Superantigen-reactive CD4+ T Cells Are Required to Stimulate B Cells after Infection with Mouse Mammary Tumor Virus

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Summary

Superantigens are defined by their ability to stimulate a large fraction of T cells via interaction with the T cell receptor (TCR) Vβ domain. Endogenous superantigens, classically termed minor lymphocyte-stimulating (Mls) antigens, were recently identified as products of open reading frames (ORF) in integrated proviral copies of mouse mammary tumor virus (MMTV). We have described an infectious MMTV homologue of the classical endogenous superantigen Mls-1^a (*Mtv-7*). The ORF molecules of both the endogenous *Mtv-7* and the infectious MMTV(SW) interact with T cells expressing the TCR Vβ6, 7, 8.1, and 9 domains. Furthermore, the COOH termini of their ORF molecules, thought to confer TCR specificity, are very similar. Since successful transport of MMTV from the site of infection in the gut to the mammary gland depends on a functional immune system, we were interested in determining the early events after and requirements for MMTV infection. We show that MMTV(SW) infection induces a massive response of Vβ6$^+$ CD4$^+$ T cells, which interact with the viral ORF. Concomitantly, we observed a B cell response and differentiation that depends on both the presence and stimulation of the superantigen-reactive T cells. Furthermore, we show that B cells are the main target of the initial MMTV infection as judged by the presence of the reverse-transcribed viral genome and ORF transcripts. Thus, we suggest that MMTV infection of B cells leads to ORF-mediated B-T cell interaction, which maintains and possibly amplifies viral infection.

Minor lymphocyte-stimulating (Mls) antigens are encoded by an open reading frame (ORF)[1] in the 3′ LTR of endogenous mouse mammary tumor virus (MMTV) (1–3). They stimulate a large proportion of T cells due to the fact that T cell reactivity to Mls antigens is determined by the usage of the V segment of the TCR β chain (4, 5). The classical (and strongest) Mls determinant, Mls-1^a, which interacts with T cells expressing Vβ6, 7, 8.1, and 9 elements (4–7), is encoded by the ORF of the *Mtv-7* proviral locus (8). We have recently found an exogenous (infectious) MMTV, MMTV(SW), with properties of Mls-1^a (9). The ORF molecules of *Mtv-7* and MMTV(SW) display an almost identical amino acid sequence, particularly in COOH termini implied to confer TCR Vβ specificity. In fact, both endogenous *Mtv-7* and infectious MMTV(SW) interact with the same TCR Vβ elements. In an MMTV-infected host, ORF-reactive T cells are deleted from both the thymic and peripheral T cell pool, although at a considerably slower rate than after expression of the endogenous *Mtv-7* (Mls-1^a) locus. Furthermore, injection of adult mice with MMTV(SW) leads to expansion of Vβ6$^+$ CD4$^+$ T cells similar to that observed after injection of Mls-1^a-expressing B cells (9). A functional immune system seems to be a prerequisite for successful transport of MMTV to the mammary gland after neonatal infection through the gut mucosa. Thus, thymectomy, absence of T cells (in nude mice), or deletion of the ORF-reactive T cells prevent virus transmission to the next generation (10–12).

While very much attention has been focused on the fate of T cells interacting with Mls, the effects of T-B cell interaction on the Mls-presenting B cell have received less attention. Generally, it was found that Mls-reactive T cell clones provide polyclonal B cell help in vitro (13–15), whereas in vivo terminal differentiation of a minor fraction of the injected Mls-1^a B cells was found (16, 17). Since B cells efficiently present ORF molecules from endogenous Mtv's, which are then recognized by ORF-specific T cells, it has been speculated that the ORF from infectious MMTVs, like

[1] *Abbreviations used in this paper:* MMTV, mouse mammary tumor virus; ORF, open reading frame.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/02/0359/08 $2.00
Volume 177 February 1993 359–366

Mls antigens, induces T-B cell interaction in order to favor virus integration and replication (18). To test this hypothesis we injected adult mice with MMTV(SW), and followed the local immune response. MMTV(SW) induces a massive response of the ORF-reactive $V\beta6^+$ $CD4^+$ T cells. Concomitant B cell accumulation and differentiation depends on the presence of the ORF-reactive $V\beta6^+$ $CD4^+$ T cells. This response coincides with the presence of reverse-transcribed viral genome and ORF mRNA in B cells. Since B cells are known to present ORF molecules from endogenous Mtv's efficiently to T cells, it might be this ORF-induced B-T interaction that maintains and possibly amplifies MMTV infection of B cells.

Materials and Methods

Mice. BALB/c and BALB/c *nu/nu* mice were purchased from HO Harlan OLAC Ltd. (Bicester, UK). Mls-1^a (*Mtv-7*) congenic BALB/c (BALB.D2) mice were bred from breeding pairs originally obtained from H. Festenstein (London Hospital Medical College) (19). MMTV(SW)-infected BALB/c mice were maintained from mice originally purchased from IFFA CREDO (L'Arbresle, France) (9). In all experiments female mice 6–10 wk of age were used.

