In Vivo Effects of a Recombinant Vaccinia Virus Expressing a Mouse Mammary Tumor Virus Superantigen

CLAUDE KRUMMENACHER,¹ HEIDI DIGGELMANN,¹ AND HANS ACHA-ORBEA^{2*}

Institute for Microbiology, University of Lausanne, 1011 Lausanne,¹ and Ludwig Institute for Cancer Research, Lausanne Branch, and Institute for Biochemistry, University of Lausanne, 1066 Epalinges,² Switzerland

Received 2 October 1995/Accepted 13 February 1996

Early after infection, the mouse mammary tumor virus (MMTV) expresses a superantigen (SAg) at the surface of B lymphocytes. Interaction with the T-cell receptor V β domain induces a polyclonal proliferative response of the SAg-reactive T cells. Stimulated T cells become anergic and are deleted from the T-cell repertoire. We have used a recombinant vaccinia virus encoding the MMTV(GR) SAg to dissect the effects of the retroviral SAg during an unrelated viral infection. Subcutaneous infection with this recombinant vaccinia virus induces a very rapid increase of V β 14 T cells in the draining lymph node. This stimulation does not require a large number of infectious particles and is not strictly dependent on the expression of the major histocompatibility complex class II I-E molecule, as it is required after MMTV(GR) infection. In contrast to MMTV infection during which B cells are infected, we do not observe any clonal deletion of the reactive T cells following the initial stimulation phase. Our data show that contrary to the case with MMTV, macrophages but not B cells are the targets of infection by vaccinia virus in the lymph node, indicating the ability of these cells to present a retroviral SAg. The altered SAg expression in a different target cell observed during recombinant vaccinia virus infection therefore results in significant changes in the SAg response.

Mouse mammary tumor virus (MMTV) is a type B retrovirus that is transmitted as an infectious milk-borne particle (8) and that causes mammary carcinomas by insertional activation of cellular proto-oncogenes (40). Germ line infections result in endogenous Mtv proviruses integrated in the genome of most mouse strains (29). The open reading frame present in the 3' long terminal repeat (LTR) of the provirus encodes a superantigen (SAg) (2, 13) which is active when it is expressed in B cells (for a review, see reference 1). An SAg is defined by its ability to stimulate a large proportion of T cells sharing a common T-cell receptor β chain variable domain (V β) (25, 46).

The *orf* gene located in the 3' LTR of the viral genome shows high sequence homology among infectious MMTV strains and endogenous Mtv loci. Contrary to the body of the protein, the last 30 amino acids at the COOH terminus are highly polymorphic. This variation correlates with the V β region specifically affected by a given SAg (1, 11). Typically, the MMTV(GR) SAg reacts with T cells bearing the T-cell receptor V β 14 segment.

Biochemical studies of the SAg protein in vitro (27) and in tissue culture (9, 10, 31) revealed the synthesis of a 47-kDa glycosylated transmembrane protein. An 18-kDa cleavage product was detected on the surface of SAg-expressing cells (47, 48). Although this product contains the hypervariable C-terminal sequence determining V β specificity (11, 49; for a review, see reference 1), the expression of this 18-kDa fragment alone is not sufficient to induce a SAg response either in vitro (14) or in transgenic mice (32).

Upon MMTV infection, B lymphocytes express low amounts of the viral SAg at their surface in the context of major histocompatibility complex (MHC) class II molecules. Presentation of the SAg by B cells leads to stimulation and proliferation of T lymphocytes bearing a defined T-cell receptor V β chain.

After expansion, the specific T cells are in a state of unresponsiveness, referred to as anergy, during which they are unable to respond to antigen or SAg stimulation. These cells are then eliminated from the T-cell repertoire by clonal deletion. During the stimulation phase, the infected B cells are activated by SAg-reactive T cells. The activation, differentiation, and expansion of the infected B cells are key steps during the life cycle of MMTV, and the presentation of a functional SAg is absolutely required to establish a productive infection (24).

Some of the MMTV SAGs have been shown to require MHC class II I-E expression for presentation to occur, whereas others can be presented in the context of I-A molecules. MMTV(GR), which is the infectious form of the endogenous locus *Mtv-2* (43), encodes one of these I-E-dependent SAGs (2, 20). Contrary to infection with other MMTVs, infection of I-E-expressing mice with MMTV(GR) or MMTV(C3H) produces a barely detectable stimulation of V β 14⁺ T cells followed by a slow deletion of these reactive cells (22, 36).

In vivo, vaccinia virus was successfully used to express heterologous antigens to generate both humoral and cytotoxic immune responses allowing protection against challenge infections (for a review, see reference 38). We produced recombinant vaccinia viruses expressing either the complete MMTV(GR) SAg or its COOH-terminal half (31). The first recombinant vaccinia virus, called vOGR1, expressed the full-length 47-kDa glycoprotein, whereas the second virus (vOGR5) produced an 18-kDa unglycosylated polypeptide in cultured cells (31). Both of these recombinant vaccinia viruses were tested for their capacity to induce an SAg response in vivo. Vaccinia virus and MMTV are completely different viruses, both in their structures and in their replication and infection cycles. As a retrovirus, MMTV generates a lifelong infection of mice, whereas vaccinia virus, which is not a natural mouse pathogen, replicates only poorly in mice. Viral replication occurs mainly at the site of subcutaneous infection; after a short primary viremia, the vaccinia virus is rapidly cleared from the blood circulation in normal mice (19). We report here that the MMTV(GR) SAg presented in vivo in a different infec-

* Corresponding author. Mailing address: Ludwig Institute for Cancer Research, Ch. des Boveresses 155, CH-1066 Epalinges, Switzerland. Phone: 41 21 692 57 23. Fax: 41 21 692 57 05.

tious context induces a response both quantitatively and qualitatively different from the one observed after MMTV infection.

