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Retrovirus-Induced Target Cell Activation in the Early Phases of Infection: the Mouse Mammary Tumor Virus Model

CARLOS ARDAVIN,[1] FRÉDÉRIC LUTHI,[2] MIKAEL ANDERSSON,[3] LEO SCARPELLINO,[3] PILAR MARTIN,[1] HEIDI DIGGELMANN,[2] AND HANS ACHA-ORBEA[3,4]*

Department of Cell Biology, Faculty of Biology, Complutense University, 28040 Madrid, Spain,[1] and Institute of Microbiology, University Hospital, University of Lausanne, 1011 Lausanne,[2] and Ludwig Institute for Cancer Research, Lausanne Branch,[3] and Institute for Biochemistry,[4] University of Lausanne, 1066 Epalinges, Switzerland

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Mouse mammary tumor virus (MMTV) infects B lymphocytes and expresses a superantigen on the cell surface after integration of its reverse-transcribed genome. Superantigen-dependent B- and T-cell activation becomes detectable 2 to 3 days after infection. We show here that before this event, B cells undergo a polyclonal activation which does not involve massive proliferation. This first phase of B-cell activation is T cell independent. Moreover, during the first phase of activation, when only a small fraction of B cells is infected by MMTV(SW), viral DNA is detected only in activated B cells. Such a B-cell activation is also seen after injection of murine leukemia virus but not after injection of vaccinia virus, despite the very similar kinetics and intensity of the immune response. Since retroviruses require activated target cells to induce efficient infection, these data suggest that the early polyclonal retrovirus-induced target cell activation might play an important role in the establishment of retroviral infections.

Mouse mammary tumor virus (MMTV) is a type B retrovirus responsible for the majority of mammary carcinomas appearing in mice in the first year of life as a result of insertional activation of host proto-oncogenes (5, 19, 20).

MMTV infects B cells initially (11, 15). After integration into the B-cell genome, MMTV expresses its superantigen (Sag) which is required to amplify the infected cells and to induce their differentiation by promoting a strong collaboration between T and B cells (1, 2, 7, 11, 15). Sags activate a very large proportion of T cells by cross-linking major histocompatibility complex class II molecules of the Sag-presenting B cells with T-cell receptor (TCR) Vβ elements. In contrast to classical antigen responses, in Sag responses, other polymorphic parts of the TCR are less important, allowing up to 30% of T cells to participate in this collaboration (1, 25, 30).

Infection with most retroviruses requires proliferation of target cells. The only exception, human immunodeficiency virus (HIV), can infect resting macrophages due to the karyophilic properties of its Gag protein. For T-lymphocyte infection, however, HIV requires activation of the T cells for infection to occur (6, 9, 29).

Based on these observations, there are two obvious possible ways for MMTV to infect its target cells, the B lymphocytes: (i) B cells preactivated at the time of infection are the targets of infection, or (ii) MMTV activates its target cells upon infection.

In order to get insights into the mechanism of infection by MMTV(SW), we have analyzed the early immune response after injection of MMTV(SW) in BALB/c mice.

Here we show that, in contrast to vaccinia virus or to classical antigens, MMTV can activate B cells polyclonally and transiently during the first few hours after virus injection. In contrast to the T-cell-dependent B-cell activation and proliferation response starting 2 days after MMTV(SW) infection, this first wave of B-cell activation is T cell independent. We also describe a similar activation of B cells after injection of another retrovirus, murine leukemia virus (MuLV).

We propose that the initial T-cell-independent B-cell activation significantly increases the efficiency of infection by MMTV and MuLV. Similar strategies may be used by other retroviruses to infect their respective target cells.

MATERIALS AND METHODS

Mice. BALB/c (*H-2*^{d}), BALB/c *nu/nu* (*H-2*^{d}), and B10.G (*H-2*^{q}) mice were purchased from Harlan Olac Ltd. (Bicester, England). *Mtv-7* congenic BALB.D2 mice were bred in our facility. In all experiments, 6- to 10-week-old female mice were used. MMTV(SW)-infected mice were originally purchased from IFFA-Credo (L'Arlabesle, France) and maintained as a breeding colony (12).