Antibodies. The following mAbs were used in this study: 14.2 (rat IgM anti-Vβ14) (20); 44.22.1 (rat IgG anti-Vβ6) (21); GK1.5 (anti-CD4) (22); F23.1 (mouse IgG2a anti-Vβ8.1-3) (23); Lyt2 (53.6.7) (anti-CD8) (24); 3.168.8.1 (rat IgM anti-CD8) (25); polyclonal rabbit $F(ab)_2$ anti-mouse Ig.

Reconstitution of Athymic BALB/c nu/nu Mice. BALB/c *nu/nu* mice were reconstituted with either $V\beta6^+$ $CD4^+$ or $V\beta14^+$ $CD4^+$ T cells as described (26). Briefly, BALB/c lymph node cells were enriched in these T cell subsets by elimination of $CD8^+$ T cells through complement lysis using the mAb 3.168.8.1 (anti-CD8). B cells and $V\beta8^+$ T cells were removed by incubation with F23.1 (anti-Vβ8.1-3) followed by goat anti-mouse Ig coupled to magnetic beads (Dynabeads; Dynal A.S., Oslo, Norway) and elimination on a magnet. The cells were then stained directly by fluoresceinated mAb 44.22 (anti-Vβ6) and biotinylated mAb 14.2 (anti-Vβ14) followed by streptavidin-PE. $V\beta6^+$ and $V\beta14^+$ cells were subsequently sorted using a FACStar Plus® (Becton Dickinson & Co., Mountain View, CA). Sorted cell populations, which were >97% pure $V\beta6^+$ $CD4^+$ or $V\beta14^+$ $CD4^+$ T cells, were washed and counted. 10^5 cells were injected intravenously per BALB/c *nu/nu* mouse. Reconstitution of recipient animals was ascertained 4 wk later by analysis of the percentage of the transferred cells among total blood lymphocytes. Mice were tail bled and leukocytes were recovered from heparinized blood samples by centrifugation through a Ficoll (Pharmacia, Uppsala, Sweden) cushion.

Milk Collection, Virus Purification, and Virus Titration. Milk was aspirated from MMTV(SW)-infected or noninfected, lactating BALB/c females after injection of 0.5 IU syntocinon/oxytocine (Sandoz, Basel, Switzerland), pooled, and stored at −70°C. A pool of milk was diluted 1:2 with PBS and centrifuged at 600 *g* for 10 min to skim. The skimmed milk serum was then diluted five times with PBS, 0.1 M EDTA, pH 7.4, centrifuged at 15,000 *g* for 1 h, and the virus pellet was resuspended in PBS. Remaining casein aggregates were removed by a quick spin at 600 *g*. The virus titers in these preparations were determined using a sandwich ELISA specific for MMTV gp52 as described before (9). Purified MMTV was diluted at 10^9 particles/25 μl and stored in aliquots at −70°C. Control milk not containing MMTV particles was treated and diluted accordingly.

Injections and Sampling. Purified MMTV (10^9 particles) or a virus-free control milk preparation were injected into the hind footpads of recipient mice. After the appropriate time the popliteal, paraaortic, and inguinal lymph nodes from the injected and noninjected footpads were isolated and pooled.

Flow Microfluorometry. Lymph node cells (10^6) were stained with anti-TCR Vβ-specific monoclonal hybridoma supernatants followed by fluoresceinated anti-rat or anti-mouse IgM or IgG antisera. B cells were detected using a polyclonal rabbit $F(ab)_2$ anti-mouse Ig directly labeled with fluorescein. PE-coupled anti-CD4 (GK1.5) (Becton Dickinson & Co.) was used in the second dimension. Biotinylated anti-CD8 (Lyt2) plus streptavidin-PE Texas red (Tandem; Southern Biotechnology Associates, Inc., Birmingham, AL) was used in the third dimension.

Leukocytes were recovered from heparinized blood samples by centrifugation through a Ficoll (Pharmacia) cushion. These cells were stained in one step with a mixture of FITC-labeled anti-TCR Vβ antibody and PE-coupled anti-CD4. For DNA analysis 3×10^6 cells were stained with FITC-conjugated antibodies as above, washed in PBS, and fixed in 70% ethanol overnight as described in (27). Cells were centrifuged at 3,000 rpm for 5 min and stained at room temperature in 1 ml PBS containing 1 mg/ml glucose, 100 Kuntz U/ml RNase A, and 50 μg/ml propidium iodide. The cells were analyzed using the Doublet Discrimination Module (Becton Dickinson & Co.). Analysis was performed on a FACScan® cell analyzer using Lysys II for data evaluation (Becton Dickinson & Co.).

Ig Estimation. Levels of IgM and IgG subclasses in culture supernatants were determined separately for each subclass by ELISA as described (28). Values were expressed (in μg/ml) by reference to a standard curve obtained with a mouse Ig reference serum (64-901; Miles Scientific, Naperville, IL). Detection limit of the individual tests was 10 ng/ml. Total Ig levels correspond to the addition of the values for IgM and each of the IgG subclasses.

