

# Viral Superantigen Drives Extrafollicular and Follicular B Cell Differentiation Leading to Virus-specific Antibody Production

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## Summary

Mouse mammary tumor virus (MMTV[SW]) encodes a superantigen expressed by infected B cells. It evokes an antibody response specific for viral envelope protein, indicating selective activation of antigen-specific B cells. The response to MMTV(SW) in draining lymph nodes was compared with the response to haptenated chicken gamma globulin (NP-CGG) using flow cytometry and immunohistology. T cell priming occurs in both responses, with T cells proliferating in association with interdigitating dendritic cells in the T zone. T cell proliferation continues in the presence of B cells in the outer T zone, and B blasts then undergo exponential growth and differentiation into plasma cells in the medullary cords. Germinal centers develop in both responses, but those induced by MMTV(SW) appear later and are smaller. Most T cells activated in the T zone and germinal centers in the MMTV(SW) response are superantigen specific and these persist for weeks in lymph nodes draining the site MMTV(SW) injection; this contrasts with the selective loss of superantigen-specific T cells from other secondary lymphoid tissues. The results indicate that this viral superantigen, when expressed by professional antigen-presenting cells, drives extrafollicular and follicular B cell differentiation leading to virus-specific antibody production.

Mouse mammary tumor virus (MMTV)<sup>1</sup> is a type B retrovirus with a life cycle that is tightly linked to the immune system. Only days after birth, suckling mice are infected by milk-borne MMTV. Infection is first detected among the B cells of the Peyer's patches. The key event then is the expression of a viral protein called superantigen (SAg) on the B cell surface; this is encoded in the 3' long terminal repeat of MMTV (reviewed in reference 1).

The superantigens encoded by MMTV are presented exclusively in the context of class II MHC and are recognized by whole families of T helper cells that have a V $\beta$  element of their TCR in common. T cells expressing the appropriate V $\beta$  undergo a SAg-induced proliferative response and in this way have the potential to provide unlimited cognate T help to MMTV-infected B cells. This strong local T-B interaction is responsible for the amplification of the infected B cell pool, allowing life-long survival of the virus within the host (2, 3). The mobility of infected lympho-

cytes is an important feature of the spread of MMTV to other organs, particularly to the mammary gland where the life cycle starts again.

The effect of SAg expression on the fate of immune cells in the periphery has been studied in detail by injection of bacterial or viral SAg into adult mice (4-8). The lymph node immune response to footpad injection of MMTV(SW) shows the following sequence of events. The B cells are preferentially infected and express a SAg that is reactive with V $\beta$ 6. CD4<sup>+</sup> T cells expressing V $\beta$ 6 subsets are then activated by the SAg and grow in number during the first 3-6 d. These activated T cells help to initiate the expansion of the infected B cells, which proliferate on day 5-6 when peak infection levels in the lymph node are reached (8). Finally, these B cells become plasma cells and reach a maximum of IgG-secreting cells on day 6 (9).

Primary responses to conventional protein antigens have been investigated in more detail. They generally require a stage of T cell priming on APCs competent in presenting antigen in combination with potent costimulation. This typically involves antigen presentation by interdigitating dendritic cells (IDC), whose precursors have taken up antigen

<sup>1</sup>Abbreviations used in this paper: IDC, interdigitating dendritic cells; MMTV, mouse mammary tumor virus; NP-CGG, haptenated chicken gamma globulin; PNA, peanut agglutinin; SAg, superantigen.

in the tissues and migrated to the T zones of secondary lymphoid organs (reviewed in reference 10). This priming process usually takes 2–4 d *in vivo* and is the main reason for the difference in the tempo of primary and secondary antibody responses (11, 12). Cognate interaction between primed T cells and B cells first takes place in the outer T zone of secondary lymphoid organs (12–14). As a result of this interaction, Ag-specific B cells start to proliferate and differentiate in parallel in follicles and in extrafollicular foci. Extrafollicular B blasts do not mutate their Ig V-region genes (15, 16), and they differentiate *in situ* into short-lived plasma cells (17, 18). In mice, this extrafollicular proliferation and differentiation occurs in the red pulp of the spleen adjacent to the T zone (13, 19) and in the medullary cord in lymph nodes (20). B cell proliferation in the follicles gives rise to germinal centers where the B blasts activate an Ig V region-directed hypermutation mechanism (16, 21, 22). These cells are then subject to a selection process, with the selected cells giving rise to long-lived antibody-producing cells (17, 23) or memory cells (24).

Some aspects of the *in vivo* immune response to MMTV(SW) closely resemble the response to MHC class II-restricted peptides. In both there is clonal expansion of Ag-reactive CD4<sup>+</sup> cells, Ag-driven collaboration between B cells and CD4<sup>+</sup> T cells, and the proliferation and differentiation of the B cells into antibody-forming cells. Other features of the SAg-induced immune response are less well characterized; some of these are addressed in this study: (a) does the response to MMTV(SW), which encodes a SAg, require T cell priming and, if so, what is the cell that induces the priming? (b) If T cell priming is required, does it occur more rapidly in the presence of an effectively unlimited number of antigen-reactive T cells? Vβ6<sup>+</sup> T cells, which are activated by the SAg of MMTV(SW), comprise some 10% of the CD4<sup>+</sup> T cells in BALB/c mouse secondary lymphoid tissues. (c) What are the sites, extent, and timing of B and T cell proliferation and differentiation in response to SAg or retroviruses *in vivo*? (d) Is the antibody production induced by injection of MMTV(SW) intrinsically polyspecific or is it mainly directed against viral antigens?

These points have been addressed in experiments that have compared the immune response to MMTV(SW) infection *in vivo* with the response to (4-hydroxy-3-nitrophenyl) acetyl conjugated to chicken gamma globulin (NP-CGG). Quantitative data on cell populations in the draining lymph node have been provided by three-color flow cytometry analysis, while the sites of T and B cell activation were determined in parallel by three-color immunohistology. The results point to a remarkable similarity between the cellular interactions that occur between SAg-dependent responses and those that are driven by recognition of peptide presented on MHC class II molecules.

## Materials and Methods

**Mice.** BALB/c mice were purchased from HO Harlan OLAC Ltd. (Bicester, UK). *Mtv7* (Mls1<sup>a</sup>) congenic BALB/c

(BALB.D2) mice were bred from breeding pairs originally obtained from H. Festenstein (London Hospital Medical College, London). MMTV(SW)-infected BALB/c mice were maintained from mice originally purchased from IFFA Credo (L'Arbesle, France).

**Antigens and Immunizations.** Groups of adult (6–12 wk) BALB/c and BALB.D2 mice were injected into both hind footpads with MMTV(SW) (~10<sup>8</sup> virus particles) purified from milk as described previously (7) or a corresponding volume of MMTV-free milk from BALB/c mice. NP (Cambridge Research Biochemicals, Northwich, UK) was conjugated to CGG (Sigma Chemical Co., St. Louis, MO) and precipitated in alum (Sigma) as described elsewhere (25). Groups of BALB/c mice were injected into both hind footpads with 25 μg alum-precipitated NP-CGG together with 5 × 10<sup>8</sup> killed *Bordetella pertussis* organisms (Lederle Labs., Gosport, UK). Control mice were injected either with alum-precipitated NP-CGG or protein-free alum precipitate with *B. pertussis* alone.

