent clonal deletion of T cells expressing V_β6 (Fig. 1). At 5 wk of age ~11% of CD4⁺ T cells were V_β6⁺ in some BALB/c IC mice, however, a considerable fraction of age-matched BALB/c IC mice contained only 6% CD4⁺ V_β6⁺ T cells (a similar observation was made by Papiernik et al. [56]). In comparison, BALB.D2 mice (which are Mls-1^a congenic BALB/c mice), had almost completely deleted CD4⁺V_β6⁺ cells from the peripheral blood at 4 wk of age (data not shown) (31). At 36 wk of age, the deletion in BALB/c IC mice had reached values similar to BALB.D2 mice (i.e.,

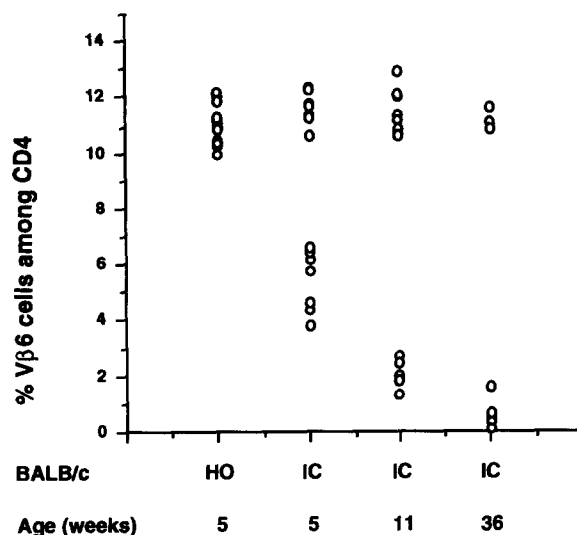


Figure 1. TCR V_β6 expression in individual BALB/c IC mice. Mice were tested for the presence of CD4⁺V_β6⁺ cells in peripheral blood. Leukocytes were isolated from heparinized blood by Ficoll density centrifugation and stained with a mixture of FITC-labeled anti-V_β6 (44.22) and PE-coupled anti-CD4 (L3T4) antibody. Analysis was performed on a FACScan®. Dots represent V_β6 values among CD4⁺ cells for individual BALB/c IC or BALB/c HO mice.

<0.5% CD4⁺V_β6⁺ T cells), whereas control values remained constant at 11% V_β6 cells.

T Cell Repertoire of Individual BALB/c Mice. The only genetic element that is known to delete V_β6⁺ T cells is Mls-1^a. Besides V_β6, however, Mls-1^a-positive mice also delete T cells expressing V_β7, V_β8.1, and V_β9 (3, 4, 10, 11). Analysis of the peripheral T cell repertoire of V_β6-deleting BALB/c IC mice showed that among the CD4⁺ subset V_β7, as well as V_β8.1, expressing T cells were also deleted (Table 1). The levels of CD4⁺V_β9⁺ T cells in BALB/c mice were too low to assess whether the deletion was significant. In

Table 1. TCR V_β Repertoire of CD4⁺ T Cells in BALB/c Mice

	BALB/c HO	BALB/c IC		BALB.D2
		3 mo	9 mo	
V _β 4	6.9 ± 0.2	8.5 ± 0.4	9.8 ± 0.2	8.9 ± 0.1
V _β 6	10.1 ± 0.2	2.9 ± 0.2	1.2 ± 0.5	0.2 ± 0.1
V _β 7	2.4 ± 0.4	1.7 ± 0.4	0.6 ± 0.2	0.6 ± 0.1
V _β 8.1	6.3 ± 0.8	4.3 ± 0.6	3.3 ± 1.5	0.9 ± 0.3
V _β 8.2	12.7 ± 0.9	12.8 ± 0.8	13.2 ± 0.7	12.5 ± 1.5
V _β 9	1.1 ± 0.2	0.8 ± 0.2	0.7 ± 0.2	0.3 ± 0.1
V _β 14	8.0 ± 1.0	8.2 ± 1.7	9.0 ± 1.1	9.2 ± 0.8

Lymph node cells were analyzed for the TCR V_β repertoire using FACS® analysis. The lymph nodes from at least three mice per group were analyzed. Data are indicated as mean ± SD.

addition, deletion of the populations expressing TCR $V_{\beta}3$, 5, and 11 (normally deleted in BALB/c mice) was observed. $V_{\beta}8.2^+$ and $V_{\beta}4^+$ T cells, which have no known Mls specificity, as well as $V_{\beta}14^+$ T cells that are known to interact with exogenous MMTVs (C3H) and (GR), were unaffected in these mice. Similarly, all other V_{β} s tested ($V_{\beta}2$, 8.3, 10, 13) were unaltered when comparing normal BALB/c mice with $V_{\beta}6$ -deleting BALB/c IC mice (data not shown). Among $CD4^+$ T cells, deletion was most obvious for $V_{\beta}6^+$ T cells, whereas $V_{\beta}7^+$ and $V_{\beta}8.1^+$ T cells were only partially deleted in 9-mo-old animals. Thus, this deletion element has the same V_{β} specificity as Mls-1^a.