Polymerase Chain Reaction. DNA was isolated from total lymph node cells or FACS®-sorted B and T cells by digestion in Tris/EDTA/SDS, pH 8.0, plus proteinase K (100 μg/ml) at 52°C overnight. DNA was purified and resuspended at a DNA equivalent of 10^6 cells per 50 μl, 20% was used per PCR reaction, corresponding to ~1 μg of genomic DNA. Cellular RNA was isolated from total lymph node cells or FACS®-sorted B and T cells after the guanidinium isothiocyanate/acid phenol procedure (29). Purified RNA was resuspended at the RNA equivalent of 10^6 cells/2 μl. The reverse transcription was performed in 1 × PCR buffer with 2 μl RNA solution as described elsewhere (30). Viral cDNA was obtained as described before (9).

Oligonucleotides to amplify all Mtv ORFs were chosen on the basis of high degree of conservation between the sequenced ORF molecules, resulting in a 898-bp PCR product: 5′ oligo, TCGTGCTCGCAGGGCTCTCAC (VJ84); 3′ oligo, GTGTCGACCCAAACCAAGTCAGGAAACCACTTG (VJ71). To specifically detect MMTV(SW), a 3′ oligonucleotide specific for the unique MMTV(SW)/*Mtv-7* ORF COOH terminus was used in combination with VJ84, yielding a 766-bp PCR product. 3′ oligo, GCGACCCCCATGAGTATATTTC (VJ83). A primer in the 5′ noncoding region at the 5′ splicing site for subgenomic mRNAs (31) was designed to specifically amplify spliced ORF mRNA: 5′ oligo, CAGGGAACTGCAGTCTCGCCTA. The 3′ oligo VJ83 described above was used to ascertain specificity for the MMTV(SW) ORF. As a control for the cDNA preparation we used primers specific for β-actin: 5′ oligo, GAGGGAAATCGTGCGTGACATCAA; 3′ oligo, GGAACCGCTCGTTGCCAATAGTGA.

DNA or cDNA was boiled for 3 min in the presence of the

oligos. The conditions for PCR were 1 min at 55°C, 1 min at 72°C, 1 min at 93°C for 40 cycles, and 7 min at 72°C in 1× PCR buffer containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.2 mM dNTP, 2 mM $MgCl_2$ (1.5 mM for the ORF mRNA and β-actin primers), 0.01% gelatin, 2.5 U Taq polymerase (AmpliTaq; Perkin Elmer Corp., Pomona, CA) using a DNA thermal cycler (Perkin Elmer Corp.). 20% of the PCR reaction was size fractionated in 1.5% agarose, stained with ethidium bromide, and visualized under UV.

Results

Acute Infection of BALB/c Mice with MMTV(SW) Induces a B Cell Response. After footpad injection of adult BALB/c mice with 10^9 MMTV(SW) particles purified from mouse milk, we observed an approximately fourfold size increase of the local (popliteal, paraaortic, and inguinal) lymph nodes. As noted previously (9) this size increase was partially due to an increase of Mls-1^a-reactive $V\beta6^+$ $CD4^+$ T cells from 10.9 ± 0.5% of CD4 in animals injected with an MMTV-free milk preparation to 24.4 ± 2.6% at day 6 after MMTV(SW) infection. In contrast non-Mls-1^a-reactive T cells such as $V\beta14^+$ $CD4^+$ did not specifically increase, displaying 9.9 ± 1.2% of $CD4^+$ T cells in control animals and 8.6 ± 1.5% at day 6 after infection. Absolute cell counts for the different $CD4^+$ T cell subsets over time are shown in Fig. 1 *A*. The T cell response in the local lymph nodes was paralleled by a specific increase of B cells from 20.0 ± 2.3% of total lymph node cells in noninfected animals to 32.7 ± 3.0% at day 6 after infection (Fig. 1 *B*). Both B and T cell responses were virus dependent since the MMTV-free milk preparation did not induce a detectable response (Fig. 1, *A* and *B*).

Using a combination of cell surface labeling and DNA content analysis, we determined the percentage of cells in cycle. Between days 4 and 6 after MMTV(SW) infection a minor fraction of B cells (2%) was found in $S+G_2+M$ phase compared with 0.8% in noninfected control lymph nodes. Slightly more (4%) $CD4^+$ T cells are in cycle at day 4 (compared with 0.6% in controls). Analysis of MMTV(SW) ORF-responsive T cells indicated an enrichment of cycling cells (8% among $V\beta6^+$ T cells).

Titration of the injected MMTV(SW) dose from 10^9 to 10^6 virus particles indicates that the B cell response is high as long as there is a significant $CD4^+$ $V\beta6^+$ T cell response (Fig. 2). This suggests that a critical virus dose is required to induce a detectable local immune response.

The B Cell Response to MMTV(SW) Depends on the Presence of Mls-1^a-reactive T Cells. BALB.D2 mice are (*Mtv-7*$^+$) Mls-1^a-congenic BALB/c mice. Therefore, they delete >95% of the T cells that react with Mls-1^a within the first 10 d after birth (32). After injection of exogenous MMTV(SW) into BALB.D2 mice the B cell response was profoundly diminished compared with the response in control BALB/c mice. Both BALB/c and BALB.D2 mice, however, responded equally well to MMTV(C3H), an exogenous MMTV that encodes an ORF molecule interacting with T cells expressing the $V\beta14$ and $V\beta15$ TCRs (2) (Table 1).