MATERIALS AND METHODS

Mice. BALB/c and C57BL/6 mice were purchased from HO Harlan OLAC (Bicester, England). In all experiments, female mice at ages of 6 to 10 weeks were used.

Antibodies. The following phycoerythrin-labeled monoclonal antibodies were used in this study: anti-CD4 (GK1.5; Becton Dickinson), anti-Ly-2/CD8 (Boehringer Mannheim), and anti-mouse immunoglobulin G (IgG) (Boehringer Mannheim). 44.22.1 directly labeled with fluorescein isothiocyanate (FITC) (rat IgG anti-V β 6) (3), 14.2 (rat IgM anti-V β 14) (33), and polyclonal rabbit F(ab')₂ anti-mouse Ig have been made in the laboratory. In cell-sorting experiments, phycoerythrin-labeled anti-mouse Ig (Boehringer Mannheim) and biotinylated anti-Mac-1 (42) and F4/80 (6) were used with streptavidin-FITC conjugate (Southern Biotechnologies).

Viruses and virus preparation. The generation of recombinant vaccinia viruses vOGR1, containing the entire GR *orf* sequence, and vOGR5, containing the C-terminal half of the GR ORF protein beginning at the fifth in-frame ATG, has been described (31). CV1 cells (African green monkey kidney cells) infected with the recombinant vaccinia virus were Dounce homogenized in cold 10 mM Tris-HCl, pH 9.0; cell fragments were eliminated by centrifugation for 10 min at 4°C and 750 $\times$ g. The supernatant is a crude stock of virus conserved at -70°C. The virus titer was determined on CV1 cells and corresponded to 2×10^8 PFU/ml for both recombinants.

Injection and sampling. Crude virus preparation corresponding to 5×10^6 PFU (25 μ l) was injected subcutaneously into the hind footpad of naive BALB/c mice; for in vivo titration, virus was serially diluted in phosphate-buffered saline (PBS) so that 25 μ l was injected each time. At different time points after infection, the draining and nondraining popliteal lymph nodes were isolated and homogenized to a single-cell suspension.

Flow cytometry. Popliteal lymph node cells (3×10^5 to 10^6) were stained with anti-T-cell receptor V β 6- or V β 14-specific FITC-labeled antibody and phycoerythrin-conjugated anti-CD4 or anti-CD8 antibody. Alternatively, B cells were labeled with FITC-anti-mouse Ig polyclonal F(ab')₂, and CD4⁺ T cells were labeled with GK1.5 antibody. Analysis was performed with a FACScan (Becton Dickinson). Large CD4⁺ T-cell blasts were selected by forward scatter analysis.

Cell separation by panning. Draining lymph nodes were collected at 19 or 44 h after infection with vOGR1 or vOGR5. After homogenization, the cells were plated in tissue culture dishes in Dulbecco modified Eagle medium plus 8% fetal bovine serum for 4 h at 37°C. Adherent cells (mostly macrophages and dendritic cells) were separated from the nonadherent cell population (mostly lymphocytes) and washed with PBS. Adherent and nonadherent cells were subjected to DNA extraction and PCR analysis.

Cell sorting by flow cytometry. Cells from a pool of six draining lymph nodes from three BALB/c mice infected in both hind footpads with 5×10^6 PFU of vOGR1 were stained as follows. B cells were labeled with phycoerythrin-conjugated anti-mouse Ig, and monocytes/macrophages were recognized by a mixture of two biotinylated antibodies (anti-Mac-1 and F4/80) and indirectly stained with streptavidin-FITC. Cells were sorted with a FACStar Plus (Becton Dickinson). B cells, macrophages, and double-negative cells (mostly T cells) were subjected to DNA extraction and PCR analysis.

DNA extraction and PCR. DNA was isolated from each cell population by digestion in 25 mM EDTA (pH 8.0), 75 mM NaCl, 0.5% sodium dodecyl sulfate, and 20 μ g of proteinase K per ml at 56°C for 4 h. DNA was extracted once with phenol, once with phenol-chloroform-isoamyl alcohol (25:24:1), and once with chloroform-isoamyl alcohol (24:1) and then was ethanol precipitated and resuspended in 10 mM Tris (pH 8.0)-0.1 mM EDTA. Positive controls were established by the addition of a variable number of GR cells to a constant larger amount of CV1 cells prior to DNA extraction according to the same protocol. The number of GR proviral copies per microgram of genomic DNA in these samples varied from 7 to 7,000 and was based on an estimated 70 copies of the GR provirus in the GR mammary tumor cell line. BALB/c DNA was extracted from the spleen or tail of a naive animal as described by Hogan et al. (26), RNase treated, and resuspended in 10 mM Tris (pH 8.0)-0.1 mM EDTA.

Genomic DNA (1 μ g or 600 ng) was used for each PCR reaction. Oligonucleotides were chosen to amplify MMTV(GR) *orf* sequences exclusively, which resulted in a 241-bp product without any of the endogenous *orf* sequences present in the BALB/c genome being amplified.