Thymic vs. Peripheral Deletion of $V_{\beta}6^+$ T Cells. Deletion of $V_{\beta}6^+$ T cells in Mls-1^{a+} strains of mice occurs very rapidly after birth in the thymus (31). In contrast, adult BALB/c IC (3 mo) mice show only a partial deletion of their $V_{\beta}6^+$ $CD4^+$ and $V_{\beta}6^+$ $CD8^+$ thymocytes (Table 2). In the periphery, $CD8^+$ $V_{\beta}6^+$ cells are deleted to a lesser degree than $CD4^+$ $V_{\beta}6^+$ T cells (Table 2). The same was true for the other V_{β} s affected in BALB/c IC mice (data not shown).

Exogenous or Endogenous MMTV. Since most (if not all) known V_{β} deletion elements are linked to MMTV, we hypothesized that the slow $V_{\beta}6$ deletion could be caused by an endogenous or exogenous MMTV.

To test the first possibility, we performed Southern blot analysis on genomic DNA obtained from $V_{\beta}6$ deleting and nondeleting BALB/c IC mice. Genomic DNA was either digested with EcoRI (data not shown) or PvuII restriction endonuclease. Both enzymes generate two diagnostic DNA fragments for most integrated viruses. Hybridization of the DNA was performed with an MMTV (GR) LTR probe.

Clearly, fragments corresponding to the BALB/c endogenous MMTVs (*Mtv-6*, -8, -9) can be identified in all the mice (Fig. 2). However, in a total of 30 BALB/c IC mice tested, no new or altered integration site that would correlate with the $V_{\beta}6$ deletion phenotype could be detected. Thus, we could exclude a germline-transmitted endogenous MMTV as the causative agent for the slow $V_{\beta}6$ deletion.

Table 2. Thymic and Peripheral Deletion of $V_{\beta}6^+$ T Cells

		Thymus	Lymph node
BALB/c HO	CD4	9.2 ± 0.2	10.4 ± 0.3
	CD8	8.3 ± 1.2	9.7 ± 0.3
BALB/c IC	CD4	4.4 ± 0.2	2.9 ± 0.7
	CD8	2.7 ± 0.5	5.9 ± 1.2
BALB.D2	CD4	0.3 ± 0.2	0.1 ± 0.1
	CD8	0.4 ± 0.3	0.2 ± 0.2

Thymocytes and lymph node cells from 3-mo-old BALB/c IC, BALB/c HO, and BALB.D2 mice were analyzed for the presence of $V_{\beta}6^+$ T cells using three-color FACS[®] analysis. $V_{\beta}6$ expression in the thymus was assessed by gating on $CD4$ or $CD8$ single-positive thymocytes. The lymph nodes and thymuses from at least three mice per group were analyzed. Data are indicated as mean ± SD.

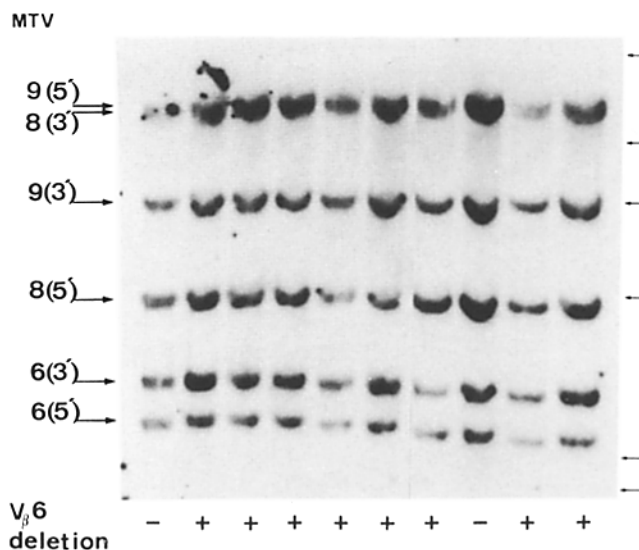


Figure 2. Pattern of endogenous *Mtv*s in BALB/c IC mice as assessed by Southern blot analysis. Tail DNA from $V_{\beta}6$ deleting (+) and nondeleting (-) BALB/c IC mice was digested by PvuII. The filter was hybridized with a MMTV (GR) LTR probe detecting two fragments for every endogenous *Mtv* contained in BALB/c mice. Arrows to the right indicate molecular weight markers of 23.1, 9.4, 6.6, 4.4, 2.3, and 2.2 kb. Arrows to the left indicate the *Mtv* fragments. The two fragments of *Mtv-7* would migrate at >20 kb (3') and 2.5 kb (5') (data not shown; 36).