$CD4^+$ $V\beta6^+$ T Cells Are Sufficient to Stimulate a B Cell Response to MMTV(SW). Nude mice lack a thymus and are therefore not able to generate a functional T cell compartment; however, they can be efficiently reconstituted with T cells (26). We reconstituted BALB/c *nu/nu* mice with either 10^5 $CD4^+$ $V\beta6^+$ or $CD4^+$ $V\beta14^+$ T cells isolated by cell sorting from lymph nodes of normal BALB/c mice. Greater than 90% of the $CD4^+$ T cells in transferred nude mice expressed the selected phenotype, whereas contamination with the alternative subset was <1%. Nude mice reconstituted

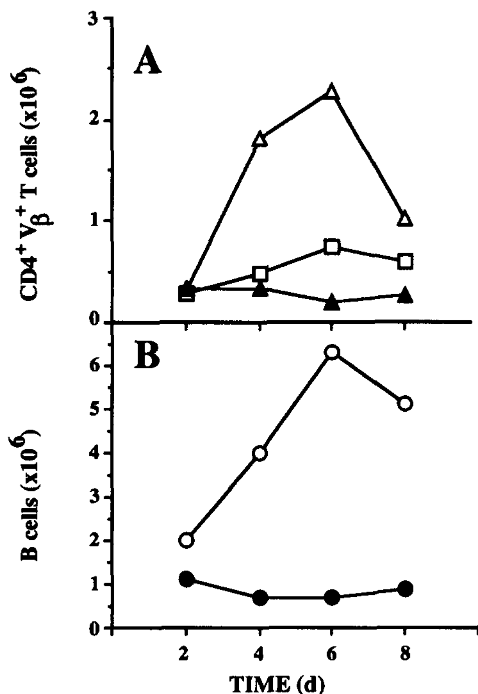

Figure 1. Local immune response to MMTV(SW). 10^9 MMTV(SW) particles were injected into one hind footpad of recipient BALB/c mice. At appropriate time points after injection the local lymph nodes (popliteal, paraaortic, and inguinal) were isolated, pooled, and analyzed for lymphocyte subsets using flow microfluorometry. (*A*) Absolute cell counts in local lymph nodes for $V\beta6^+$ $CD4^+$ T cells (△) and $V\beta14^+$ $CD4^+$ T cells (□) after MMTV(SW) infection and for $V\beta6^+$ $CD4^+$ T cells (▲) in mice injected with a MMTV-free milk preparation. Data represent mean values from two to four animals per time point. (*B*) B cell response upon MMTV(SW) injection (○) and upon injection of the control milk preparation (●). Data represent mean values derived from the same animals as above.

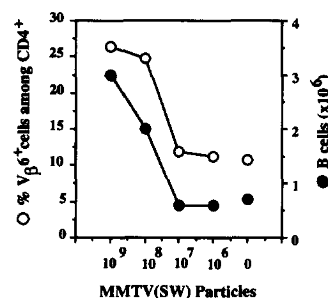

Figure 2. Effect of MMTV(SW) dose on T and B cell responses. Response of $V\beta6^+$ T cells indicated as percentage of $CD4^+$ T cells (○) and concomitant B cell response (●) (indicated as absolute cell counts in popliteal, paraaortic, and inguinal lymph nodes) upon titration of the MMTV(SW) dose. Data represent mean values of two animals at day 4 after injection of MMTV(SW).

Table 1. *B Cell Response to MMTV in Mls-1ᵃ Congenic Mice*

Virus injection	BALB/c		BALB.D2	
	n	B cells*	*n*	B cells
None	9	0.7 ± 0.5	7	1.1 ± 0.7
MMTV free	3	0.7 ± 0.3	2	0.9/0.6
MMTV (C3H)	3	3.4 ± 1.3	3	3.5 ± 1.2
MMTV (SW)	4	6.9 ± 1.4	4	2.2 ± 1.1

* Absolute numbers (× 10^6) (±SD) of B cells in popliteal, paraaortic, and inguinal lymph nodes 6 d after injection of 10^9 MMTV particles or an MMTV-free milk preparation.

with either Vβ14⁺ or Vβ6⁺ CD4⁺ T cells had 8.6 ± 3.9% CD4⁺ T cells or 9.0 ± 4.0% CD4⁺ T cells among total lymph node cells, respectively. In comparison, normal BALB/c mice have ~60% CD4⁺ T cells in peripheral lymph nodes, 10% of which are either Vβ6⁺ or Vβ14⁺.