MAbs. The following monoclonal antibodies (MAbs) were used in this study: H1-2F3 (31) (anti-CD69, biotin conjugated), GL1 (anti-B7-2, biotin conjugated) (Pharmingen, San Diego, Calif.), H129.19 (anti-CD4, phycoerythrin conjugated) (Boehringer, Mannheim, Germany), 53-6.7 (anti-CD8, phycoerythrin conjugated) (Boehringer), 44-22.1 (3, 21) (anti-Vβ6, fluorescein conjugated), and 14.2 (17) (anti-Vβ14, fluorescein conjugated).

Virus isolation and in vivo treatment. MMTV(SW) was purified from milk as described previously (12). Virus was removed from MMTV(SW)-infected milk by collecting the supernatant after centrifugation at 10,000 × *g* for 1 h. Wild-type vaccinia virus was grown, and the titers of the virus were determined as described previously (16). Mice were given a 10-μl injection in the hind footpad with MMTV(SW) (10^9 virus particles, virus titers determined as described (12). Alternatively, mice were injected with vaccinia virus (2×10^6 PFU or 10 μl/footpad), MuLV (5×10^4 PFU), or lipopolysaccharide (LPS) from *Escherichia coli* (20 μg/footpad) (Sigma, San Diego, Calif.). At the indicated times, the draining popliteal lymph nodes were removed and single-cell suspensions were prepared.

Flow cytometry. Lymph node cells (10^6) were stained with biotinylated anti-CD69 or anti-CD86 (B7-2), followed by streptavidin-tricolor (Caltag, San Francisco, Calif.), phycoerythrin-conjugated anti-CD4, and anti-CD8 MAbs and fluorescein-conjugated anti-Vβ6 or anti-Vβ14 MAbs. $CD4^+$ and $CD8^+$ cells were separated in the FL2 channel by using appropriate dilutions of the two MAbs. Analysis was performed on a FACScan (Becton Dickinson & Co., Mountain View, Calif.) cell analyzer with Lysis II software for data evaluation. Dead cells were excluded based on their forward and side scatter characteristics.

$CD69^+$ and $CD69^-$ B cells from popliteal lymph nodes were sorted at different times after MMTV(SW) injection on a FACStar Plus (Becton Dickinson & Co.) flow cytometer after double staining with biotinylated anti-CD69, followed by streptavidin-phycoerythrin (Caltag) and fluorescein-conjugated anti-B220 (Caltag). After reanalysis, the sorted cell populations had a purity of >98% (data not shown).

* Corresponding author. Mailing address: Ludwig Institute for Cancer Research, Lausanne Branch, University of Lausanne, 1066 Epalinges, Switzerland. Phone: 21 692 57 10. Fax: 21 653 44 74. E-mail: hacha@eliot.unil.ch.

Cell cycle. Cell cycle stages were determined by staining cell nucleus DNA with propidium iodide (50 μg/ml), 0.1% Triton X-100, and 0.1% sodium citrate as described previously (18).

PCR. DNA from 50,000 cells (determined by fluorescence-activated cell sorting) was amplified with the 5′ oligonucleotide MS10 (AGGTGGGTCACAAT CAACGGC) which reacts with all the open reading frame (ORF) molecules and with the MMTV(SW) and Mtv-7-specific oligonucleotide VJ83 (GCGACCCCC ATGAGTATATTTC) complementary to long terminal repeat positions 705 to 725).

The conditions for the PCR were as follows: one cycle consisting of 5 min at 94°C, 1 min at 60°C, and 1 min at 72°C; 30 cycles, with 1 cycle consisting of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C; and finally, an extension step for 10 min at 72°C in 1× PCR buffer containing 20 mM Tris-HCl (pH 8.55), 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 150 μg of bovine serum albumin per ml, 0.2 mM each of the four deoxynucleoside triphosphates; 2.5 U of *Taq* polymerase (Biotaq; Bioprobe Systems) and each oligonucleotide (25 μM) were added to the PCR mixture.