Exogenous MMTVs can be inherited through transmission from mother to offspring via milk. This maternal transmission of MMTV (C3H) has been shown to result in deletion of $CD4^+$ $V_{\beta}14^+$ T cells (33). We therefore bred BALB/c IC female mice that had deleted $V_{\beta}6^+$ T cells with nondeleter BALB/c IC males. The offspring of these crosses had all inherited the $V_{\beta}6$ -deleter phenotype (data not shown). To directly test milk from this mother for exogenous MMTV, we used an ELISA system to detect the MMTV envelope protein gp52 in milk. As shown in Fig. 3, milk from this $V_{\beta}6$ -deleting BALB/c IC mother contained as much gp52 as milk obtained from lactating C3H/OuJ mice,

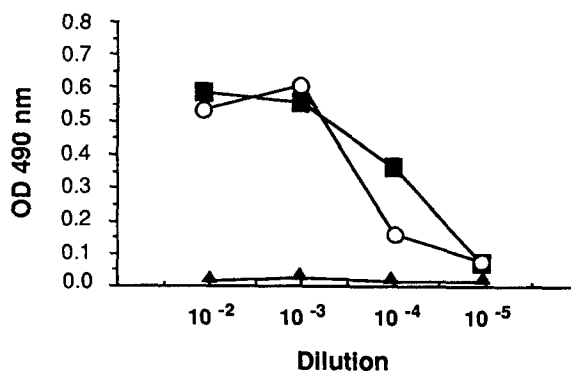


Figure 3. Presence of MMTV in milk samples. Milk samples were tested for the presence of exogenous MMTV. Polyclonal sheep anti-gp52 IgG was used as a capture antibody for MMTV contained in C3H milk (○), BALB/c IC milk (■), or BALB/c HO milk (▲). Rabbit anti-gp52 was then used to detect the immobilized MMTV.

which contain high titers of MMTV (C3H). All BALB/c IC deleter females tested showed high titers of MMTV in their milk (data not shown), whereas no gp52 was detected in BALB/c HO milk. This is in agreement with earlier reports that normal BALB/c mice do not contain detectable MMTV particles in milk (57). Since 1 pg of MMTV corresponds to ~1,000 virus particles (40), and purified MMTV (GR) was used as a standard, we could estimate the detection limit of the ELISA to be $<2 \times 10^4$ MMTV particles per microliter of milk. Milk from C3H or from $V_{\beta 6}$ -deleting BALB/c IC mice contained usually between 10^8 and 10^9 MMTV particles per microliter of milk. From now on we refer to this new MMTV as MMTV (SW) (see Discussion).

In Vivo Response to MMTV (SW). To test whether the MMTV (SW) particles could affect $V_{\beta 6}^+$ T cells in vivo,

Table 3. The Exogenous MMTV (SW) and Mls-1^a B Cells Induce CD4⁺ $V_{\beta 6}^+$ T Cell Increase In Vivo

Injection	No.	Percent V_{β} among CD4 ⁺ T cells		
		$V_{\beta 6}$	$V_{\beta 8.2}$	$V_{\beta 14}$
No injection	1	11.1	12.0	10.0
	2	12.0	12.0	9.4
	3	11.8	14.0	11.5
BALB/C HO milk	1	10.9	13.2	10.8
	2	10.5	12.5	9.9
C3H-milk	1	9.3	11.4	12.6
	2	9.5	11.5	11.5
	3	9.1	11.3	13.5
BALB/c IC milk	1	<u>27.8</u>	7.5	6.7
	2	<u>30.7</u>	7.6	6.7
	3	<u>30.3</u>	ND	8.1
BALB/c HO B cells	1	11.6	13.2	ND
	2	13.1	ND	10.8
	3	12.2	ND	10.6
BALB/c IC B cells	1	12.2	13.6	ND
	2	13.8	ND	11.0
	3	12.8	ND	11.0
BALB.D2 B cells	1	<u>43.8</u>	7.7	ND
	2	<u>31.2</u>	ND	8.7
	3	<u>39.4</u>	ND	8.3

Milk (20 μ l) containing 4×10^9 to 10^{10} virus particles or 6×10^6 splenic B cells were injected into the footpads of BALB/c HO mice. After 4 d, the percentage of cells expressing the indicated TCR V_{β} chains among the CD4⁺ cells in the popliteal and inguinal lymph nodes was determined by FACS[®] analysis. Results from individual mice are shown. Values showing significant increases are underlined.

we injected 20 μ l milk, i.e., $\sim 4 \times 10^9$ virus particles, in the hind foot pads of normal adult BALB/c HO mice. As controls, BALB/c HO mice were either injected with milk containing MMTV (C3H) (4×10^9 to 4×10^{10} particles) or milk from control BALB/c HO mice (not containing detectable MMTV particles).