After injection of MMTV(SW) or MMTV(C3H) into these reconstituted nude mices we assessed the immune responses in the local lymph nodes and compared these with control BALB/c *nu/nu* mice (displaying 0.6 ± 0.5% CD4⁺ T cells among total lymph node cells). An efficient B cell response is induced whenever the appropriate combination of ORF-specific CD4⁺ T cell and MMTV is present, e.g., CD4⁺ Vβ6⁺ T cells and infection with MMTV(SW) (Table 2). As seen before for BALB.D2 mice a small B cell response is seen in nonreconstituted nude mice, possibly reflecting a minor T cell–independent B cell response. Alternatively, residual reactive T cells in BALB.D2 mice and/or ORF-reactive T cells arising spontaneously in nude mice may contribute to this response.

MMTV(SW) Infection Induces Ig Secretion by B Cells. When 5 × 10^5 lymphocytes were cultured for 3 d after in vivo MMTV(SW) infection, we observed production of Ig (IgM + IgG) in supernatants of lymphocytes from infected but not from uninfected animals. Preliminary experiments indicated that maximal secretion of Ig by B cells from local lymph nodes occurred 6 d after injection of MMTV(SW) (not shown).

Ig secretion by lymph node cells from BALB/c mice increased from 0.1 μg/ml (uninfected animals, 21.5% B cells present in culture) to 15 μg/ml by lymphocytes from infected animals (35.4% B cells). However, the absence of Mls-1ᵃ-reactive T cells in BALB.D2 mice decreased the Ig production ~10-fold (1.5 μg/ml) (Fig. 3 *A*). Upon MMTV(SW) infection of reconstituted BALB/c *nu/nu* mice, the presence of only CD4⁺ Vβ6⁺ T cells restored high Ig production (20 μg/ml) (85.1% B cells in culture) compared with nonreconstituted BALB/c *nu/nu* (0.5 μg/ml) (91.2% B cells) or Vβ14⁺ CD4⁺ reconstituted nude mice (2 μg/ml) (84.0% B cells) (Fig. 3 *B*).

The Ig subclass distribution in the supernatants derived from infected BALB/c lymph node cells revealed a predominant IgG response. Among the IgG subclasses the IgG2a isotype was found at a 10-fold higher amount than the other IgG subclasses. This response was greatly diminished when ORF-reactive CD4⁺ T cells were absent as in the case of BALB.D2, BALB/c *nu/nu*, and Vβ14⁺ CD4⁺ reconstituted nude mice (Fig. 4).

In Vivo MMTV(SW) Infects B Cells Predominantly. Analysis of lymph node cells for MMTV(SW) ORF DNA by the PCR indicated that reverse-transcribed viral genome was detectable in draining lymph nodes 4 d after virus injection (Fig. 5 *A*). Thus, the presence of a reverse-transcribed viral genome coincided with a CD4⁺ Vβ6⁺ T and B cell response as shown in (Fig. 1). Since nondraining lymph nodes were still MMTV(SW) negative 3 wk after local infection, virus spread might be very inefficient. Control PCR specific for all Mtv's present, i.e., exogenous and endogenous Mtv's (*Mtv* 6, 8, and 9 in BALB/c mice), indicated that they could be amplified with equal efficiency from all the different samples (Fig. 5 *A*).

To determine the phenotype of MMTV(SW)-infected cells we performed PCR analysis on FACS®-sorted B and T cells from lymph node cells 6 d after infection. As shown in Fig. 5 *B*, B cells are the primary target of MMTV(SW) infection. Only a very weak signal was detected in the T cell prepara-

Table 2. *Immune Response Induced by MMTV in Either Vβ6⁺ CD4⁺ or Vβ14⁺ CD4⁺ T Cell–reconstituted BALB/c nu/nu Mice*

Injection	BALB/c *nu/nu* reconstituted with:					
	No cells		Vβ14⁺ CD4⁺ T cells		Vβ6⁺ CD4⁺ T cells	
	B cells*	CD4⁺*	B cells	Vβ14⁺ CD4⁺	B cells	Vβ6⁺ CD4⁺
None	2.3 ± 1.4	0.03 ± 0.02	2.9 ± 0.8	0.3 ± 0.1	2.1 ± 0.4	0.3 ± 0.1
MMTV(C3H)	2.6 ± 1.3	0.02 ± 0.02	<u>9.9 ± 3.9</u>	0.6 ± 0.2	3.9 ± 1.8	0.3 ± 0.1
MMTV(SW)	3.4 ± 0.5	0.02 ± 0.02	4.0 ± 0.7	0.2 ± 0.1	<u>10.5 ± 2.2</u>	<u>0.8 ± 0.2</u>

* Absolute cell counts (× 10^6) (±SD) in popliteal, paraaortic, and inguinal lymph nodes from three to five mice determined between days 4 and six after injection of 10^9 MMTV particles. Statistically significant increases are underlined.