The specific product was detected by liquid hybridization as described previously (16) with 20% of the PCR mixture being hybridized in 150 mM NaCl–2.5 mM EDTA with 50 fmol of a ^{32}P-labelled internal probe common to various ORF sequences, MS11 (CAAGGAGGTCTAGCTCTGGCG). The conditions for denaturation and hybridization were 5 min at 98°C, 15 min at 66°C, and a rapid cooling to 4°C. The reaction products were separated by size on a 2% agarose gel, dried on DE81 paper (Whatman), and autoradiographed at −70°C overnight with an intensifying screen.

PCR products were separated on a 2% agarose gel, and the dried gel was autoradiographed on Kodak X-OMAT films (Eastman Kodak Company, Rochester, N.Y.).

RESULTS

MMTV(SW)-induced B-cell activation. CD86 (B7-2) and CD69 are very early activation markers which appear on the surfaces of lymphocytes within minutes of activation. At different times after MMTV(SW) injection, the draining lymph nodes were removed and the expression of these early activation markers on the different lymphocyte subsets was analyzed. As shown in Fig. 1a, the majority of B cells expressed these two markers 18 h after virus injection, and the percentage of cells expressing these activation markers was dose dependent (data not shown). Peak expression was reached at 18 h, and the percentages of activated B cells returned to control levels between 21 and 29 h. During this first phase of activation, neither the MMTV(SW) Sag-reactive $V\beta6^+CD4^+$ nor $V\beta6^+CD8^+$ T cells showed any significant up regulation of CD69, and no increase in proliferating cells was detected as assessed by cell cycle analysis (Fig. 1b). By this method we routinely observed 3 to 4% of lymph node cells in cycle in control lymph nodes and peripheral blood lymphocytes and could not detect an increase in the percentage of cells in cycle during the first 2 days after virus injection. Similar results were obtained with bromodeoxyuridine (BrdU) incorporation (data not shown). These results do not exclude the possibility that a small fraction of the CD69-expressing B cells enters the cell cycle after interaction with the virus. Alternatively, preactivated B cells might be the major target of MMTV infection (see Discussion). After 65 h, a second wave of expression of CD86 (B7-2) and CD69 became apparent, which was paralleled by an increase in CD69 expression by the Sag-reactive $V\beta6^+CD4^+$ T cells. Neither $CD4^+$ T cells expressing other TCR Vβs nor $CD8^+$ T cells showed a significant activation. In contrast to the first wave of B-cell activation, this second wave of B-cell and Sag-reactive T-cell activation was accompanied by a dramatic increase in cycling cells (Fig. 1b).

To ensure that this early B-cell activation was not due to a MMTV(SW)-independent effect of milk, we tested milk preparations from noninfected BALB/c mice [MMTV(SW)-free BALB/c milk] and supernatants of MMTV(SW) milk after removing the virus by centrifugation [supernatant of MMTV(SW)-infected BALB/c milk]. None of these control milk preparations induced a significant increase in the percentage of $CD69^+$ B cells or TCR $V\beta6^+CD4^+$ T cells (Table 1).