To follow the events after virus challenge, we analyzed the popliteal and inguinal lymph nodes. Results obtained with exogenous virus indicated that 4 d after injection was an appropriate time point to analyze the local immune response.

Milk containing the C3H virus induced only a marginal increase of TCR $V_{\beta 14}$ -expressing T cells in the local lymph nodes of the injected footpad compared with noninjected control mice. No alteration in the V_{β} profile was observed with control milk. However, injection of the milk from $V_{\beta 6}$ deleters induced a strong specific increase of $V_{\beta 6}$ cells from 10% to $\sim 30\%$ of CD4⁺ T cells (Table 3).

Since it is a distinct feature of Mls-1^a B cells to induce a strong proliferative response of T cells from Mls-1^a-nonexpressing mice (1, 28–30), we similarly injected Mls-1^a B cells into the footpad. Again, CD4⁺ $V_{\beta 6}^+$ T cells increased when BALB/c HO mice were injected with BALB.D2 (Mls-1^a) splenic B cells. No specific increase, however, was observed when B cells from BALB/c IC $V_{\beta 6}$ deleters or BALB/c HO mice were injected (Table 3). Furthermore, B cells from $V_{\beta 6}$ deleters were unable to stimulate a proliferative response of $V_{\beta 6}^+$ cells in vitro or to induce IL-2 production by the $V_{\beta 6}^+$ T cell hybrid RG-17 (data not shown).

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Table 4. MMTV (SW) Induces Deletion of CD4⁺ $V_{\beta 6}^+$ T Cells

Injection	No.	Time after injection				
		4 d	14 d	40 d		
BALB c HO milk	$V_{\beta 6}$	1	9.5	11.2	10.8	
		2	9.9	11.1	11.1	
	$V_{\beta 14}$	1	9.6	9.4	10.2	
		2	9.6	9.1	9.8	
	C3H milk	$V_{\beta 6}$	1	10.3	11.4	12.7
			2	11.2	13.0	12.6
$V_{\beta 14}$		1	8.3	7.2	5.5	
		2	8.8	6.8	4.8	
BALB/c IC milk	$V_{\beta 6}$	1	6.7	3.3	1.5	
		2	7.2	3.6	1.6	
	$V_{\beta 14}$	1	9.8	10.3	ND	
		2	11.7	10.7	11.5	

Mice were injected with milk as described in the legend to Table 3. At the indicated time points the percentage of $V_{\beta 6}^+$ and $V_{\beta 14}^+$ T cells in the CD4⁺ subset was determined in the peripheral blood by FACS[®] analysis. Results of individual mice are shown.

Mls-1^a that upon injection in vivo, the initial proliferation of T cells is followed by a state of nonresponsiveness and partial peripheral deletion of the reactive T cells (13, 58, 59). Thus, we tested blood samples various times after virus injection for the presence of CD4⁺V β 6⁺ and CD4⁺V β 14⁺ T cells. At 4 d, a small but significant reduction of V β 6⁺ T cells was observed in mice injected with MMTV (SW)-containing milk (Table 4). This could have been due either to recruitment of these cells to the local lymph node and/or the onset of clonal deletion. The reduction of CD4⁺V β 6⁺ T cells in peripheral blood reached 75% after 2 wk and was almost complete after 6 wk (1.5% of CD4⁺ T cells). MMTV (C3H) did not induce a specific reduction of CD4⁺V β 14⁺ cells 2 wk after injection. After 6 wk, however, C3H milk induced a specific reduction of CD4⁺V β 14⁺ T cells, which did not exceed 50%. Values for CD4⁺V β 6⁺ and CD4⁺V β 14⁺ T cells remained unchanged in mice injected with control milk. Thus, both viruses induced deletion of the expected V β s from the peripheral T cell pool upon local injection into the footpad.

Purification of the Exogenous Virus and Cloning of MMTV (SW) orf. We partially purified the novel MMTV contained in milk of V β 6 deleters. Reverse transcription was used to prepare cDNA from the viral RNA. The orf molecule in the 3' LTR has recently been shown to mediate the V β specificity in both exogenous MMTV (C3H) (39) and (GR) (32). Therefore, we used the PCR with primers to conserved regions spanning the orf coding region to amplify a 1.1-kb stretch of the 3' LTR containing the entire orf sequence. The PCR products were then cloned and sequenced. In addition to BALB/c IC milk, a PCR product was obtained from C3H milk. However, no PCR product was obtained from the control BALB/c HO milk.