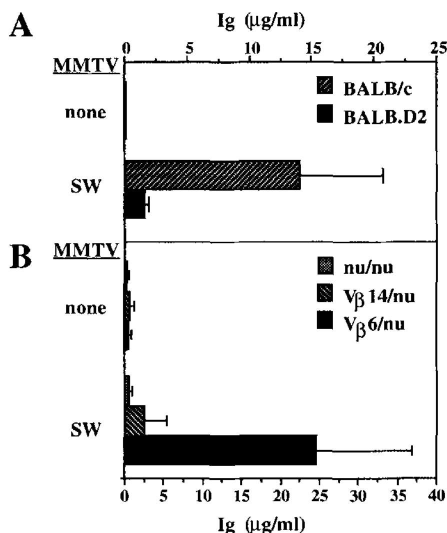

Figure 3. Ig secretion by MMTV(SW)-infected lymph node cells. Lymph node cells were isolated 6 d after infection with MMTV(SW), and 5 × 10^5 cells were cultured for 3 d in 200 µl of complete DMEM supplemented with 5% FCS without any further stimulation. Total Ig (IgG + IgM) levels in supernatants were subsequently determined using isotype-specific sandwich ELISAs as described in Materials and Methods. Supernatants were derived from: (*A*) infected and noninfected BALB/c or BALB.D2 mice; and (*B*) infected and noninfected BALB/c *nu/nu* (*nu/nu*) controls or BALB/c *nu/nu* mice reconstituted with either Vβ14$^+$ CD4$^+$ T cells (Vβ14/*nu*) or Vβ6$^+$ CD4$^+$ T cells (Vβ6/*nu*).

tion, which could be due to crosscontamination, although the FACS®-sorted cell preparations were >99% pure B or T cells upon reanalysis. Again endogenous Mtv ORFs are amplified equally well from the different DNA samples (Fig. 5 *B*).

Analysis of MMTV(SW) ORF mRNA Expression. To address the issue whether ORF mRNA is present during the MMTV(SW)-induced local immune response in vivo, we used a PCR primer within the 5′ noncoding region at the 5′ splicing

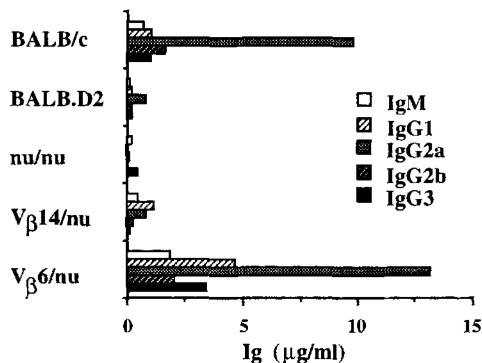

Figure 4. Isotype pattern of Ig production by MMTV(SW)-infected lymph node cells. Lymph node cells from MMTV(SW)-infected BALB/c, BALB.D2, BALB/c *nu/nu* (*nu/nu*), and Vβ14$^+$ CD4$^+$ (Vβ14/*nu*) or Vβ6$^+$ CD4$^+$ (Vβ6/*nu*) reconstituted nude mice were isolated and cultured as described above. Ig subclass distribution was determined using isotype-specific sandwich ELISAs as described in Materials and Methods.

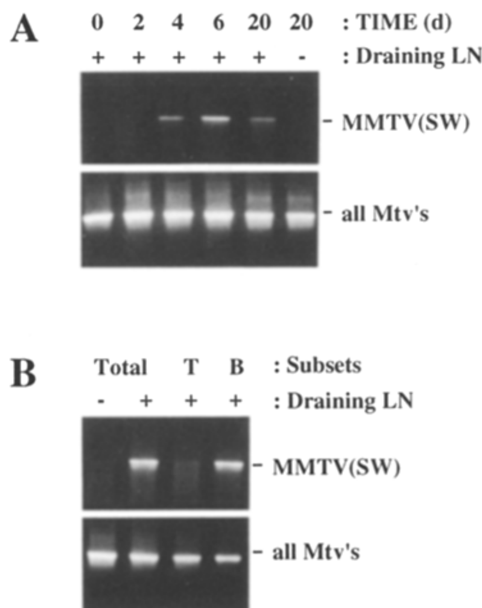

Figure 5. Analysis of MMTV(SW) infection by PCR. (*A*) Lymph node cells from draining (popliteal and paraaortic) or nondraining (axillary) lymph nodes (*LN*) were isolated at the indicated time points after injection of MMTV(SW) into the footpad of BALB/c mice. Lymphocyte DNA was isolated and subjected to PCR analysis specific for MMTV(SW) (and *Mtv-7*) ORF DNA yielding a 766-bp PCR product. As a control we amplified all Mtv ORFs present in BALB/c mice, including endogenous *Mtv*'s 6, 8, and 9, yielding a 898-bp PCR product. (*B*) At day 6 after infection lymph node cells were separated into B and T cells (both populations were >99% pure) using FACS®. Isolated DNA was then subjected to PCR specific for MMTV(SW) ORF DNA or all Mtv ORFs as described above.