**Tissue Preparation.** The thymidine analog 5-bromo-2'-deoxyuridine (BrdU) (Sigma) was administered as 2 mg in saline *i.p.* 2.5 h before sacrifice. This was to label cells in S phase of the cell cycle. The animals were killed by CO<sub>2</sub> asphyxiation, when blood was taken and the draining popliteal lymph nodes were removed. Those tissues used for immunohistology were wrapped in aluminium foil, snap-frozen in liquid N<sub>2</sub>, and stored at –70°C in grip-sealed polythene bags until sections were cut. The frozen lymph nodes were embedded in OCT compound (Miles, Inc., Elkhart, IN) in a defined orientation and 5 μm cryostat sections were mounted onto 4-spot glass slides. These were air dried for 1 h, then fixed in acetone at 4°C for 20 min, dried, and stored in sealed polythene bags at –20°C.

**Flow Cytometry Reagents and Antibodies.** Flow cytometry reagents were as follows: anti-Vβ6-FITC or biotin (44.22.1) (26), anti-Vβ14-FITC (14.2) (27), anti-CD4-PE (GK1.5) (Boehringer Mannheim GmbH, Mannheim, Germany), anti-CD8-PE (Ly-2) (Boehringer Mannheim), anti-B220-FITC or PE (6A3-6B2) (Caltag, San Francisco, CA), anti-IgD-biotin (AMS 9.1) (PharMingen, San Diego, CA), peanut agglutinin (PNA) biotin (Sigma, Buchs, Switzerland), streptavidin-Tricolor (Caltag), anti-BrdU-FITC (Becton Dickinson & Co., Palo Alto, CA).

**Flow Cytometry.** One of the two draining popliteal lymph nodes was used for analysis of cell subsets and their proliferation, whereas the other was processed for histology. To analyze the surface markers, lymph node cells were labeled with different combinations of antibodies listed above and analyzed on a FACScan® (Becton-Dickinson & Co., Mountain View, CA) using Lysis II software for data evaluation. To analyze cells in S phase of the cell cycle on the basis of BrdU uptake, cells were first surface labeled using PE and Tricolor-conjugated reagents. After washing, cells were resuspended in cold 0.15 M NaCl and fixed by dropwise addition of cold 95% ethanol. Cells were left 0.5 h on ice, washed, and incubated in 100 μl PBS containing 1% paraformaldehyde and 0.01% Tween-20 overnight. Cells were pelleted, then incubated with 15 U/ml DNAase I (Pharmacia Biotech AG, Duebendorf, Switzerland) in 0.15 M NaCl, 4.2 mM MgCl<sub>2</sub>, pH 5, for 30 min at 37°C. Cells were washed, incubated for 30 min with anti-BrdU-FITC (Becton Dickinson), washed again, and analyzed on FACScan®.

**Histological Reagents and Antibodies.** The rat mAbs anti-Vβ6 (44.22.1), anti-Vβ14 (14.2), anti-CD3 (17A2), anti-CD4 (H129), and anti-B220 (14.8) were used as culture supernatants. Sheep anti-IgD (Binding Site Ltd., Birmingham, UK) and mouse anti-BrdU (Dako Ltd., High Wycombe, UK) were used as purified

IgG. The following biotinylated reagents were used: rat anti-syndecan-1 and mouse anti-MHC II (14-4-4S) (PharMingen); rabbit anti-MMTV(SW) gp52 and rabbit preimmune sera were gifts from F. Luethi (Lausanne, Switzerland); PNA (Sigma). NP-binding cells and CGG-binding cells were identified using NP conjugated to rabbit IgG and biotinylated CGG, respectively. As a second stage, where alkaline phosphatase was to be used, nonbiotinylated antibodies were treated with one of the following biotinylated antibodies: rabbit anti-rat Ig, swine anti-rabbit Ig, or goat anti-mouse Ig (Dako Ltd.). Sheep anti-mouse IgD was detected using peroxidase-conjugated donkey anti-sheep Ig (Binding Site Ltd.). Biotinylated reagents were detected with StreptABComplex alkaline phosphatase.

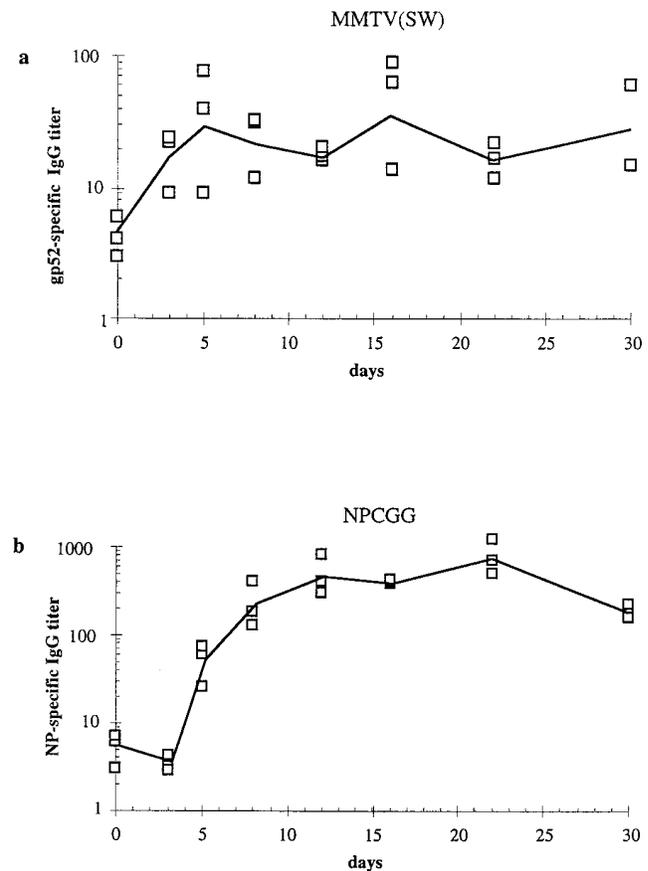
**Immunohistological Staining.** Tissue sections were double-stained for IgD with CD3, CD4, V $\beta$ 6, V $\beta$ 14, syndecan-1, and PNA and also for B220, MHC II, and MMTV gp52 localization. Both NP and CGG-binding B cells were localized in tissue sections as described in reference 19. Slides were washed for 10 min in Tris-buffered saline (pH 7.6) (TBS) before adding predetermined dilutions of the primary Ab in TBS. These were incubated for 1 h at room temperature, then washed extensively in two changes of buffer. The second reagents were added to the sections and incubated for 45 min. After further washes, StreptABComplex alkaline phosphatase was added and the sections incubated for 30 min. Enzyme substrates were made up as previously described in reference 28. Sections were developed first for peroxidase activity using 3-3' diaminobenzidine (Sigma), then for alkaline phosphatase using the substrate Naphthol AS-MX phosphate and chromogen Fast Blue BB salt; levamisole (Sigma) was used to block endogenous alkaline phosphatase activity.