Sequence analysis revealed that the 3' LTR from the novel exogenous MMTV (SW) is very similar to other described MMTV sequences at the 5' end of the orf (Fig. 4). However, a stretch with a unique sequence was found at the 3' end, altering the last 21 amino acids of the putative orf protein completely.

Cloning of Mtv-7 orf (Mls-1^a). Since the novel MMTV (SW) has the identical V β specificity as Mls-1^a, and a strong linkage between endogenous Mls-1^a and Mtv-7 has been observed (32, 36), we attempted to clone Mtv-7. To this end, genomic DNA isolated from BALB.D2 spleen was digested with the restriction endonuclease EcoRI and size fractionated on an agarose gel. DNA was subsequently isolated from gel slices corresponding to the 3' ends of Mtv-9 (10.0 kb), Mtv-7 (11.7 kb), and Mtv-6 (16.7 kb). (38) PCR analysis was then performed with primers that can amplify all known MMTV orf sequences.

Nucleotide sequences of clones derived from PCR products obtained from the Mtv-9 fraction were nearly identical to the published Mtv-9 LTR orf sequence (Fig. 5, and data not shown). However, clones obtained from the Mtv-7 and Mtv-6 fractions showed striking differences at the 3' end of the LTR, as compared with previously published sequences (Fig. 4). The putative Mtv-7 sequence was very similar to

the exogenous virus sequence described above. At the extreme 3' end of the orf coding region, which is very different from all known MMTV orf sequences (and is thought to mediate the V β specificity), Mtv-7 and the novel virus display extremely high sequence homology, except for only three conservative amino acid differences (Fig. 5).

Discussion

With the discovery that Mls antigens are encoded by endogenous *Mtv* proviral loci, experiments using exogenous MMTV to analyze infection, tolerance development, and the interplay between the immune system and cancer development have become possible. This sort of analysis has been hampered by the fact that only two exogenous MMTVs have been characterized in sufficient detail so far, namely the MMTVs (C3H) and (GR) (32, 39). Both of these viruses behave very similarly to the Mls-like antigens that are slow deleters and weak stimulators in a mixed lymphocyte reaction. They can be presented only by MHC I-E molecules and affect T cells expressing V β 14. With the description of MMTV (SW) in this article, a virus with biological properties very similar to Mls-1^a, we found the exogenous counterpart of a strong Mls determinant.

One of the most prominent features of Mls-1^a is the strong proliferative response of T cells from Mls-1^a-negative mice to Mls-1^a-expressing B cells (1, 28–30). In that respect, Mls-1^a is the strongest of all the Mls determinants. In this study we show that this feature, however, was not found in B cells from mice maternally infected with the Mls-1^a-like exogenous MMTV (SW). Several explanations could account for this discrepancy. (a) The amino acid sequence comparison between the exogenous and the endogenous virus indicates a few minor differences at the COOH terminus of the orf molecule. However, these changes in the COOH terminus of the exogenous (*Mtv-7*-like) orf compared with *Mtv-7* are conservative, and thus unlikely to account for an inefficient interaction with the TCR V β . The two most likely explanations are: (b) the frequency of MMTV-infected B cells could be too low to induce a stimulation. In control mixing experiments, 3% Mls-1^a expressing BALB/D2 B cells are required to induce a detectable mixed lymphocyte reaction (H. R. MacDonald, unpublished observations). (c) The expression level of the MMTV orf protein could be too low to induce a strong stimulation. However, injected in vivo, the novel virus is almost as efficient as Mls-1^a B cells at triggering V β 6⁺ T cells. In comparison, exogenous C3H virus is not able to stimulate V β 14-expressing T cells in the same assay. By this criterion, the novel MMTV encodes a strong Mls determinant.

It is striking that, in vivo, MMTV (C3H) and the novel MMTV induce a slow deletion of T cells expressing their target V β s in mice that take up the virus by suckling. Although both the thymus and the periphery show deletion of V β 6⁺ T cells in infected mice, peripheral deletion of CD4⁺ V β 6⁺ T cells is more profound, suggesting that some