site for subgenomic mRNA's (31) in combination with the MMTV(SW) (and *Mtv-7*) ORF-specific primer described above. This combination should allow a PCR amplification from spliced MMTV(SW) ORF mRNAs, but not from the unspliced MMTV(SW) genome or the integrated MMTV-(SW) DNA or the endogenous *Mtv-7*, since the two target sequences are separated by ~8 kb containing the *gag-pol-env* genes. As shown in Fig. 6 *A*, the ORF mRNA-specific primers indeed do not yield a detectable PCR product from BALB/c and BALB.D2 DNA or from the unspliced (genomic) MMTV-(SW) cDNA. The latter result demonstrates that the mature virion does not contain spliced ORF mRNA species. The control PCR amplification using primers located within the ORF molecule, however, gives the expected band at 0.7 kb from the *Mtv-7*$^+$ BALB.D2 DNA and from the unspliced MMTV(SW) cDNA (Fig. 6 *A*). To demonstrate MMTV(SW) ORF expression we analyzed cellular RNA isolated from lymph node cells after MMTV(SW) infection for the presence of spliced ORF mRNAs. As shown in Fig. 6 *B*, no MMTV(SW) ORF mRNA was amplified from noninfected BALB/c mice, whereas a signal was readily detected from RNA obtained from lymph node cells 6 d after infection. Upon separation by FACS® into T and B cells (both populations were >99.5% pure) ORF mRNA was almost exclusively amplified from the RNA obtained from the B cell popula-

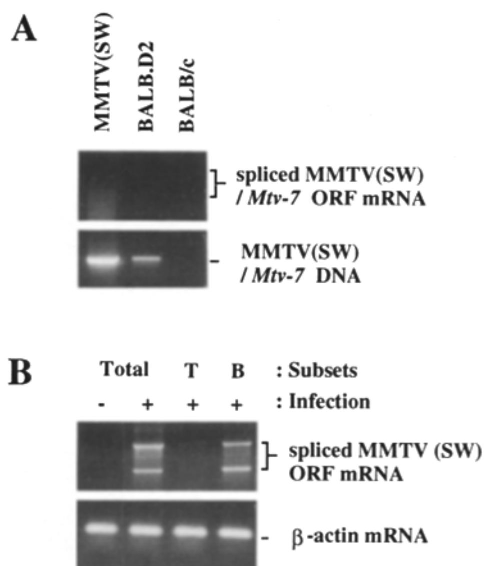

Figure 6. Analysis of MMTV(SW) ORF mRNA expression by PCR. (*A*) Specificity of the PCR for spliced ORF mRNA. A primer at the 5′ splicing site for subgenomic mRNAs together with the MMTV(SW) (and *Mtv-7*) ORF-specific primer described in Fig. 5 were tested on genomic DNA from BALB/c and BALB.D2 mice and on the cDNA derived from the MMTV(SW) genome in which the splice site and the ORF are separated by ~8 kb of gag-pol-env sequences. The quality of these preparations was confirmed by primers that amplify a 766-bp stretch within the MMTV(SW) or *Mtv-7* ORF, which do not require a splicing event. The quality of the BALB/c DNA was confirmed with primers that amplify all endogenous Mtv's (see Fig. 5 *B*). (*B*) Lymph node cells from draining (popliteal and paraaortic) lymph nodes were isolated at day 6 after injection of MMTV(SW) into the footpad of BALB/c mice. Lymph node cells were then separated into B and T cells using FACS® to a purity of >99.5%. Lymph nodes from noninfected BALB/c mice were used as a control. After RNA isolation, the prepared cDNA was subjected to PCR analysis specific for spliced MMTV(SW) ORF mRNA. The observed PCR products were 1.0 and 1.8 kb. The quality of the cDNAs was confirmed by control PCR specific for β-actin mRNA.

tion. ORF mRNA was detected as early as day 4 (not shown). PCR amplification with primers specific for β-actin mRNA demonstrated that all RNA preparations were of comparable quality.

Since the oligos used for amplification of ORF transcripts do not amplify the entire 3′ nor most of the 5′ untranslated region, the PCR product is ~0.65 kb shorter than the corresponding transcript. Thus, the 1.0-kb PCR product fitted the prediction for a putative ORF transcript that has been described as an mRNA of ~1.7 kb (33). Surprisingly, we observed a second PCR product of 1.8 kb (Fig. 6 *B*) that would correspond to an mRNA of ~2.5 kb. The specificity of both transcripts for MMTV(SW) is indicated by the fact that no such amplification products were obtained from uninfected BALB/c RNA and from DNA samples (Fig. 6, *A* and *B*). Preliminary sequence analysis indicates that the 1.0-kb PCR product corresponds to the putative ORF transcript (33). The longer transcript might be related to a 1.85-kb transcript found in EL-4 thymoma cells, taking into account a 491-bp deletion found in the 3′ LTR of the EL-4 proviral genome (34).

Discussion

Infection with MMTV occurs vertically after transmission from mother to offspring by the milk. Several lines of evidence suggest that the immune system and particularly T cells play a crucial role for MMTV infection and transport to the mammary gland. However, early MMTV infection events, including the role of the superantigen activity in viral infection and that of the different lymphocyte subsets as putative intermediate virus hosts, are still poorly understood.