Slides were washed in two changes of distilled H<sub>2</sub>O for 10 min before treating for 20 min with 1 M HCl at 60°C. This not only exposes the incorporated BrdU within the tissue but also destroys the activity of previously bound immunohistological reagents. Slides were returned to water, then two changes of buffer, before anti-BrdU was added. This was detected with biotinylated goat anti-mouse Ig, then StreptABComplex-alkaline phosphatase. Enzyme activity was demonstrated using Naphthol AS-MX phosphate with Fast Red TR salt (Sigma).

**ELISA Techniques.** Plastic plates with 96 flat-bottomed wells were coated overnight at 4°C with 5  $\mu$ g/ml of either NP-BSA or recombinant viral envelope protein gp52 in carbonate buffer (pH 9.6). Plates were washed with PBS plus 0.05% Tween-20, then blocked with 1% BSA. After washing, dilutions of the test sera were added and incubated during 4 h at room temperature. A balanced mixture of four mouse IgG anti-gp52 mAb and a high titer anti NP immune serum were used as controls to produce standard calibration curves to determine antibody titers. Bound Ab was detected using biotinylated anti-mouse IgG (Amersham Rahn, Zurich, Switzerland) followed by streptavidin-conjugated alkaline phosphatase (Boehringer Mannheim GmbH). Plates were developed with *p*-Nitrophenyl-phosphate (Sigma) and absorbance read at 405 nm. Serum titers were calculated using the calibration curve to give an OD<sub>50</sub> of the standard controls.

## Results

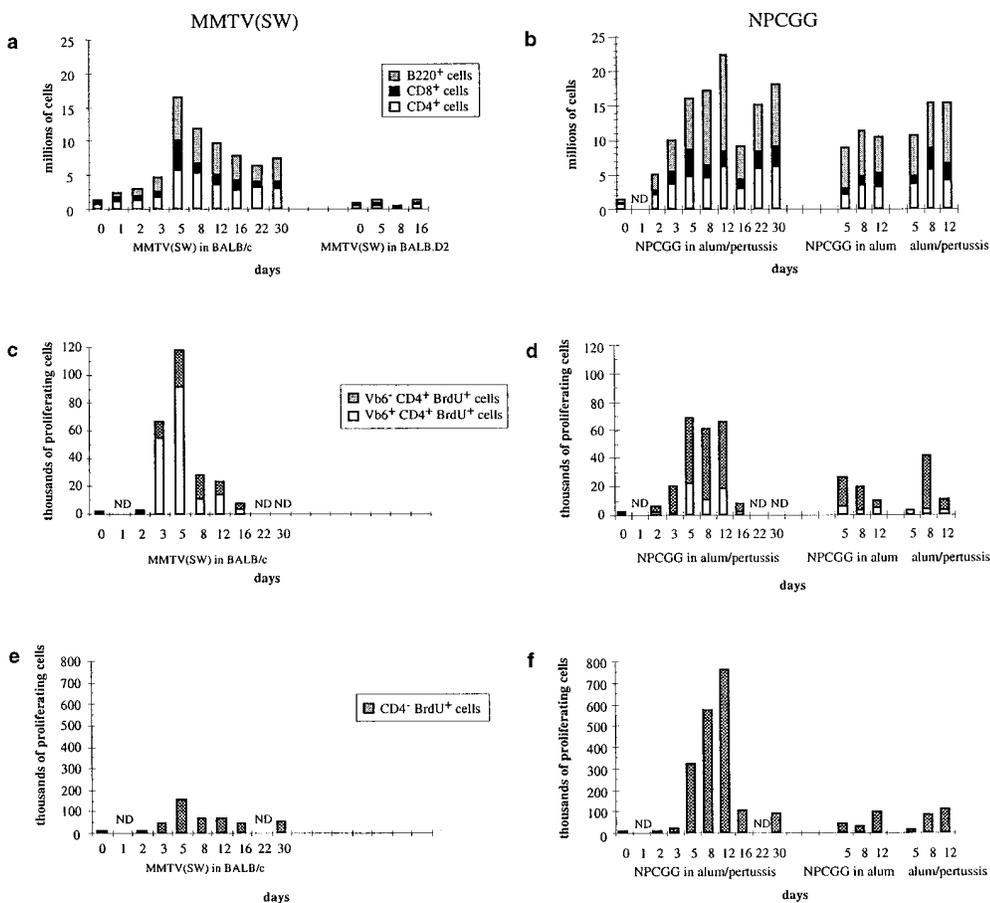
**The Experimental Approach.** BALB/c mice were injected into both hind footpads with milk-purified infectious MMTV(SW) or alum-precipitated NP-CGG with killed *B. pertussis* microorganisms. The specific serum antibody titers of these immunized mice were measured and



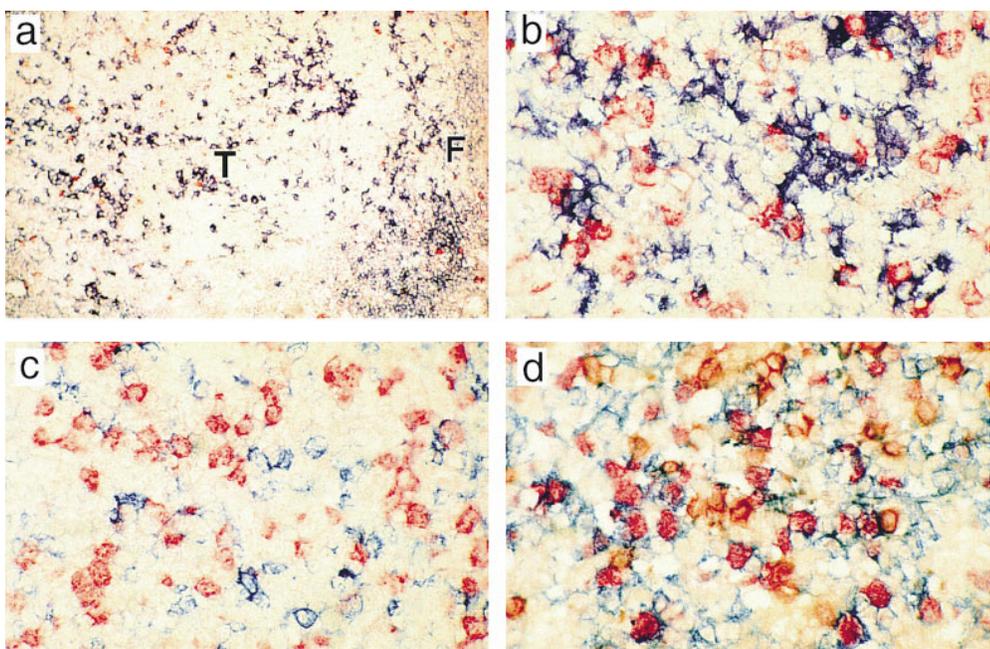
**Figure 1.** Kinetics of antigen-specific serum IgG antibody responses following immunization with MMTV(SW) or NP-CGG into both footpads. (a) shows the titers against bacterially derived recombinant viral envelope protein gp52 of mice injected with MMTV(SW), and (b) shows the anti-NP titers in mice immunized with alum-precipitated NP-CGG plus *B. pertussis*. Shown are the individual values (squares) as well as the mean (line).

the immune responses in the draining popliteal LN were compared. To assess the proliferative response, mice were given a pulse of BrdU i.p. 2.5 h before death to identify and localize cells that had been in S phase of the cell cycle over this period. One draining popliteal LN was used to quantify cellular subsets by flow cytometry; the contralateral draining LN was used to colocalize these subsets by immunohistology. With both methods, cells were stained for cell differentiation and proliferation markers between day 0 and day 30 after antigen challenge. The antibody responses are shown in Fig. 1, while the flow cytometry data are shown in Figs. 2 and 4 and the histological analyses in Figs. 3, 5, 6, 7.