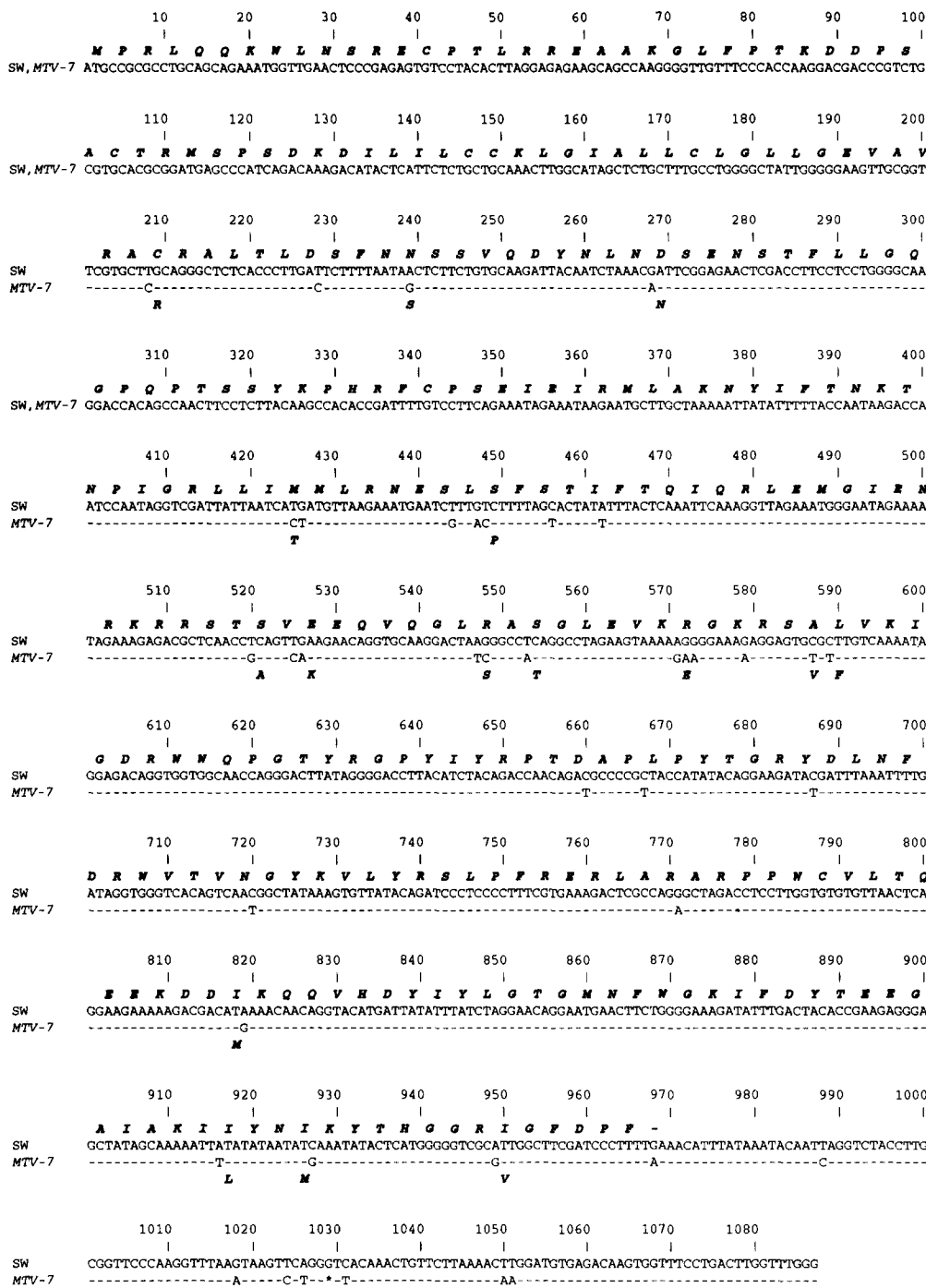


Figure 4. Nucleotide and amino acid sequences of MMTV (SW) orf and *Mtv-7* orf. In the *Mtv-7* nucleotide sequence, the nucleotides identical with the MMTV (SW) sequence are indicated in dashes. Below the *Mtv-7* nucleotide sequence, the differences in the *Mtv-7* orf amino acid sequences are indicated. The amino acids are written in the single-letter code. The sequences of the oligonucleotides used for amplification are underlined. The sequence data are available from EMBL under accession numbers X65339 and X65340.

virus-infected cells may recirculate to the thymus in the neonate.

With both viruses it takes several months until the deletion is complete. When introduced into adults by foot pad injection, the C3H virus again induces a slow, 50% reduction of $V\beta 14^+$ cells, whereas the novel Mls-1^a-like virus induces a very rapid and almost complete deletion of $V\beta 6^+$ T cells. This apparent discrepancy might be explained by a very inefficient uptake of the latter virus through the gut, and

argues against a weak stimulation potential of the exogenous virus. Since virus uptake may be dependent upon the envelope protein gp52, detailed analysis of the gp52 sequence may be required to test this hypothesis.