We show here that B cells are the primary target for MMTV infection since ORF transcripts and the bulk of reverse-transcribed viral genome are present in B cells. Since we show that the mature virus particle does not contain ORF transcripts and it is believed that ORF protein is not present in the mature virion (35), it is likely that after infection, de novo ORF protein expression and presentation in the context of MHC class II antigens (36) by infected B cells leads to stimulation of ORF-reactive $CD4^+$ T cells. B cells are then stimulated by these activated $CD4^+$ T cells. This sequence of events is supported by the fact that cycling T cells are observed before cycling B cells in the local response. The hypothesis that MMTV(SW)-infected B cells present the ORF molecule to T cells, thereby inducing direct B-T cell interaction, is consistent with several lines of evidence. (*a*) $CD4^+$ T cells respond in vitro to ORF molecules from endogenous Mtv's presented by B cells in the context of MHC class II molecules. The interaction can be blocked by antibodies to MHC class II, CD4, or the TCR (for review see reference 37). (*b*) B cells are polyclonally inducible using Mls as the target antigen (13). Direct Th–B cell interaction was required for the initial B cell activation step, whereas activation of bystander B cells not expressing the Mls determinant has been shown by some (14) but not by others (13).

After MMTV infection the presence of ORF-reactive $CD4^+$ T cells was both necessary and sufficient to induce a response among B cells. This B cell stimulation may lead to an increased probability of reinfection and/or may indicate that a rare subset of infected B cells is amplified. This latter point is consistent with the data showing a low percentage of proliferating B cells. In any case an activated B cell should provide an appropriate milieu to transcribe the MMTV genome, as has been demonstrated for germline-transmitted, endogenous Mtv's (38).

After accumulation in the local lymph nodes some B cells undergo differentiation and secrete antibodies. The Ig subclass distribution in culture supernatants of lymphocytes from infected animals implies a T cell– and ORF-dependent Ig production. This is supported by the finding that infected BALB/c *nu/nu* mice do not produce significant amounts of IgG and that efficient IgG production in nude mice can be reconstituted by MMTV ORF-reactive Th cells. A predominant IgG2a secretion by infected BALB/c lymph node cells suggests that IFN-γ-producing Th type I cells (39) are induced upon MMTV infection. In fact IFN-γ production has been demonstrated in Mls-specific Th cell clones (40, 41) and in secondary in vitro Mls responses (H. R. MacDonald, unpublished results). In this respect it is interesting to note that

the transiently elevated serum IgG2a levels found after neonatal injection of Mls-1ª-bearing B cells could be reversed by anti-IFN-γ antibodies (17).

The IgG2a subclass of antibodies has generally been found as specific (antiviral) as well as nonspecific (polyclonal) antibodies elicited in mice by viral infections (42). After MMTV(SW) infection anti-MMTV antibodies were found at low but comparable titers in supernatants of both infected BALB/c and BALB.D2 mice (not shown), indicating that the viral superantigen probably does not play a role in the MMTV-specific antibody response. However, the massive IgG2a response of BALB/c or Vβ6$^+$ CD4$^+$ reconstituted nude mice is dependent on the viral superantigen since BALB.D2, nude, or Vβ14$^+$ CD4$^+$ reconstituted nude mice secrete only low amounts of IgG2a antibodies. Thus, polyclonal IgG2a production depends on the presence of both the superantigen and CD4$^+$ T cells reactive with it.

Recently Golovkina et al. (12) showed that deletion of superantigen-reactive T cells induced by transgenic expression of a superantigen led to reduced transport of the corresponding exogenous MMTV to the mammary gland. These data have been interpreted by others to mean that superantigen-reactive T cells are the primary target for MMTV infection (43). However, our experiments show that B cells are the primary target of infection and that the absence of superantigen-reactive T cells does not allow efficient stimulation of presumably infected B cells. Similarly, earlier data published by Tsubura et al. (11) indicate that T cells but not B cells from infected mice can transfer MMTV infection to nude mice. Again our experiments suggest that the absence of T cells in nude mice does not allow efficient stimulation of infected B cells. Thus, infected B cells alone may not be able to transfer MMTV to the mammary gland in nude mice. With regard to the latter study it should be noted that we assayed for T cell infection very early (6 d) after local infection. However, Tsubura et al. (11) reported MMTV transport to the mammary gland of nude mice after transfer of T cells isolated 6–7 mo after infection. Infection of T cells may therefore be a late event in MMTV transmission.

Continuous deletion of ORF-reactive CD4$^+$ T cells in infected mice (9) may reflect an ongoing T cell stimulation due to persistence of MMTV(SW)-infected B cells. Thus, MMTV ORF-induced, T cell–dependent B cell stimulation would assure the maintenance and possibly the amplification of the virus and increase the probability of infecting the mammary gland. The absence of superantigen-reactive T cells may deprive the intermediate virus host and thus the virus from this amplification step and therefore lead to an inefficient infection.

We thank Pierre Zaech and Christian Knabenhans for FACS® analysis and Dr. Ursula Bommhardt for the β-actin primers. We are grateful for the helpful discussions with Drs. Heidi Diggelmann and Ed Palmer.

This work was partially supported by grants from the Swiss National Science Foundation to S. Izui (31-28782.90) and to H. Acha-Orbea (31-32271.92). H. Acha-Orbea is a recipient of a START fellowship from the Swiss National Science Foundation.

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Received for publication 24 July 1992 and in revised form 16 October 1992.

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