The 5' oligonucleotide, AGGTGGGTACAATCAACGGC (MS10; GR LTR 705 to 725), is common to various Mtv *orf* sequences, and the 3' oligonucleotide, AATAATGTTCTATCAGTCCAGC (MS8; GR LTR 925 to 905), is specific for the GR *orf* sequence.

The conditions for PCR were 5 min at 95°C, 1 min at 63°C, and 1 min at 72°C followed by 40 cycles of 30 s at 94°C, 30 s at 63°C, and 1 min at 72°C in 1 $\times$ PCR buffer [20 mM Tris-HCl (pH 8.55), 16 mM (NH₄)₂SO₄, 2.5 mM MgCl₂, 150 μ g of bovine serum albumin per ml, 0.2 mM (each) deoxynucleoside triphosphates, 25 μ M oligonucleotide, 1.5 U *Taq* polymerase (Biotaq; Bioprobe Systems)]. A DNA thermocycler (Perkin-Elmer Corp.) was used for the PCR. The specific

FIG. 1. Local immune response to vOGR1 and vOGR5. A total of 5×10^6 PFU was injected into one or both footpads of naive BALB/c mice. At different time points after infection, popliteal lymph node (draining and nondraining) cells were isolated and analyzed by flow cytometry for the percentages of V β 14⁺ CD4⁺ T cells. The percentages of V β 14⁺ cells in the CD4⁺ T-cell population in the draining lymph node after vOGR1 or vOGR5 infection are represented. As a control, the contralateral popliteal nondraining lymph node from vOGR1-infected mice was used. Error bars show standard deviations.

product was detected by liquid hybridization, with 40% of the PCR reaction mixture being hybridized in 150 mM NaCl-2.5 mM EDTA with 50 fmol of one of the following specific ³²P-labeled internal probes common to various Mtv *orf* sequences: MS11 (CAAGGAGGTCTAGCTCTGGCG [MMTV(GR) LTR 787 to 767]) or MS3 (TACCAAGGAAGGGGAGTGGC [MMTV(GR) LTR 887 to 907]). The conditions for denaturation and hybridization were 5 min at 98°C, 15 min at 66°C (MS11) or 65°C (MS3), and a rapid cooling to 4°C. Half of the reaction product was size separated on a 2% agarose gel, dried on DE81 paper (Whatman), and autoradiographed at -70°C overnight with an intensifying screen.

RESULTS

Local immune response to recombinant vaccinia viruses vOGR1 and vOGR5. Wild-type or recombinant viruses (5×10^6 PFU) were injected into the hind footpads of BALB/c mice. A strong anti-vaccinia virus immune response was noticed in the draining popliteal lymph node of the mice after vOGR1, vOGR5, or wild-type WR vaccinia virus infection. A drastic size increase of the draining lymph node was observed independent of the presence of SAg-derived sequences. Four days after the infection with the wild-type WR vaccinia, we observed a larger proportion of B cells in the draining lymph node compared with that observed after infection with both recombinants. That effect mirrors a decrease in the virulence of the two recombinants compared with that of the wild-type virus. This phenomenon is known for vaccinia virus thymidine kinase-negative mutants used in vivo (12). Both recombinants seem to have overall similar virulences. In contrast, only vOGR1 was able to induce a strong and specific SAg response of V β 14⁺ CD4⁺ T cells (Fig. 1). The percentage of this particular subset of T cells started to increase 6 h after infection and reached its maximum after 12 h. This effect was visible until day 5; at day 6, the level of V β 14⁺ CD4⁺ T cells was back to normal. During the peak of the reaction, the percentage of reactive T cells in the CD4⁺ population was 2- to 2.5-fold higher than that in the control lymph nodes. This increase of the V β 14 subset of T cells was localized in the draining popliteal lymph node, and we never observed it in the contralateral nondraining lymph node used as an internal control.

The vOGR5 recombinant, which contains only the COOH-terminal half of the SAg, did not trigger such an SAg response. After infection with this particular virus and despite an equiv-

FIG. 2. Effect of vOGR1 dose on T cells in a local lymph node. The response of Vβ14⁺ cells is indicated as a percentage of CD4⁺ T cells (■). Variations in the percentages of CD4⁺ T cells in the lymph node draining the infection with vOGR1 (□) or vOGR5 (△) are indicated in comparison with those for the nondraining control lymph node (100%). Data represent mean values with standard deviations for three draining lymph nodes at day 2 after the injection of vOGR1 or vOGR5 in the footpad.

alent size increase of the draining lymph node, the percentage of Vβ14⁺ cells in the draining lymph node remained unaffected compared with that in controls (Fig. 1). To verify the specificity of this reaction, the Vβ6⁺ T-cell population was analyzed in all experiments. The level of this subset was not directly affected by the presence of the MMTV(GR) SAg in vaccinia virus; a slight decrease in percentage was observed during the augmentation of Vβ14⁺ CD4⁺ T cells because of compensation.

In contrast to the cases with MMTV or bacterial SAGs, injection of the recombinant vaccinia virus never led to a detectable deletion of the SAg-reactive T cells. Between 6 days and 1 year following the injection of vOGR1, no deletion of SAg-reactive Vβ14⁺ CD4⁺ T cells was observed in the draining and nondraining lymph nodes compared with the situations with vOGR5-injected or naive BALB/c mice (Fig. 1).