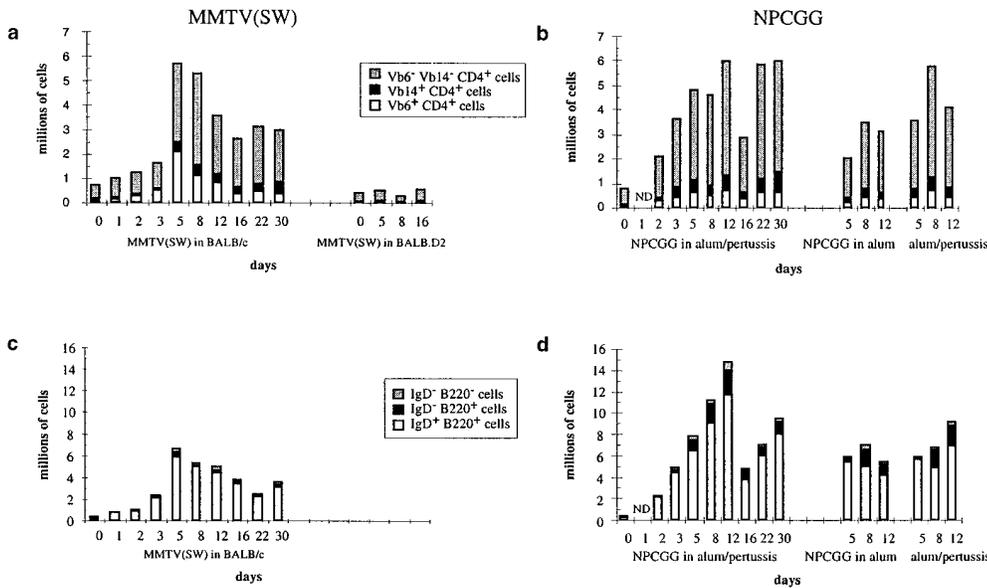
**Serum Antibody Responses Indicate that Ag-specific T and B Cells Interact Efficiently.** Serum antibody titers were assessed against recombinant viral envelope protein gp52 (Fig. 1 a) and NP (Fig. 1 b). Specific IgG antibody to both gp52 and NP are detectable respectively from day 3 and day 5, peaking around day 5 and day 8. Thereafter, the antibody titers remain relatively constant through day 30.



**Figure 2.** Flow cytometric analysis of T and B cell numbers and proliferative responses. BALB/c mice were immunized into both footpads with MMTV (SW) (a, c, e), or alum-precipitated NP-CGG with *B. pertussis* (b, d, f). Cell suspensions of the draining popliteal LN were analyzed at different times after immunization. a and b show the mean numbers of B220<sup>+</sup> cells, CD4<sup>+</sup> cells, and CD8<sup>+</sup> cells, per popliteal LN. c and d show the mean numbers of CD4<sup>+</sup>, Vβ6<sup>+</sup>, and CD4<sup>+</sup>, Vβ6<sup>-</sup> cells that had incorporated BrdU during the 2.5 h before the nodes were removed. e and f show the mean number of CD4<sup>-</sup> cells that had incorporated BrdU. Extrapolation from histology suggests that the CD4<sup>-</sup> blasts are mainly B blasts. a also shows results for BALB.D2 mice immunized with MMTV(SW) while b, d, f also show the results for BALB/c mice immunized with alum-precipitated NP-CGG without *B. pertussis* or mice given protein-free alum precipitate plus *B. pertussis*. Three mice were analyzed for each immunization at each timepoint.



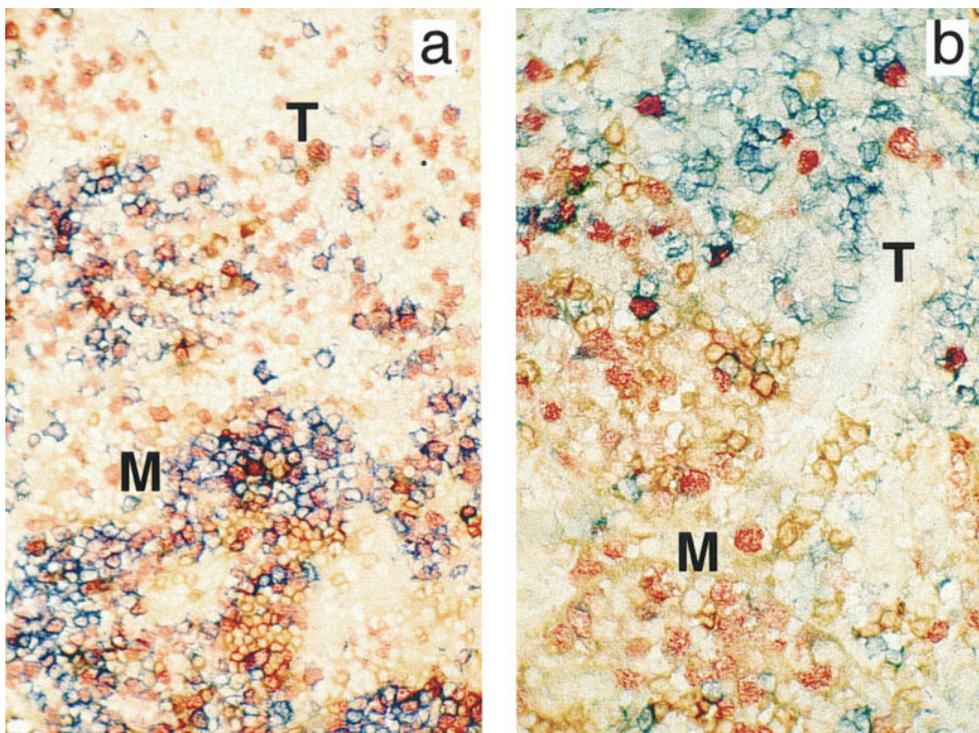
**Figure 3.** Histological localization of cells proliferating within the T zone (T) of lymph nodes draining MMTV(SW) infection. Cells incorporating BrdU during a 2.5-h pulse are identified by red staining nuclei in all panels. a (×100) shows MHC class II expression (blue) 2 d after challenge with MMTV(SW); large interdigitating cells are seen in the T zone where very few cells are proliferating. The smaller class II<sup>+</sup> cells at the edge of the panel are follicular (F) B cells. b-d (×500) show the T zone from a lymph node taken 3 d after infection with MMTV(SW). At this stage there are large numbers of BrdU<sup>+</sup> cells in that area. These are associated with IDC stained blue for MHC class II expression in b; they are not obviously associated with B cells stained blue for B220 expression in c and they are largely Vβ6<sup>+</sup> as indicated by blue staining in d where recirculating IgD<sup>+</sup> B cells are stained brown.