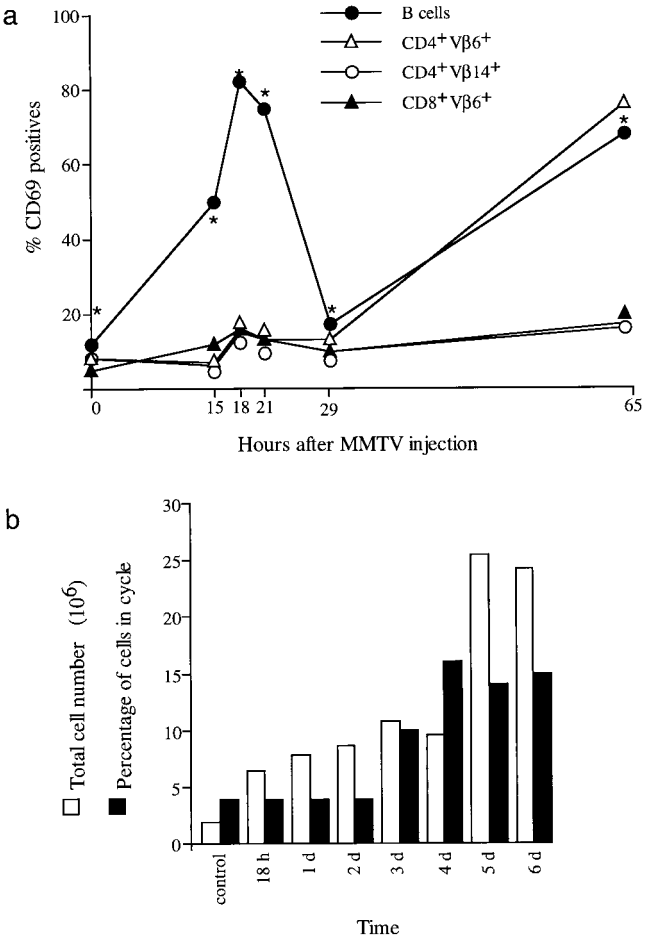

FIG. 1. (a) Kinetics of B- and T-cell activation in the draining popliteal lymph nodes after injection of MMTV(SW) in the footpads of BALB/c mice. The first phase of B-cell activation did not result in a CD69 up regulation in T cells, whereas the second wave of B-cell activation was paralleled by activation of Sag-reactive $CD4^+V\beta6^+$ T cells. The percentage of $CD69^+$ cells is shown at different times for B cells, $CD4^+V\beta6^+$ T cells, $CD4^+V\beta14^+$ T cells, and $CD8^+V\beta6^+$ T cells. The percentage of $B7.2^+$ B cells is indicated (asterisks). Each symbol represents the mean of the results corresponding to two lymph nodes. This experiment was performed four times with similar results. (b) Analysis of the total cell number and percentage of cells in cell cycle in the draining lymph node after infection by MMTV(SW). d, days.

Based on these results, we conclude that a fast and transient B-cell activation was induced by MMTV(SW), which was not accompanied by activation of T cells. Sag-reactive T cells only became activated 24 to 48 h after virus injection as a result of Sag presentation by the infected B cells in agreement with our previous data (15).

T-cell independence of early MMTV(SW)-induced B-cell activation. In order to determine whether this first wave of B-cell activation was T cell dependent, we injected T-cell-deficient BALB/c *nu/nu* mice and B10.G ($H\text{-}2^q$) mice with MMTV(SW). It has been shown previously that mice expressing the $H\text{-}2^q$ major histocompatibility complex haplotype are unable to present MMTV(SW) Sags (14). As shown in Fig. 2, both types of mice exhibited the first wave of B-cell activation, like BALB/c mice, whereas the second wave of activation was absent.

TABLE 1. Virus-dependent immune response after injection of MMTV(SW)-infected BALB/c milk and control preparations[a]

Injection	% CD69+ B cells		% Vβ6+ CD4, day 5
	Day 1	Day 5	
PBS	6.3	ND	13.5
MMTV-free BALB/c milk	7.2	3.8	13.0
MMTV(SW)-infected BALB/c milk	**70.4**	**22.9**	**34.9**
Supernatant of MMTV(SW)-infected BALB/c milk	7.4	3.8	13.7

[a] BALB/c mice were injected with phosphate-buffered saline (PBS), milk preparations from infected BALB/c mice [MMTV(SW)-infected BALB/c milk] and noninfected BALB/c mice [MMTV(SW)-free BALB/c milk], or supernatant of milk containing MMTV(SW) after the virus was removed by centrifugation [supernatant of MMTV(SW)-infected BALB/c milk]. The values for the virus-dependent immune response after injection of MMTV(SW)-infected BALB/c milk are shown in boldface type; the other preparations injected are control preparations. Early (day 1) and late (day 5) B-cell activation was evaluated by expression of CD69; stimulation of Sag-reactive T cells was estimated by determining the mean percentage of $CD4^+V\beta6^+$ T cells at day 5. Injection of MMTV(SW)-free BALB/c milk or supernatant of MMTV(SW)-infected BALB/c milk had no effect on B- or T-cell stimulation, while MMTV(SW)-infected milk induced strong B-cell activation at day 1 and stimulation of B cells and Sag-reactive T cells at day 5.

These results clearly show that the first wave of B-cell activation is T cell independent.