Dose dependence. Titration of the injected vOGR1 dose from 5×10^6 to 1.6×10^3 PFU indicated that the maximal increase in the percentage of Vβ14⁺ CD4⁺ T cells in the draining lymph node was achieved with the injection of 5×10^4 PFU ($19.2\% \pm 1.5\%$ versus 10%), and these T cells could still be detected after infection with 5×10^3 PFU ($12.1\% \pm 0.8\%$ versus 9.9%) upon analysis at day 2 (Fig. 2). At that time, the level of Vβ14 cells never exceeded 25% of the CD4 population, even when 1,000-fold the minimal effective dose was injected.

The percentage of total CD4⁺ T cells in the draining lymph node was elevated compared with that in control nondraining lymph nodes when vOGR1 was injected at a dose greater than 5×10^5 PFU; it was reduced (80% of control) when 1.6×10^5 or fewer PFU was used (Fig. 2). In contrast, this correlation was not observed when vOGR5 was used, since a high dose (5×10^6 PFU) reduced the percentage of total CD4 cells in the draining lymph node to 84% of the CD4 cell population in the nondraining lymph node.

I-E dependence. MMTV(GR) SAg has been shown to be I-E dependent (2, 20). In order to test whether SAg produced after

vaccinia virus infection follows the same behavior, BALB/c (I-E-expressing) and C57BL/6 (I-E-nonexpressing) mice were injected with 5×10^6 PFU of vOGR1 and the Vβ14⁺ percentage of CD4⁺ T cells was analyzed at day 2 after infection (Fig. 3). As described above, a twofold increase of the percentage of Vβ14⁺ CD4⁺ T cells in draining lymph nodes compared with that in control nondraining lymph nodes was observed for BALB/c mice. When forward scatter analysis was used during flow cytometry to select for enlarged CD4⁺ blasts, the level of Vβ14-positive cells in this subpopulation was found to have increased by four- to fivefold (44.3 versus 10.7%), a result also observed in other experiments. After infection of I-E-negative C57BL/6 mice, the response of Vβ14⁺ T cells towards the SAg was less prominent but clearly visible. In the total CD4⁺ population of the draining lymph node, the increase was low but significant (10.7 versus 7.9%). In the selected CD4⁺ blasts, the 2.5-fold increase (25.5 versus 9.2%) indicated the effect of the SAg carried by vOGR1 in I-E-negative mice. As a control, the Vβ6⁺ T-cell subset was not directly affected by the SAg (data not shown). The decrease of this subset in the draining lymph node was indirect and due to the increase in the numbers of Vβ14⁺ CD4⁺ T cells or T-cell blasts.

Analysis of in vivo SAg-presenting cells. Analysis of lymph node cells for the presence of GR *orf* sequences in recombinant vaccinia virus DNA by PCR indicated that similar numbers of viral DNA molecules were present in draining lymph nodes shortly after infection with both recombinants. The signal is specific for the GR *orf* sequence introduced by the recombinant vaccinia virus, since none of the endogenous Mtv *orf* sequences of the BALB/c genome were amplified. The presence of the MMTV(GR) *orf* gene in the draining lymph node was already detected 4 h after infection and parallels the time course of the Vβ14⁺ T-cell response. To determine the phenotype of vaccinia virus-infected cells, we performed PCR

FIG. 3. Comparison of local immune responses to the vOGR1 SAg of BALB/c (I-E-positive) and C57BL/6 (I-E-negative) mice infected with 5×10^6 PFU of vOGR1. The responses of Vβ14⁺ cells (top) and Vβ6⁺ cells (bottom) are indicated as percentages of total CD4⁺ T cells or large CD4⁺ blasts in local draining (open bars) and nondraining (solid bars) lymph nodes. Data represent mean values with standard deviations for three draining lymph nodes at day 2 after the injection of vOGR1.

FIG. 4. PCR analysis of vOGR infection. (A) Lymph node cells from draining popliteal lymph nodes were isolated at the indicated time points after the injection of 5×10^6 PFU of vOGR1 or vOGR5 into the footpads of BALB/c mice. Cells were separated by panning on petri dishes, and DNA was isolated from both the adherent (A) and nonadherent (NA) cell populations. DNA (1 μ g) was subjected to PCR amplification specific for MMTV(GR) *orf* DNA. The PCR product was then hybridized with internal probe MS11. Negative controls are no DNA (lane 1), BALB/c spleen DNA (lane 2), and CV1 cell DNA (lane 11). Positive controls (lanes 12 to 15) contain different copy numbers (7 to 7,000) of GR proviral sequences present in DNA extracted from CV1 and GR cells mixed at different ratios. (B) Cells from a pool of six draining lymph nodes were collected 2 days after infection and stained for B cell- and macrophage-specific markers. The cells were sorted with a FACS to obtain pure populations. DNA from total lymph node cells (To) and from sorted populations of B cells (B), macrophages (M Φ), and double-negative cells referred to as T cells (T) was extracted. DNA (600 ng) from sorted cells and 1 μ g of DNA from total LN cells were used for PCR amplification and liquid hybridization with internal probe MS3. The negative control (lane 1) was 1 μ g of BALB/c tail DNA.