**Figure 4.** Quantitative analysis of T and B cell subsets within the draining popliteal LN responding to MMTV(SW) (a, c), or alum-precipitated NP-CGG plus *B. pertussis* (b, d). Cell suspensions were analyzed by flow cytometry as in Fig. 2. Values in a and b represent mean numbers of cells/node in different CD4<sup>+</sup>, Vβ subsets and those in c and d mean numbers of CD4<sup>+</sup>CD8<sup>-</sup> cells/node sorted on the basis of their expression of IgD and B220. The same control responses were included as for Fig. 2. Three mice were analyzed for each immunization at each time-point.

*T Cell Priming, the Extrafollicular B Cell Response, and Nonspecific Accumulation of Recirculating Lymphocytes in the Nodes Draining the Site of Challenge with Antigen.* The first phenomenon observed after footpad challenge with MMTV(SW) or NP-CGG is a relatively nonselective increase in the number of lymphocytes in the draining nodes after injection of either of the test antigens (Fig. 2, a and b). The numbers of B cells, CD4<sup>+</sup> T cells, and CD8<sup>+</sup> T cells in the draining LN double in the first 2 d after MMTV(SW) infection or NP-CGG injection, but this is not associated with proliferation (Fig. 2, c–f, Fig. 3 a). By the end of day 3 after immuniza-

tion CD4<sup>+</sup> T cell proliferation is marked in the response to both antigens (see Fig. 2, c and d). In the MMTV(SW)-immunized mice this is mainly associated with Vβ6<sup>+</sup> T cells (Figs. 2, c and 3 d); it is seen throughout the T zone and is associated with MHC class II<sup>+</sup> IDC (Fig. 3 b) rather than B220<sup>+</sup> B cells (Fig. 3 c). The T zone proliferative response of CD4<sup>+</sup> T cells continues through day 5 when the proportion of Vβ6<sup>+</sup> T cells in the nodes draining MMTV(SW) infection reaches its highest, at ~30% of all CD4<sup>+</sup> T cells (Fig. 4 a). On day 5, much of the T cell proliferation is in association with B220<sup>+</sup> B cells on the edge of the T zone



**Figure 5.** Photomicrographs showing extrafollicular B cell proliferation in response to MMTV(SW). Draining LN were taken 5 d after infection with MMTV(SW) and stained for IgD (brown), BrdU incorporation (red), and in blue either syndecan-1 in a (×250) or Vβ6 in b (×500). Syndecan-1<sup>+</sup> plasmablasts are largely seen proliferating within the medullary cords (M). At this stage, Vβ6<sup>+</sup> T cells comprise ~30% of T cells and are almost all located in the T zone (T) where some are in cell cycle. Very few Vβ6<sup>+</sup> cells are in the medulla and some of those that are there are likely to be in the efferent lymphatic channels rather than the medullary cords.

and less with IDC; this may reflect a shift from the T cell priming stage to one where primed T cells are proliferating after making cognate interaction with B cells that are expressing SAg.

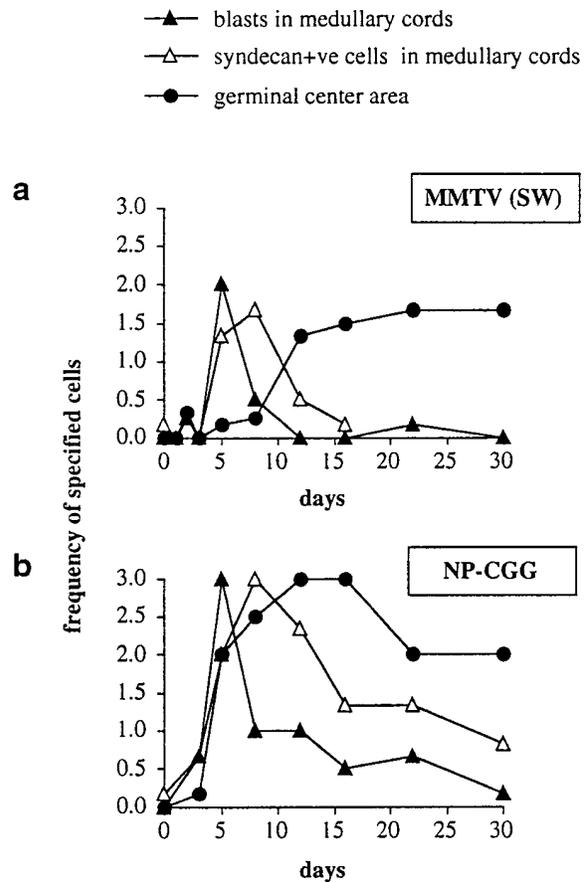
There is some B cell proliferation on day 3 (see Fig. 2, *e* and *f*) but this is far more marked on day 5 when it is mainly associated with B220<sup>+</sup> cells in the medullary cords; many of these blasts have started to express syndecan-1 (Fig. 4, *c* and *d*; 5 *a*, 6, *a* and *b*). By day 8, the cells in the medullary cord blasts are syndecan-1<sup>+</sup> B220<sup>-</sup> nonproliferating plasma cells with cytoplasmic IgM or IgG (data not shown). Most of these plasma cells are gone by day 12 in the MMTV(SW) response; they are more persistent in the response to NP-CGG (Fig. 6, *a* and *b*). At the height of the B cell proliferative response to MMTV(SW) in the medullary cords there are abundant V $\beta$ 6<sup>+</sup> T cells in the T zone but very few of these cells are associated with the proliferating B cells in the medullary cords (see Fig. 5 *b*). This reflects the finding that antigen-specific T cells are not obvious in extrafollicular foci of B cell proliferation in the spleen during responses to protein antigen (29, 30).

In parallel with B cell proliferation in the medullary cords, there is further accumulation of surface sIgD<sup>+</sup> B cells in the nodes. This peaks at day 5, but increased numbers of these cells are present through 30 d after immunization with either antigen (see Fig. 4, *c* and *d*). The bulk of the sIgD<sup>+</sup> B cells are in the follicles and the increased number of these cells seems likely to be due to an increased capacity of the node to accommodate recirculating B cells.

**Follicular B and T Cell Responses.** Germinal center formation is already visible by day 5 after immunization with NP-CGG in the draining nodes (Fig. 6 *b*). Overt germinal centers do not appear in the MMTV(SW)-infected mice until day 12 (Fig. 6 *a*); despite their late appearance and rather small size (Fig. 7 *b*), they persist over weeks and are occasionally still observed after 120 d (data not shown). Some clusters of PNA<sup>+</sup> cells were seen in the follicles of draining nodes 5 d after footpad injection of MMTV(SW), but these had gone by day 8. It seems possible that the first cohort of activated B cells that migrate to the follicles in these mice undergo apoptosis within a short period. Possible reasons for this are discussed below.

The retention of antigen on follicular dendritic cells is important in the selection of B cells that have mutated their Ig V-region genes in germinal centers (reviewed in reference 31). In the mice immunized with NP-CGG, antigen was clearly seen on follicular dendritic cells in the draining nodes from day 8. The viral envelope protein gp52 was also visibly localized on follicular dendritic cells 8 d after immunization with MMTV(SW) (data not shown). In both instances, the localization of antigen coincided approximately with the peak of the extrafollicular antibody response against the immunizing antigen. The delay in the appearance of fully developed germinal centers in the MMTV(SW)-infected mice does not seem to be attributable to lack of antigen on follicular dendritic cells.