Preferentially infected B cells which were activated by MMTV(SW). MMTV(SW)-infected cells carrying the retroviral cDNA can be detected by PCR. In order to assess whether there was a correlation between early B-cell activation and infection, $CD69^+$ and $CD69^-$ B cells were sorted from infected BALB/c mice by flow cytometry 18 to 30 h after high (10^9 particles) or low (10^8 particles) doses of MMTV(SW). At these time points, 20 to 60% of B cells expressed CD69. As shown in Fig. 3, retroviral DNA could be detected in the $CD69^+$ B cells but not in the nonactivated $CD69^-$ B cells 18 h after injection at the higher virus dose only. After 30 h, both

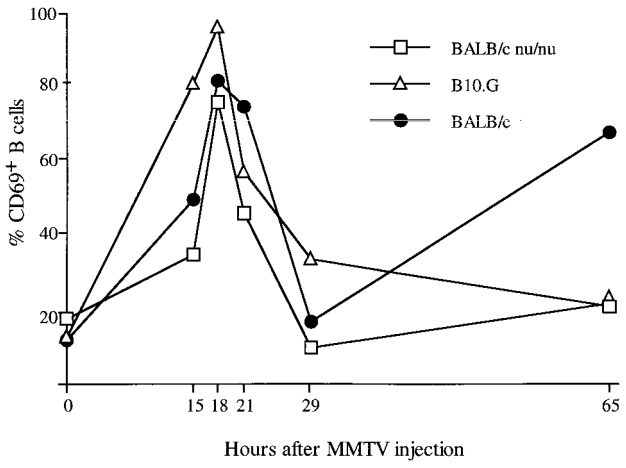

FIG. 2. T-cell independence of initial B-cell activation. The kinetics of B-cell activation was measured by analyzing expression of CD69 after MMTV(SW) injection in BALB/c, BALB/c *nu/nu*, and B10.G (*H-2*^{q}) mice. Whereas the early B-cell activation is T cell independent, the second phase of activation depends on T-cell help and therefore is absent in T-cell-deficient mice (BALB/c *nu/nu*) and in mice unable to present MMTV(SW) Sags (B10.G). Each symbol represents the mean of the results corresponding to two lymph nodes. This experiment was performed four times with similar results.

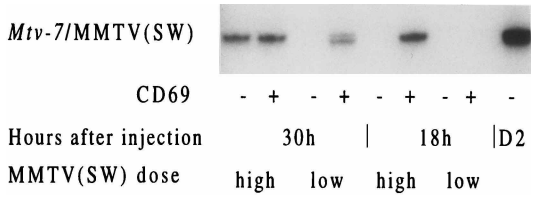

FIG. 3. Preferential infection of activated B cells. $CD69^+$ and $CD69^-$ B cells from popliteal lymph nodes of BALB/c mice were sorted by flow cytometry 18 or 30 h after MMTV(SW) injection. DNA from 30,000 cells was amplified with MMTV(SW)-specific oligonucleotides. The high MMTV(SW) dose was 10^9 virus particles, and the low dose was 10^8 virus particles. DNA obtained from BALB.D2 splenocytes (indicated as D2) containing two copies of *Mtv-7* per cell was used as a positive control. The PCR products of MMTV(SW) and *Mtv-7* migrate to the same position.

virus doses induced detectable infection in the $CD69^+$ population. The detection of MMTV in $CD69^-$ B cells 30 h after high-dose virus injection is most likely due to down regulation of CD69 after 20 to 30 h in the infected B cells. Lower doses lead to a slightly slower kinetics of appearance and disappearance of CD69 expression on B cells and explains the different result obtained with lower doses. Estimations based on standard curves indicated that only a very small proportion of activated B cells was infected. Similar results were obtained for BALB/c *nu/nu* and B10.G mice (data not shown).