analysis on lymph node cell subpopulations (Fig. 4). Adherent and nonadherent cells were separated by panning on petri dishes. Under these conditions, lymphocytes remain in suspension and the adherent cells consist mostly of macrophages and dendritic cells. A signal specific for the GR *orf* sequence was observed in the adherent cell fraction and was very low or absent in the nonadherent cell population (Fig. 4A). With regard to the low copy number of viral DNA detected in sorted macrophages (10 to 100 copies per 600 ng of DNA), we could estimate that a maximum of 1 in 10^2 to 1 in 10^3 macrophages was infected. One day after infection, the intensities of the signals for vOGR1- and vOGR5-infected animals were comparable; 2 days after infection, however, the PCR signal for vOGR5 was clearly lower than that for vOGR1 (Fig. 4A). To confirm the type of cells infected within the lymph node, we performed cell sorting with a fluorescence-activated cell sorter (FACS) on the basis of B-cell- and monocyte/macrophage-specific surface marker expression, with such surface markers as Ig or Mac-1 and F4/80, respectively, being used. B lymphocytes were 95% pure, with less than 0.3% contaminating macrophages after sorting. Mac-1- or F4/80-expressing cells were purified to 90%, with less than 2% contaminating B cells. Double-negative cells, mainly T cells, were 99% pure after the FACS procedure. MMTV(GR) *orf*-specific sequences were detected in the draining lymph node (Fig. 4B, lane 2) of infected BALB/c mice but not in the DNA of uninfected animals (lane 1). In the selected populations, the vaccinia virus genome was detected in monocytes/macrophages, whereas B and T lymphocytes appeared not to be infected (Fig. 4B, lane 4 versus lanes 3 and 5). This result clearly indicates that macrophages are the main targets of infection by vaccinia virus in lymph nodes in which the SAg reaction occurs.

DISCUSSION

MMTV(GR) is the infectious virus produced by the spontaneously activated *Mtv-2* proviral locus of GR mice (43). It has been shown that the *orf* gene product of both the infectious and the endogenous forms of this virus induces a very weak SAg activity (2, 20). Clonal deletion of the SAg-responsive T cells was very slow in MMTV(GR) transgenic animals (2) and

in MMTV(C3H)- or MMTV(GR)-infected mice (20, 36). Infection of BALB/c mice in the footpad with MMTV(C3H), a virus encoding an SAg closely related to the GR SAg with the same V β specificity (11), induced only a weak stimulation in the draining lymph node (22).

The expression of the MMTV(GR) SAg by a recombinant vaccinia virus was able to induce a strong and specific SAg response of V β 14⁺ CD4⁺ T cells in vivo. This response was rapidly detectable and reached a peak level about 12 h after the infection. Most likely, this initial increase was due to trapping and retention of SAg-reactive T cells in the draining lymph node. The very rapid effect of the SAg might reflect the biological differences of viral gene expression in infections with vaccinia virus and MMTV. In the naturally occurring infection by MMTV, the retroviral genome must first be reverse transcribed and integrated before SAg synthesis and presentation can occur. For integration, dividing cells are most likely required. In contrast, vaccinia virus early genes are transcribed immediately after the entry of the virus into the cell. In the recombinant vaccinia viruses that were used in this study, SAg expression is driven by the 7.5K (7,500-molecular-weight) early-late vaccinia virus promoter, which allowed the detection of the product of the SAg gene as soon as 2 h after the infection of cultured CV1 cells with vOGR1 (30).

However, several observations suggest that the slower kinetics of the SAg response after retroviral infection cannot be due only to the time it takes to express the viral protein at the surface of B cells. Injection of *Mtv-7*-expressing congenic B cells or infection with an MMTV harboring the same SAg gene results in the same slow response (5) compared with the extremely rapid response seen after recombinant vaccinia virus infection. Since vOGR1 expresses higher amounts of SAg (31) than MMTV or endogenous *Mtv*, the amount of SAg produced might also play a role in accelerating the stimulation.

With MMTV, the activation of SAg-reactive T cells starts at around day 3 and reaches peak levels around days 4 to 6, and thereafter clonal deletion of the SAg-reactive T cells is observed throughout the life of the mouse. The lifelong deletion is explained by the continued presence of MMTV-infected cells during the whole life of the infected mouse. For vaccinia virus-expressed MMTV SAg, the increase in the percentage of

SAG-reactive T cells is measurable 6 h after injection and peaks at between 12 h and 4 days, and the percentages return to preinjection levels by day 6. No signs of clonal deletion are observed. The absence of a chronic infection of vaccinia virus in these mice might account for the lack of long-term deletion.

When responses to bacterial SAGs (such as staphylococcal enterotoxin B [SEB]) and retroviral SAGs expressed by MMTV or by vaccinia virus recombinants were compared, several differences were observed. After the injection of soluble SEB into the footpad of a mouse, an immediate activation of SAG-reactive T cells followed by a reduction of this T-cell subset after 24 h was observed (17, 44). The initial deletion described for SEB injection has not been observed after footpad infection with MMTV strains SHN, C3H, and SW (22, 35) or with vaccinia virus expressing MMTV SAGs (Fig. 1). For the bacterial SAGs, a strong increase of the SAG-reactive T cells peaking at day 3 was observed, and thereafter a partial clonal deletion can be measured for 1 to 2 months. SEB is most likely present in very high concentrations early after inoculation and decreases rapidly thereafter. However, high amounts of vOGR1 (100 times the minimal stimulatory dose) during the first hours of infection do not induce any deletion before or directly after the stimulation phase.