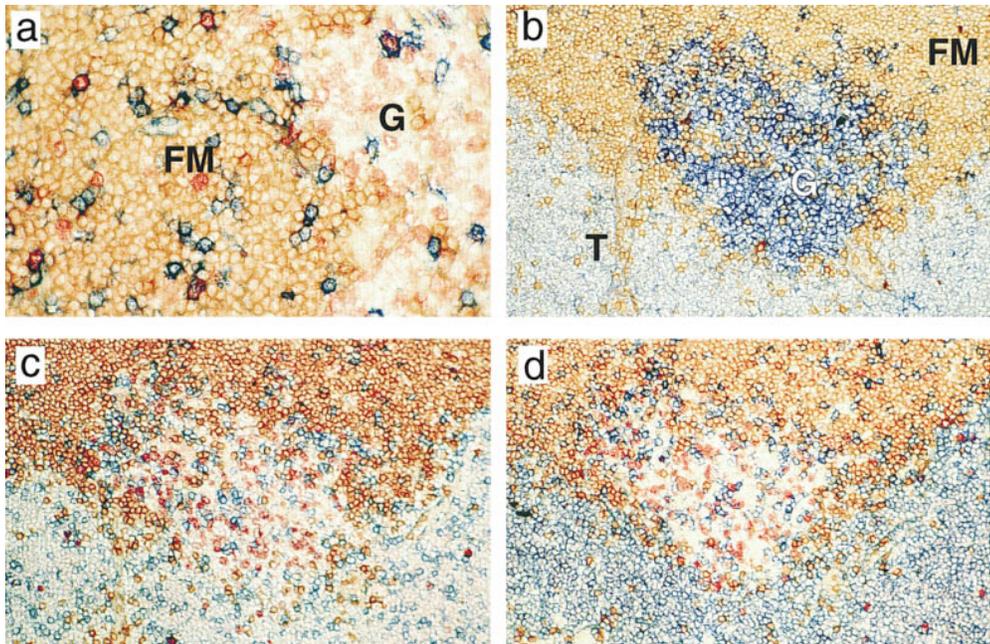
T cells accumulate in the follicles in both groups of mice from day 3 onward. The T cells associated with germinal



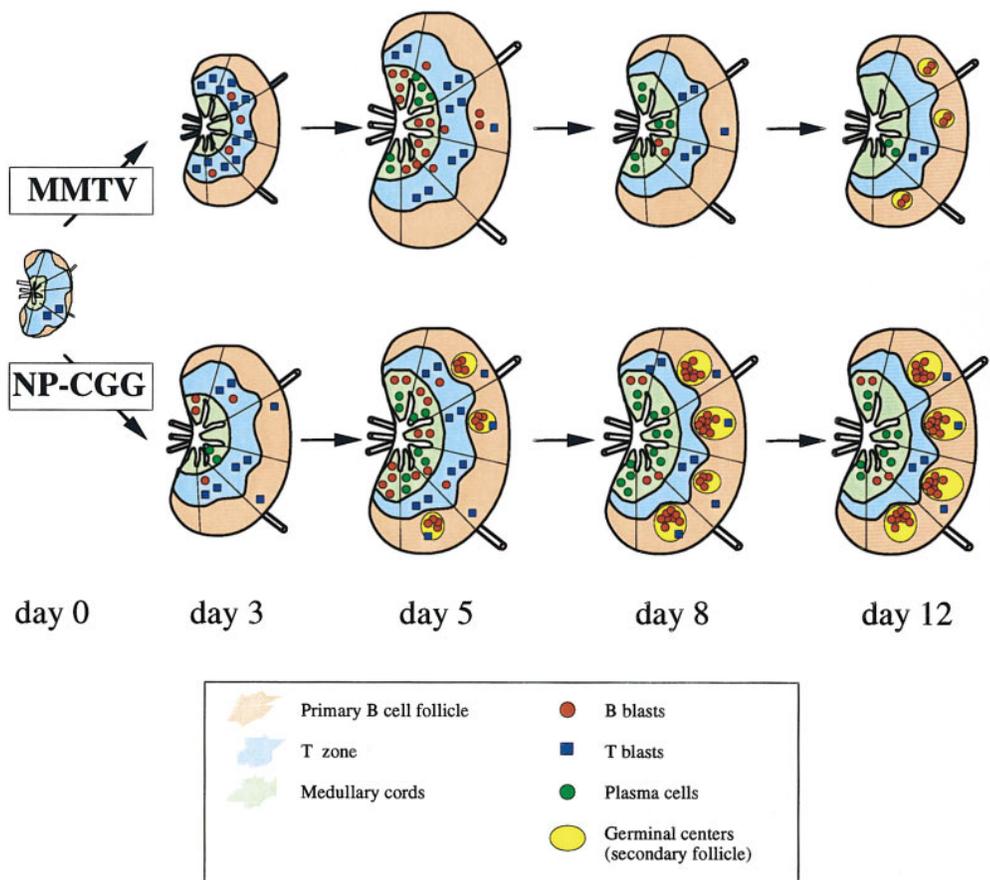
**Figure 6.** Semiquantitative histological analysis of B cell proliferation and differentiation within the draining LN following immunization with (a) MMTV(SW) or (b) NP-CGG. Slides from the histological study were assessed by two people independently as the extent of reaction in arbitrary units (zero = the level seen in nonimmunized mice, and 3 = a maximum response) and the scores for three mice in each group at each timepoint are averaged. Plasma cells and plasmablasts were identified as being syndecan-1<sup>+</sup>; blasts are defined as BrdU<sup>+</sup> cells. Germinal centers were identified as PNA<sup>+</sup>B220<sup>+</sup>IgD<sup>-</sup> areas.

centers in MMTV(SW)-infected mice are mainly V $\beta$ 6<sup>+</sup> (Fig. 7, *c* and *d*) and are notable for their lack of proliferation. In contrast, the T cells that appear in the follicles in response to NP-CGG do proliferate; this T cell proliferation is in the follicular mantle as well as in the germinal center (Fig. 7 *a*). This histological finding is reflected in the persistence of BrdU-labeled CD4<sup>+</sup> T cells identified by flow cytometry in lymph node cell suspensions from NP-CGG-immunized mice taken on day 8 and day 12 (see Fig. 2 *d*) in contrast with the minimal proliferation at that stage in the MMTV(SW)-infected mouse lymph node suspensions (see Fig. 2 *c*). In common with previous findings, T cell proliferation is mainly associated with the first 2 wk of the primary follicular response (30).