Lymphocyte activation after injection of vaccinia virus, LPS, or MuLV. In order to determine whether similar early B-cell activation occurs after injections of mitogens or other viruses, we injected mice with another murine retrovirus (MuLV) or alternatively with either vaccinia virus or the polyclonal B-cell activator LPS. The retrovirus MuLV exhibited the first phase of polyclonal B-cell activation with kinetics similar to MMTV, but activation levels of B cells were high throughout the observation period (Fig. 4). We never observed a strong T-cell activation (Fig. 4). Injection of vaccinia virus led to lymph node size increases with kinetics and cell numbers similar to those of MMTV(SW) (data not shown) and induced a polyclonal B-cell activation. However, vaccinia virus induced a much slower up regulation of CD69 in B cells than MMTV(SW) which was comparable to the second, T-dependent activation phase observed with MMTV(SW). Only after 48 h did a significant percentage of B express CD69, and this B-cell activation was accompanied by an increase in CD69 expression in T cells. On the other hand, LPS showed a very fast and simultaneous induction and down regulation of activation markers on B cells and $CD4^+$ as well as $CD8^+$ T cells.

Increase of cell number after MMTV(SW) injection: attraction and retention versus cell division. Analysis of the percentage of B cells which express CD69 and the absolute number of B cells in the draining lymph node during the first hours after MMTV(SW) injection is shown in Fig. 5. By 6 h after injection, a large proportion of B cells expressed this activation marker and the absolute B-cell number had increased three- to fivefold over that of the noninjected mice. This initial increase of the number of B cells cannot be explained by cell division exclusively, as the shortest reported B-cell cycling time is 6 h and no massive cell proliferation was detectable at this time point. No detectable increase in the percentage of BrdU-positive cells was detected after a 12-h BrdU pulse in vivo (data not shown). The increase in B-cell numbers after virus injection is most likely due to recruitment and/or retention of B cells in the draining lymph node. This is further supported by the finding that during the first 24 h, no increase in the percentage of

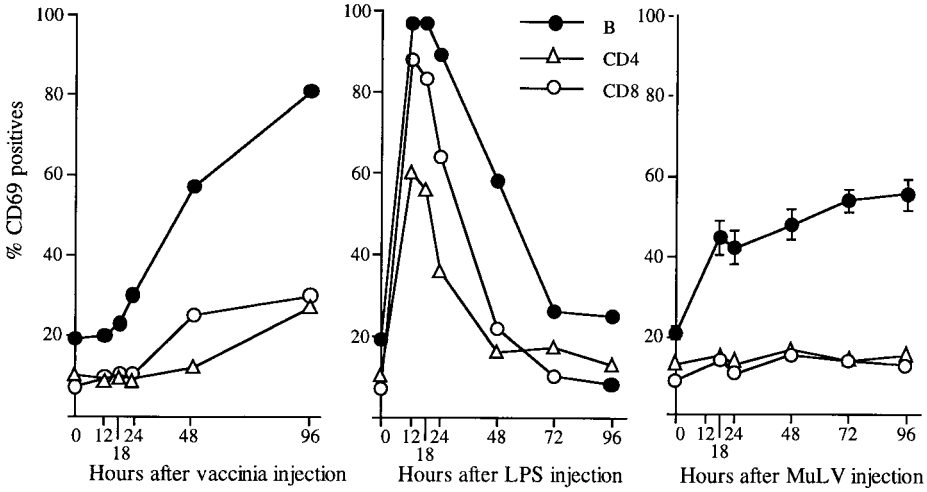

FIG. 4. Kinetics of B- and T-cell activation in the draining popliteal lymph node after injection of vaccinia virus or LPS or MuLV in the footpads of BALB/c mice. The percentage of $CD69^+$ cells at different times for B cells, $CD4^+$ T cells, and $CD8^+$ T cells is shown. Each symbol represents the mean of the results corresponding to two lymph nodes except for the experiment with MuLV for which the mean and standard deviation of triplicate measurements are shown. This experiment was performed three times with similar results.

proliferating cells was detected by cell cycle analysis as already shown (Fig. 1b).