Infectious MMTVs and endogenous MtvS have been classified into two groups on the basis of the efficiency of their SAG effects. The first group induces a strong response independent of the expression of the MHC class II I-E molecules; members of this group are the infectious MMTV strains SW, SHN, C4, V, and JYG and endogenous *Mtv-1*, -6, and -7. Members of the second group strictly require the expression of I-E to exert a weak SAG effect followed by a slow deletion of the reactive cells. In this group are the two closely related exogenous MMTV strains GR and C3H and endogenous *Mtv-2*, -8, -9, -11, and -13. In mice expressing I-E molecules, infection with MMTV strains GR or C3H leads to poorly detectable stimulation of V β 14 cells (22); after vOGR1 infection, the extent of the increase of the responsive population (2.5-fold) is analogous to the one observed upon stimulation with a strong SAG, e.g., those of MMTV strains SW and SHN (22, 35). In the context of vaccinia virus infection, the expression of the weak (GR) SAG has stimulatory effects similar to those of a strong SAG. In addition, the recombinant vaccinia virus is able to stimulate V β 14 T cells in the absence of MHC class II I-E molecules *in vivo*. The ability of a certain infectious or endogenous MMTV SAG to trigger a weak or a strong response might depend on its level of expression or its affinity for MHC class II molecules. The expression of the MMTV(GR)-derived SAG from a strong vaccinia virus promoter can overcome the strict I-E dependence, and the SAG is correctly presented, although with a reduced efficiency. Our results clearly indicate that an SAG which was thought to be strictly I-E dependent can be presented in the absence of I-E and suggest that the I-E dependence of MMTV SAGs is mainly quantitative and not qualitative.

The minimal amount of vaccinia virus able to initiate an SAG response in the infection model presented here was very low (Fig. 2). In addition, the number of vaccinia virus DNA copies detected by PCR indicated that not more than 1 in 10² to 1 in 10³ macrophages is infected (when more than a 1,000-fold-higher dose is used) (Fig. 4). This result suggests that a very low number of SAG-presenting cells can achieve a strong stimulation, provided that they express a sufficient amount of SAG.

During natural MMTV infection or after inoculation of the retrovirus into the footpad, SAG expression occurs mainly in B cells (24), in which transcription of the SAG gene was shown to be positively regulated (15, 34). Expression of SAG in B cells

fills an essential biological function during the course of MMTV infection (1, 24) by amplifying the number of and inducing the differentiation of infected B lymphocytes (23). B cell-deficient mice do not allow exogenous MMTV SAG presentation, but they are susceptible to endogenous SAGs which have to be presented by other unidentified cells (7). It has been previously shown that endogenous Mtv SAGs are not effectively presented by macrophages (37, 45). Vaccinia virus is able to infect a broad range of cell types *in vitro*, including B and T lymphocytes and macrophages (4, 28). As the transcription of vaccinia virus genes in the cytoplasm of the infected cells uses mainly the viral machinery (41), the expression of SAG under the control of a vaccinia virus early promoter is not restricted to B cells. It has been shown that after intraperitoneal and subcutaneous injections of vaccinia virus into mice, macrophages were the major target of infection *in vivo* and supported productive infection (16). It has also been reported that after vaccinia virus infection *in vitro*, primary macrophages do not produce virus but undergo morphological changes and cell death (39). In the latter case, the block in viral infection would occur at the level of DNA replication, allowing early gene products to be expressed and detected on the surface of the macrophage (39). In order to determine the targets for vaccinia virus infection in our experiments, we separated macrophages from B and T lymphocytes by panning on culture dishes or cell sorting according to the expression of specific surface markers. We analyzed DNA from each population by PCR to detect specifically the SAG sequence present in the viral genome. This experiment clearly demonstrated the presence of viral sequences in macrophages but not in B or T lymphocytes. As macrophages also express MHC class II molecules at their surface, they can potentially present the MMTV SAG to T cells; our data suggest that such cells possess all the necessary elements to strongly stimulate CD4⁺ T lymphocytes with a retroviral SAG. The lack of the presentation of endogenous Mtv SAGs by macrophages should therefore be attributed to qualitative and/or quantitative differences in the production of SAGs in these cells.

When B cells are used as antigen-presenting cells in primary immune responses, the responding T cells are often anergized (18, 21). This fact might be one of the reasons for the clonal deletion of SAG-reactive T cells after MMTV injection. It is not clear which cells are the predominant presenting cells for bacterial SAGs, but it is likely that in this response, B cells also predominate. Recombinant vaccinia virus, in contrast, infects nearly exclusively macrophages/monocytes in the lymph nodes and never shows any signs of the deletion of the SAG-reactive T cells. Although macrophages do not present endogenous Mtv SAGs, our results indicate that they can efficiently present retroviral SAGs when such SAGs are expressed by a recombinant vaccinia virus.

It is unlikely that the short survival time of the virus explains the lack of the induction of deletion. Spread of the infection occurred, since small amounts of virus were detected in lymph nodes and the spleen for more than 1 week after injection, although no significant specific stimulation was observed in the spleen. Moreover, a partial anergy was observed after several weeks in the draining and nondraining lymph nodes of the mice infected with vOGR1. These observations showed that most if not all reactive T cells had access to SAG. Therefore, it is possible that the type of presenting cells may be decisive for the induction of the deletion whereas a chronic infection would be required for the maintenance of the deletion. Further experiments are needed to address this point.

From that perspective, the expression of retroviral SAGs in unrelated viral infections can provide a useful tool for the

dissection of the different effects of SAgS in correlation with quantitative and qualitative differences in expression and presentation.

ACKNOWLEDGMENTS

We thank Pierre Zaech for sorting B cells and macrophages and Riccardo Wittek for providing the vaccinia virus strain as well as the transfer vector.