Typically, the response to MMTV(SW) injection is associated with depletion of V $\beta$ 6<sup>+</sup>, CD4<sup>+</sup> T cells from nonresponding lymphoid organs (7). In contrast with this, V $\beta$ 6<sup>+</sup> T cells persist in the responding popliteal node through day 30. These are distributed both in germinal centers where



**Figure 7.** Photomicrographs of stained sections to show germinal centers formed during responses to NP-CGG in *a* and MMTV(SW) in *b-d* within the draining LN. *a* ( $\times 250$ ) shows a LN taken 8 d after immunization with NP-CGG stained for IgD (brown), CD3 (blue) and BrdU (red) incorporation. Proliferating T cells can be seen both within the IgD<sup>-</sup> germinal center (G) and the surrounding IgD<sup>+</sup> follicular mantle (FM). *b-d* ( $\times 160$ ) are serial sections from a draining node 22 d after infection with MMTV(SW) stained for IgD (brown), BrdU (red) and blue staining which shows PNA in *b*, Vβ6 in *c*, and CD3 in *d*, respectively. Almost all the T cells within the PNA<sup>+</sup> germinal center (G) and around 10% of the T cells in the T zone (T) express Vβ6. Very few follicular Vβ6 T cells are BrdU<sup>+</sup>; this applied to T cells in follicles at all stages of the response to MMTV(SW).



**Figure 8.** Summary of the major events of the lymph node immune response to either MMTV(SW) superantigen or to NPCGG. This sketch summarizes the responses that were quantified by flow cytometry (Figs. 2 and 4) and localized by immunocytochemistry. (Figs. 3, 5-7). Emphasis is put on the sites of T and B cell activation and differentiation with symbols representing the relative number of cells in a given compartment.

V $\beta$ 6<sup>+</sup> cells are the dominant T cell type and in the T zones where they comprise about 10% of T cells (Fig. 7, *c* and *d*). In addition, there is still substantial nonspecific T cell retention in the T zone of draining nodes 30 d after immunization with either antigen (see Fig. 2, *a* and *b*).

**Control Responses.** To test whether the addition of *B. pertussis* to NP-CGG was responsible for the early germinal center formation, mice were injected with alum-precipitated antigen in the absence of *B. pertussis*. B cell proliferation was well established by day 5 (see Fig. 2 *f*), although this was at a considerably lower level than that seen when *B. pertussis* was included. Histological analysis confirms that antigen-specific B cell proliferation occurred both in the follicles and medullary cords, and germinal center formation was not delayed compared with that seen when *B. pertussis* was given with the antigen. This microorganism enhances the level of the specific (and nonspecific) immune responses to NP-CGG without affecting the timing or pattern of the response. Another group of mice was immunized with *B. pertussis* in combination with a protein-free alum precipitate. This induced nonspecific retention of T and B cells in the draining nodes (see Fig. 2 *b*) and was associated with a small follicular and medullary cord reaction, which was somewhat delayed compared with the reaction to NP-CGG. The response to NP-CGG alone or *B. pertussis* plus protein-free alum precipitate was appreciably smaller than the response to the combination of alum-precipitated NP-CGG plus *B. pertussis*.

The immune reactions observed in response to MMTV(SW) are a consequence of the retroviral infection process and the expression of viral proteins, most notably of the SAg. To separate SAg-induced from anti-viral effects MMTV(SW) was injected into BALB.D2 mice. These animals are congenic with BALB/c mice but constitutively express the *Mtv7* SAg that binds V $\beta$ 6. BALB.D2 mice consequently lack the V $\beta$ 6 T cells that react with the SAg of MMTV(SW). No significant increase in T or B cell numbers was noted in the draining nodes of BALB.D2 mice on day 5, day 8, and day 16 after the injection of MMTV(SW) (see Fig. 2 *a*). The histological appearance of these nodes was similar to those of nonimmunized mice and to groups of mice injected with virus-free milk, with no obvious proliferative responses and no germinal center formation. These findings confirm the crucial role of the SAg for all the events of lymphocyte proliferation and differentiation described for MMTV(SW) infection in BALB/c mice. It also indicates that the other viral proteins by themselves are not inducing any measurable immune response in this host.

## Discussion

The lymphoid architecture involved in specific immune responses has been investigated mainly using model antigens and rarely with infectious agents. Here, we compared the histological appearance of lymph nodes after subcutaneous injection of a T-dependent haptenated protein (NP-CGG) with a murine retrovirus (MMTV) that expresses a SAg soon after infection. We show that the extrafollicular

T and B cell activation during both responses are remarkably similar. Both antigens induced germinal centers, although these were smaller and appeared later in the response to MMTV(SW). The sequence of the responses to these two antigens are summarized diagrammatically in Fig. 8.

**The Role of SAg in the Response to MMTV(SW).** The BALB/c and BALB.D2 mouse strains are congenic for a single *Mtv* locus, *Mtv7*. Due to *Mtv7* SAg expression, BALB.D2 lack T cells reactive with MMTV(SW) and therefore cannot mount a SAg response to this virus strain. We have taken advantage of this strain difference to separate the SAg-induced effects from conventional responses to other MMTV proteins. As these proviruses are inherited as self-antigens, all laboratory mice should be tolerant to most MMTV peptides. The BALB/c genome contains three endogenous *Mtv* loci (*Mtv6*, *Mtv8*, and *Mtv9*); only some of these encode complete envelope protein (32, 33), but all of them encode functional SAGs. Tolerance of T cells to the endogenously expressed *Mtv* SAg has clearly been demonstrated by neonatal deletion of specific V $\beta$  T cell subsets (reviewed in reference 1). As a consequence, BALB.D2 mice, lacking V $\beta$ 6-expressing T cells, fail to respond to the V $\beta$ 6-specific MMTV(SW) but not to the V $\beta$ 2-specific MMTV(C4) or V $\beta$ 14-specific MMTV(C3H) (2; our unpublished results). It is not clear yet whether these animals display T cell tolerance to endogenously encoded viral envelope proteins. Here, we show that BALB.D2 mice, contrary to BALB/c mice, fail to mount a proliferative T and B cell response to infectious MMTV(SW), suggesting tolerance to the viral SAg as well as to the viral peptides. These results suggest that SAg is an essential element of the response of BALB/c mice to MMTV(SW) and not simply an interesting and efficient alternative to conventional help evoked by these peptides.

**The Specificity of the B Cell Response to MMTV(SW).** As antibodies specific for viral envelope protein gp52 were produced from day 3 after immunization, it seems that a proportion of the B cells that were induced to proliferate in the medullary cords were specific for viral envelope antigen. Relatively few B cells seed extrafollicular foci in the spleen during T cell-dependent responses (15) and these go through 6–7 cell cycles before differentiating to become plasma cells (16, 19). The medullary cord responses to MMTV(SW) and NP-CGG seem similar to the responses in splenic extrafollicular foci in which small numbers of activated B cells initiate the response. If this is the case, specific antibody production would only be expected if there is a bias towards the activation of B cells that have specificity for MMTV(SW) envelope protein. Two factors may contribute to the specificity of the response: (*a*) virus entry to the B cells may be greatly facilitated if the B cells express surface immunoglobulin that is specific for viral envelope proteins, and (*b*) appropriate engagement of the B cell receptor by antigen leads B cells to migrate to the T zones of secondary lymphoid organs where they seek interaction with primed T cells specific for the processed antigen. B cells that have not engaged antigen through their antigen-specific receptors do not appear to interact with primed T cells.

Memory B cells in the spleen, which are resident in the marginal zones, move rapidly to the T zone after they have picked up antigen from the marginal zone blood sinusoids (28). On reaching the T zone they make rapid cognate interaction with primed T cells, producing Ig switch transcripts within 12 h of encountering antigen (19). Then the B blasts move into the red pulp or follicles where they proliferate further.