DISCUSSION

Injection of MMTV(SW) in BALB/c mice induces a biphasic B-cell activation. We show here that within the first 24 h after injection, B cells undergo a T-cell-independent, transient activation as measured by up regulation of activation markers CD69 and CD86 (B7-2). Surface expression of CD69 on B cells reached peak levels by 18 h after MMTV(SW) injection and declined after 24 h. Similar kinetics of down regulation of CD69 after polyclonal T-cell activation were previously described (reviewed in reference 26). Both, down regulation of CD69 after activation and cell death of partially activated B cells may contribute to the decrease in the percentage of CD69-positive cells. A second phase of B-cell activation starting after 30 to 48 h after injection of MMTV(SW) is accompanied by the activation of MMTV(SW) Sag-reactive $V\beta6^+$ T cells and by the active cell proliferation of both the B-cell and $V\beta6^+$ T-cell compartments. In fact, this second B-cell response is dependent on Sag-reactive T cells and involves the presentation of MMTV(SW)-encoded Sags on the surfaces of the MMTV(SW)-infected B cells to the Sag-specific T-helper cells. This Sag-driven B-cell activation and proliferation, which greatly increases the number of MMTV(SW)-infected cells, has been demonstrated to be required for an efficient virus infection (10, 15).

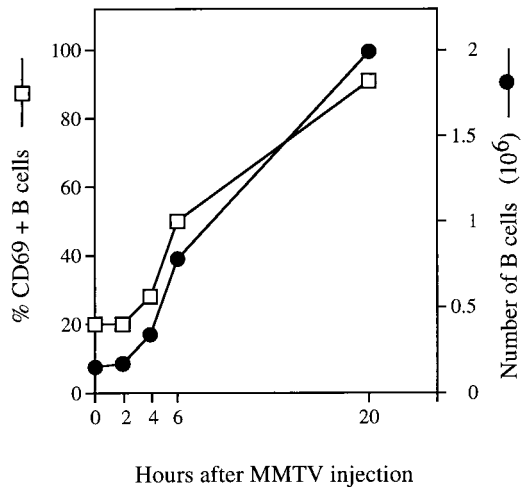

FIG. 5. B-cell response during the first hours after MMTV(SW) injection in BALB/c mice. The percentage of $CD69^+$ B cells and the absolute number of B cells in the draining popliteal lymph nodes are represented. Each symbol represents the mean of the results corresponding to two lymph nodes. This experiment was performed three times with similar results.

The first wave of polyclonal B-cell activation was not paralleled by an increase in the percentage of proliferating B cells and/or T-cell activation. There is precedence to such direct B-cell activation by viral surface proteins, as T-cell-independent activation of virus-specific B cells was observed after injection of vesicular stomatitis virus particles, which probably can cross-link virus-specific immunoglobulins on the B-cell surface (4). Alternatively, polyclonal B-cell activation can result from MMTV entry into the target cells. This is, however unlikely, as in this case a large proportion of the activated cells should contain viral DNA, and we have shown that whereas 50% of the B cells were activated 18 h after MMTV injection, only a very small fraction of the activated cells had detectable MMTV DNA. In fact, previous studies have shown that only about 1 in 10,000 B cells in the draining lymph node had detectable viral DNA 24 h after virus injection despite the presence of up to 80% activated B cells (13, 15).

One possible explanation for the low frequency of infected cells is that only cells already in cycle before contact with the virus can be infected. However, poor infection of mice having high numbers of activated B cells or B cells in cycle argue against this requirement (8a).

It has been clearly established that most retroviruses require the host cell to be in cycle for integration of viral cDNA to occur (23, 28). Infection by HIV is independent of host cell proliferation due to the karyophilic properties of the viral preintegration complex but nevertheless requires T-lymphocyte activation for infection to occur (6, 9). Interestingly, the

second murine retrovirus tested, MuLV, behaved very similarly to MMTV in the first phase of polyclonal B-cell activation.

The first transient B-cell activation phase observed after MMTV injection does not occur during the infection by vaccinia virus (as shown in this study) or other viruses which have been demonstrated to display a B-cell superstimulatory mitogenic potential. Vaccinia virus infections induce strong cytokine responses, and it therefore seems unlikely that the observed effects are due to direct cytokine effects. Instead, murine cytomegalovirus, influenza A virus, and herpesvirus exhibit a B-cell activation pattern similar to the second activation phase induced by MMTV, i.e., involving cell proliferation and, in some cases, T-cell help (8, 22, 24). Besides, it has been reported that infection with Epstein-Barr virus correlates with the up regulation of the B-cell activation marker CD23 during the 24 h after infection before the onset of B-cell proliferation (27). Therefore, efficiency of some virus infections could rely on the induction of a fast B-cell activation process during the early phases of infection.

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