This work was supported by grants from the Swiss National Science Foundation (SNSF 31-30849.91 to H.D. and SNSF 31-32271.91 and 31.42468.94 to H.A.-O.). H.A.-O. is a recipient of a START career development award (31-27145.89) from the Swiss National Science Foundation.

REFERENCES

- Acha-Orbea, H., and H. R. MacDonald. 1995. Superantigens of mouse mammary tumor virus. *Annu. Rev. Immunol.* **13**:459–486.
- Acha-Orbea, H., A. N. Shakhov, L. Scarpellino, E. Kolb, V. Müller, A. Vessaz-Shaw, R. Fuchs, K. Blöchliger, P. Rollini, J. Billotte, M. Sarafidou, H. R. MacDonald, and H. Diggelmann. 1991. Clonal deletion of V-beta14-bearing T cells in mice transgenic for mammary tumor virus. *Nature (London)* **350**:207–211.
- Acha-Orbea, H., R. M. Zinkernagel, and H. Hengartner. 1985. Cytotoxic T cell clone-specific monoclonal antibodies used to select clonotypic antigen-specific cytotoxic T cells. *Eur. J. Immunol.* **15**:31–36.
- Alonso, J. M., J. Rodriguez, E. Vinuela, G. Kroemer, and C. Martinez-A. 1991. Highly efficient expression of proteins encoded by recombinant vaccinia virus in lymphocytes. *Scand. J. Immunol.* **34**:619–626.
- Andersson, M., and H. Acha-Orbea. 1994. The primary in vivo immune response to Mls-1 (Mtv-7 sag). Route of injection determines the immune response pattern. *Immunology* **83**:438–443.
- Austyn, J. M., and S. Gordon. 1981. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur. J. Immunol.* **11**:805–815.
- Beutner, U., E. Kraus, D. Kitamura, K. Rajewsky, and B. T. Huber. 1994. B cells are essential for murine mammary tumor virus transmission, but not for presentation of endogenous superantigens. *J. Exp. Med.* **179**:1457–1466.
- Bittner, J. J. 1936. Some possible effects of nursing on the mammary gland tumor incidence in mice. *Science* **84**:162.
- Blöchliger, K., and H. Diggelmann. 1992. Expression of the mouse mammary tumor virus ORF gene in cultured cells. *Intern. Rev. Immunol.* **8**:339–357.
- Brandt-Carlson, C., and J. S. Butel. 1991. Detection and characterization of a glycoprotein encoded by the mouse mammary tumor virus long terminal repeat gene. *J. Virol.* **65**:6051–6060.
- Brandt-Carlson, C., J. S. Butel, and D. A. Wheeler. 1993. Phylogenetic and structural analyses of MMTV LTR ORF sequences of exogenous and endogenous origins. *Virology* **193**:171–185.
- Buller, R. M. L., G. L. Smith, K. Cremer, A. L. Notkins, and B. Moss. 1985. Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. *Nature (London)* **317**:813–815.
- Choi, Y., J. W. Kappler, and P. Marrack. 1991. A superantigen encoded in the open reading frame of the 3' long terminal repeat of mouse mammary tumour virus. *Nature (London)* **350**:203–207.
- Choi, Y., P. Marrack, and J. W. Kappler. 1992. Structural analysis of a mouse mammary tumor virus superantigen. *J. Exp. Med.* **175**:847–852.
- Corley, R. B., F. E. Lund, T. D. Randall, L. B. King, S. Doerre, and D. L. Woodland. 1992. Mouse mammary tumor proviral gene expression in cells of the B lineage. *Semin. Immunol. Ser.* **4**:287–296.
- Dales, S., and B. G. T. Pogo. 1981. Biology of poxviruses. *Virol. Monogr.* **18**:1–109.
- D'Amiano, L., K. M. Awad, and E. L. Reinherz. 1993. Thymic and peripheral apoptosis of antigen-specific T cells might cooperate in establishing self tolerance. *Eur. J. Immunol.* **23**:747–753.
- Eynon, E. E., and D. C. Parker. 1992. Small B cells as antigen-presenting cells in the induction of tolerance to soluble protein antigens. *J. Exp. Med.* **175**:131–138.
- Fenner, F., R. Wittek, and K. R. Dumbell. 1989. The orthopoxviruses, p. 1–432. Academic Press, Inc., San Diego, Calif.
- Ferrick, D. A., K. Cho, L. Gemmell-Hori, and D. W. Morris. 1991. Genetic analysis of the effects of mtv-2 on the T cell repertoire in the WXG-2 mouse strain. *Int. Immunol.* **4**:805–810.
- Fuchs, E. J., and P. Matzinger. 1992. B cells turn off virgin but not memory T cells. *Science* **258**:1156–1159.
- Held, W., A. N. Shakhov, G. Waanders, L. Scarpellino, R. Luethy, J.-P. Kraehenbühl, H. R. MacDonald, and H. Acha-Orbea. 1992. An exogenous mouse mammary tumor virus with properties of Mls-1a (Mtv7). *J. Exp. Med.* **175**:1623–1633.
- Held, W., G. A. Waanders, H. Acha-Orbea, and H. R. MacDonald. 1994. Reverse transcriptase-dependent and -independent phases of infection with mouse mammary tumor virus: implications for superantigen function. *J. Exp. Med.* **180**:2347–2351.
- Held, W., G. A. Waanders, A. N. Shakhov, L. Scarpellino, H. Acha-Orbea, and H. R. MacDonald. 1993. Superantigen-induced immune stimulation amplifies mouse mammary tumor virus infection and allows virus transmission. *Cell* **74**:529–540.