In the case of MMTV(SW), 60% of all local B blasts were found to be infected 6 d after footpad injection (2). In this study, we demonstrate that these B blasts, and therefore the infection, localize to the medullary cords. It will be interesting to determine the fate of these B blasts and their role in virus persistence.

As previously discussed, T cells should be tolerant to endogenously produced MMTV(SW) envelope protein. As B cells are continuously produced from hemopoietic cells in the marrow, there can be expected to be a steady output of cells specific for envelope protein. B cells that have specificity for antigens expressed by cells in the marrow may be deleted in situ (34, 35). On the other hand, most newly produced virgin B cells leave the marrow and migrate to the outer T zone of the secondary lymphoid organs (36, 37); if on the way they have taken up antigen not bound to cells and they encounter primed helper T cells in the T zone, they can be recruited into an antibody response (38). The MMTV(SW) envelope protein-specific B cells may be representatives of these cells. If the antigen-charged cells fail to find T help they stay in the T zone for up to 3 d, after which time they die (39). This may be the situation if endogenously encoded envelope protein is presented, for T cell tolerance to viral peptides presumably exists. After MMTV infection of B cells and SAg expression efficient T help can be obtained. The SAg response therefore might override B cell tolerance to viral antigens.

*The Requirement for Conventional Priming of T Cells.* Recirculating T cells migrate between the T zones of secondary lymphoid organs (40, 41). In the T zones they screen the surface of IDC; if they find MHC class II-peptide or MHC class II-SAg they recognize they are selectively retained and activated. This process seems to be fundamental to T cell priming in most situations (10). Although B cell-depleted mice show reduced growth of the number of antigen-specific T cells after primary immunization they still show T cell priming (42, 43). A requirement of IDC for priming has to be distinguished from the major role B cells can play in the subsequent expansion of clones of primed T cells (30, 42). This effect might have been important in the continued proliferation of V $\beta$ 6<sup>+</sup> T cells seen on day 5 after immunization with MMTV(SW). Evidence has been presented showing that virgin T cells are switched off, rather than primed on encounter with antigen-presenting B cells (reviewed in 44).

There appears to be a requirement for IDC for T cell priming in the response to MMTV(SW) SAg. V $\beta$ 6<sup>+</sup> T cells were first seen to proliferate in the T zone in association with IDC rather than B cells. In addition, SAg-specific virgin T cells are present in virtually unlimited numbers (10%

of all T cells); the delay until the third day before T cell proliferation can not plausibly be attributable to a lack of SAg-specific T cells. Although this delay could be due to the low frequency of initially infectable B cells, the rapid onset of the medullary cord blast reaction after the onset of T cell proliferation suggests B cells specific for viral envelope protein were ready and waiting for encounter with primed T cells. In conclusion, our evidence suggests that virgin V $\beta$ 6<sup>+</sup> T cells are initially not activated by SAg-expressing B cells but dendritic cells. We are currently testing this hypothesis.

It is not clear how SAg is acquired by the IDC that prime V $\beta$ 6<sup>+</sup> T cells in the MMTV(SW) response. After injection of MMTV(SW), infection classically has been associated first with B cells (8). Endogenous *Mtv* SAg can be presented in mice congenitally lacking B cells (45, 46), although the capacity of spleen cells from these mice to induce proliferation of T cells expressing the appropriate V $\beta$  was found to be 100-fold less than that of mice with B cells (46). Similar data are lacking for the expression of exogenous MMTV SAg and it remains to be shown whether IDC are infected, or perhaps acquire SAg passively from other cells that have been infected. Soluble SAg has been described (47–49), as has the secretion of loaded MHC class II molecules by B cells (50).

*The Follicular Responses.* The follicular response to NP-CGG was visible 5 d after immunization in parallel to the extrafollicular B cell response. Germinal centers induced by MMTV(SW) were unusual in that they were delayed by several days and were smaller (Fig. 8). There were signs of isolated PNA-binding cells in the follicles of the MMTV(SW)-infected mice 5 d after immunization, but none on day 8. This raises the possibility that the follicular response is aborted by a massive output of virus from B blasts proliferating in the medullary cords at that time. Temporary dissolution of established germinal centers induced by soluble antigen has been reported by a number of groups (51–53). By day 8, the production of antibody against viral envelope protein could result in the clearance of most virus in the extracellular fluid; after this, germinal centers might be expected to emerge. This hypothesis is supported by the recent histological analysis of VSV infection in mouse spleen where virus-specific germinal center started to arise from day 6 onward; this virus is thought to be cleared within 24 h after infection (54). It is also possible that the T cell priming or the interaction between primed T cells and MMTV(SW)-infected B cells in the T zone promotes extrafollicular B cell growth but is in some way ineffective in inducing germinal center formation. The IgG2a dominance of the early antibody response to MMTV (8, 9) may reflect IFN- $\gamma$  production and bias towards a T<sub>H</sub>1 response and against early germinal center formation. If this is the case, the effect is short-lived, for germinal centers were established by day 12 after infection and then persisted for weeks. These were still present at 30 d after immunization and they may provide foci of chronically infected B cells. This is supported by the finding that mice deficient in B7 interactions, that are unable to form germinal centers, show

poor virus dissemination and do not complete the virus life cycle (55).

The T cells in the germinal centers in MMTV(SW) mice were predominantly  $V\beta 6^+$ , implying that centrocytes are infected by virus and their selection is SAg driven. This raises the interesting possibility that selection of centrocytes could be based on SAg expression rather than the presentation of peptides from antigen picked up from follicular dendritic cells. This may not be the case, because the persistence of antibody against viral envelope proteins suggests that antigen-specific cells continue to hold some selective advantage. Perhaps germinal center B cells *in vivo* have to bind antigen through their surface Ig as well as receiving signals from local T cells. Viral envelope protein was found in the present study to be bound to follicular dendritic cells from day 8 after immunization.

*Regulation of the Number of Antigen-reactive T Cells.* The extrafollicular growth of SAg-reactive T cells on the third to eighth day after infection is followed by massive systemic loss of  $V\beta 6^+$  T cells in the second week (7), but the present study shows that  $V\beta 6^+$  T cells in the follicles and T zones of the responding node are spared; this suggests

regulation of numbers rather than indiscriminate deletion. Stimulation with specific antigen has been reported previously to block SAg-induced deletion of T cells (56–58).

The combination of immunohistochemistry and flow cytometry has provided a powerful approach to pinpoint the activation and differentiation stages of lymphocytes in a primary immune response *in situ*. The results suggest that the MMTV(SW)-encoded superantigen leads to cognate interactions similar to those induced by MHC peptide. IDCs seem to play a major role in priming SAg-specific T cells. It is followed by a cognate interaction between primed T cells and infected virus-specific B cells at the edge of the T zone. Those B cells migrate into the medullary cords, where they clonally expand and differentiate into plasma cells in the virtual absence of T cell help. This extrafollicular B cell differentiation results in the amplification of the virus infection as well as in the secretion of virus envelope-specific antibodies. Later, interaction occurs between virus-infected B cells and SAg-specific T cells in germinal centers. The sustained follicular response may be important in maintaining virus infection and completing the virus life cycle.

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