The incidence of mammary tumors induced in BALB/c mice foster nursed to C3H mothers is very high (80–90%) at 1 yr of age (60). In contrast, in our BALB/c IC colony we did not observe a single mammary tumor so far. Even old (>1 yr) breeders ($n = 20$) that had several litters did not

MTV	V β -deletion	250	260	270	280	290	300	310	320
MTV-8	11	PPFRERLARARPPWC	VL	TQEEKDDIKQ	QVHDYIYL	GTGMNVWGKIF	PHYTKEGAVARQ	LEHISADTF	GMSYNG
MTV-9	5, 11	-----	-----	-----M-----	-----	-----	-----	-----L-----	-----
MTV-11	11	-----	-----FS-----	-----M-----	-----	-----	-----	-----	-----
MTV-6	5, 3	-----	-----M-----	-----	-----	-----IH*-V-YNSR-E-KRHII	-----K-LPLAF	-----	-----
MTV-1	3	-----	-----S-----	-----	-----	-----*IH*-V-YNSR-E-KRHII	-----K-LP	-----	-----
C3H	14	-----	-----M-S-----	-----M-----	-----	-----HF-----*	-----T--GLI--Y--K-Y----	-----YE	-----
GR	14	-----	-----M--EK-----	-----M-----	-----	-----HF--V--*	-----T--GLI--Y--K-Y----	-----YD	-----
BR-6	?(14?)	S-----	-----M-----N-----	-----	-----	-----SSI-----*	-----RT--ALI--Y--K-Y----	-----YD	-----
MTV-17	?	-----	-----K-----E-----	-----	-----	-----R-RI--K--RCI	-----DIR--K	-----	-----
GR-K	?	-----	-----S-----M-----	-----	-----	-----V-R--RDLNVF-K*SR-EVQKHLID*	-----IKALPL--	-----	-----
SW	6, 7, 8, 1, 9	-----	-----	-----	-----	-----F-----D--E--I-KI IYN-KYTHG-RIGDFPF	-----	-----	-----
MTV-7	6, 7, 8, 1, 9	-----	-----	-----M-----	-----	-----F-----D--E--I-KI-YNMKYTHG-RVGFDPF	-----	-----	-----

Figure 5. Amino acid sequence comparison of the known orf sequences. The COOH-terminal 70 amino acids, which contain the most polymorphic residues, are shown. The orf sequences are grouped in families as depicted on the left, according to the deletion of the indicated TCR V β -expressing T cells. The sequences for BR-6, *Mtv-1*, -8, -11, -17, and for MMTV (GR), (C3H), and (C3H-K) are from the literature (29, 32, 73-79). The *Mtv-6*, -7, -9, and MMTV (SW) orf sequences were derived in this study.

develop tumors. Thus, on the same genetic background, mammary tumor induction is only observed with MMTV (C3H), although MMTV (SW) deletes V β s as profoundly as MMTV (C3H). This suggests that the tumor-inducing capacity of MMTV is not reflected in its efficiency to delete V β s.

The question about the origin of this exogenous virus remains open. BALB/c IC mice were derived from BALB/c J mice, when a breeding colony was established in 1988 at IFFA Credo. A genetic contamination is very unlikely since the endogenous MMTV copies are identical to standard BALB/c mice and because the colony is regularly tested for skin graft rejection with BALB/c J skin. No rejections have been observed. Mice with Mls-1^a-like determinants have never been reported in the BALB/c J colony. Thus, the most likely means of MMTV infection is maternally through milk. The BALB/c IC breeding pairs were derived from BALB/c J mothers by Caesarian section and fostered to outbred Swiss foster mothers. Therefore, two major possibilities might explain the origin of this exogenous Mls-1^a-like virus: one is that it represents the original MMTV, which upon integration is found as *Mtv-7* in several laboratory mouse strains. Since many outbred Swiss mice have the H-2^q haplotype,

which is not able to present Mls antigens (15), the exogenous virus would not have been detected by analysis of V β expression (3, 4, 10). Alternatively, MMTV (SW) originates from *Mtv-7*. After formation of infectious particles from the integrated provirus, production of infectious particles in milk can occur. Several examples for this possibility exist in the literature. *Mtv-1* and *Mtv-2* can form infectious virus particles that are then transmitted to offspring through the milk and through the germline (61-63). Normal mice not containing *Mtv-1* and *Mtv-2* can form mammary tumors, which are most likely due to other endogenous MMTV, after 1 yr of age (57). Furthermore, treatment of laboratory mouse strains or mouse cell lines with irradiation and/or carcinogens can lead to formation of infectious MMTV particles (64-68). Although further experiments are required to definitively settle this question, it seems likely that the virus was transmitted to BALB/c IC mice from outbred Swiss mice. Hence, we propose the designation MMTV (SW) for this virus.