- Herman, A., J. W. Kappler, P. Marrack, and A. M. Pullen. 1991. Superantigens: mechanism of T cell stimulation and role in immune responses. *Annu. Rev. Immunol.* **9**:745–772.
- Hogan, B., F. Costantini, and E. Lacy. 1986. Manipulating the mouse embryo: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Korman, A. J., P. Bourgaire, T. Meo, and G. E. Rieckhof. 1992. The mouse mammary tumor virus long terminal repeat encodes a type II transmembrane glycoprotein. *EMBO J.* **11**:1901–1905.
- Koszinowski, U., F. Kruse, and R. Thomssen. 1975. Interactions between vaccinia virus and sensitized macrophages in vitro. *Arch. Virol.* **48**:335–345.
- Kozak, C., R. Callahan, G. Peters, V. Morris, R. Michalides, J. Dudley, M. Green, M. Davison, O. Prakash, A. Vaidya, J. Hilgers, A. Verstraeten, N. Hynes, H. Diggelmann, D. Peterson, J. C. Cohen, C. Dickson, N. Sarkar, and R. Nusse. 1987. A standardized nomenclature for endogenous mouse mammary tumor viruses. *J. Virol.* **61**:1651–1654.
- Krummenacher, C. 1995. Ph.D. thesis. University of Lausanne, Lausanne, Switzerland.
- Krummenacher, C., and H. Diggelmann. 1993. The mouse mammary tumor virus long terminal repeat encodes a 47 kDa glycoprotein with a short half-life in mammalian cells. *Mol. Immunol.* **30**:1151–1157.
- Lambert, J.-F., H. Acha-Orbea, E. Kolb, and H. Diggelmann. 1993. The 3' half of the mouse mammary tumor virus orf gene is not sufficient for its superantigen function in transgenic mice. *Mol. Immunol.* **30**:1399–1404.
- Liao, N., and D. H. Raulet. 1992. Expression of the Mls-1a superantigen results in an increased frequency of V-beta 14⁺ T cells. *J. Immunol.* **149**:1151–1155.
- Lund, F. E., and R. B. Corley. 1991. Regulated expression of mouse mammary tumor proviral genes in cells of the B lineage. *J. Exp. Med.* **174**:1439–1450.
- Luther, S., A. N. Shakhov, I. Xenarios, S. Haga, S. Imai, and H. Acha-Orbea. 1994. New infectious mammary tumor virus superantigen with Vbeta-specificity identical to staphylococcal enterotoxin B (SEB). *Eur. J. Immunol.* **24**:1757–1764.
- Marrack, P., E. Kushnir, and J. Kappler. 1991. A maternally inherited superantigen encoded by a mammary tumor virus. *Nature (London)* **349**:524–526.
- Molina, I. J., N. A. Cannon, R. Hyman, and B. T. Huber. 1989. Macrophages and T cells do not express Mlsa determinants. *J. Immunol.* **143**:39–44.
- Moss, B. 1993. Poxvirus vectors: cytoplasmic expression of transferred genes. *Curr. Opin. Genet. Dev.* **3**:86–90.
- Natuk, R. J., and J. A. Holowczak. 1985. Vaccinia virus proteins on the plasma membrane of infected cells. Infection of peritoneal macrophages. *Virology* **147**:354–372.
- Nusse, R., and H. E. Varmus. 1982. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* **31**:99–109.
- Rohrman, G., and B. Moss. 1985. Transcription of vaccinia virus early genes by a template-dependent soluble extract of purified virions. *J. Virol.* **56**:349–355.
- Springer, T., G. Galfre, D. S. Secher, and C. Milstein. 1979. Mac-1: a macrophage differentiation antigen identified by monoclonal antibody. *Eur. J. Immunol.* **9**:301–306.
- Van Nie, R., A. A. Verstraeten, and J. De Moes. 1977. Genetic transmission of mammary tumour virus by GR mice. *Int. J. Cancer* **19**:383–390.
- Wahl, C., T. Miethke, K. Heeg, and H. Wagner. 1993. Clonal deletion as direct consequence of an in vivo T cell response to bacterial superantigen. *Eur. J. Immunol.* **23**:1197–1200.
- Webb, S. R., A. Okamoto, Y. Ron, and J. Sprent. 1989. Restricted tissue distribution of Mlsa determinants: stimulation of Mlsa-reactive T cells by B cells but not by dendritic cells or macrophages. *J. Exp. Med.* **169**:1–12.
- White, J., A. Herman, A. M. Pullen, R. Kubo, J. W. Kappler, and P. Marrack. 1989. The Vbeta-specific superantigen staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. *Cell* **56**:27–35.
- Winslow, G. M., P. Marrack, and J. W. Kappler. 1994. Processing and major histocompatibility complex binding of the mtv7 superantigen. *Immunity* **1**:23–33.
- Winslow, G. M., M. T. Scherer, J. W. Kappler, and P. Marrack. 1992. Detection and biochemical characterization of the mouse mammary tumor virus 7 superantigen (Mls-1a). *Cell* **71**:719–730.
- Yazdanbakhsh, K., C. G. Park, G. M. Winslow, and Y. Choi. 1993. Direct evidence for the role of COOH terminus of mouse mammary tumor virus superantigen in determining T cell receptor Vbeta specificity. *J. Exp. Med.* **178**:737–741.