Since most of the divergence in MMTV orf sequences resides in the COOH terminus of the putative orf protein, it has been argued that the COOH terminus confers V β specificity (27, 32, 39). It was recently shown that the orf molecule of *Mtv-7* is responsible for deletion of Mls-1^a-reactive T cells (68a) and that the orf molecule of *Mtv-9* is responsible for deletion of V β 5 and V β 11 T cells (69). For *Mtv-7* (and the homologous novel exogenous MMTV [SW]), the COOH terminus is very different from previously published orf sequences. An additional argument for the importance of the COOH terminus in determining the V β specificity is that orf of *Mtv-6*, a gene encoding Mls-3^a (a deletion element for TCR V β 3), is identical to the orf sequence of *Mtv-1*, another V β 3 deleter (Fig. 5). The orf molecules that are implicated in clonal deletion of T cells fall into four groups, each specific for a particular subset of T cells expressing (a) specific V β region(s). With the nine MMTV orf molecules sequenced so far, which correspond to one of four TCR V β specificities, an excellent correlation exists between the COOH-terminal orf sequence and TCR V β specificity. In addition, we have shown in experiments using transgenic mice that a variant of MMTV (C3H) with a dramatically altered orf COOH terminus, failed to induce deletion of TCR

	24	25	26	27	28	29	30	31	32	n	Identity	Similarity
<i>Mtv-7</i> orf	FDYTEEG	AI	AK	ILY	NK	YTHG	GRV	GFDP	F	12		
SW orf	FDYTEEG	AI	AK	ILY	NK	YTHG	GRV	GFDP	F	12		
Calpactin p11	NQK	DPL	AVDK	IMK	DL	DQCR	DGK	VGF	QFS	28	25%	64%
18A2	FKRT	DEAA	AFQK	VMS	NL	LD	SNR	DNE	VDFQ	29	24%	66%

Figure 6. Amino acid sequence homology between *Mtv-7* orf, MMTV (SW) orf, and S100 proteins. Similarity between sequences was determined using the program Bestfit from the Genetics Computer Group (GCG) package (80). A stretch of 28 and 29 amino acids was analyzed for Calpactin p11 and 18A2, respectively. Numbering of amino acids is according to Fig. 5 for orf molecules and reference 69 for S100 proteins. Identical amino acids are shown in bold characters and asterisks indicate the COOH termini of the orf molecules. The helix-loop-helix motif of S100 proteins described in reference 70 is superimposed onto the *Mtv-7* and MMTV (SW) orf sequences.

V β 14. The key residues within the COOH terminus required for orf-TCR interaction remain to be defined.

Analysis of sequence homology of entire orf molecules did not reveal any significant homologies with sequences in the database. When the unique COOH terminus of the *Mtv-7* and MMTV (SW) orfs were compared with the "Swissport" data base, however, significant homologies to two members of the S100 protein family were found: the calpactin L chain p11 and 18A2 (see Fig. 6) (for review see reference 70). S100 proteins display a common structural feature termed EF hand. This is a helix-loop-helix structure that has in its original form calcium-binding capacity (71). The homology of *Mtv-7* and MMTV (SW) orf is located in the COOH-terminal EF hand spanning the first E helix (nine amino acids) and the loop (12 amino acids). The COOH-terminal orf residue (phenylalanine) corresponds to the first amino acid of the second, F helix (Fig. 6). Biochemical evidence suggests that this particular EF hand in the calpactin L chain does not bind calcium anymore (72).

These findings might suggest that the *Mtv-7* and MMTV (SW) orf molecules have a COOH terminus forming a helix-loop configuration. Since the polymorphic COOH terminus

has been implicated in V β specificity of the orf molecules, it might be this loop of 12 amino acids that confers the binding to the V β domain of the TCR.

The existence of exogenous MMTV (and their endogenous counterparts) with different TCR V β specificity and kinetics of clonal deletion provides a powerful tool to investigate the different phases of peripheral and thymic tolerance mechanisms. The timing of deletion can be controlled with dose, route of infection, and choice of virus. Neonatal and adult immune response and tolerance can be compared. In addition, after local administration, the spread of the virus in the immune system and to the mammary gland can be analyzed in much greater detail.

With the finding of this Mls-1^a-like exogenous MMTV (SW), it seems likely that many more exogenous MMTV viruses may exist in the wild (and laboratory) mouse population. Analysis of the milk of different strains of mice will give indications about the frequency and heterogeneity of such viruses. Potentially, MMTVs with many different TCR specificities can be found. Sequence analysis of the orf cDNA and comparison with TCR deletion patterns will give insight into important residues for TCR and MHC interaction.

We thank P. Zaech and C. Knabenhans for FACS[®] analysis, N. Jeanguenet for performing the ELISA, and IFFA Credo for providing valuable information and mice. We are grateful for the helpful discussions with H. Diggelmann and P. Rollini.

H. Acha-Orbea is a recipient of a START fellowship from the Swiss National Science Foundation.

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Received for publication 27 December 1991 and in revised form 26 February 1992.

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