Induction of Long-lived Germinal Centers Associated with Persisting Antigen after Viral Infection

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Summary

Vesicular stomatitis virus (VSV) induces an early T cell-independent neutralizing IgM response that is followed by a long-lived, T cell–dependent IgG response. We used the specific amplification factor of several 100× of VSV-virions for immunohistology to analyze the localization of VSV-specific B cells at different time points after immunization. At the peak of the IgM response (day 4), VSV-specific B cells were predominantly present in the red pulp and marginal zone but not in the T area. These B cells were mostly stained in the cytoplasm, characterizing them as antibody secreting cells. By day 6 after immunization, germinal centers (GC) containing surface-stained VSV-specific B cells became detectable and were fully established by day 12. At the same time, large VSV-specific B cell aggregates were present in the red pulp. High numbers of VSV-specific GC associated with persisting antigen were present 1 mo after immunization and later, i.e., considerably longer than has been observed for haptens. Some GC, exhibiting follicular dendritic cells and containing VSV-specific, proliferating B cells were still detectable up to 100 d after immunization. Long-lived GC were also observed after immunization with recombinant VSV-glycoprotein in absence of adjuvants. Thus some anti-virally protective (memory) B cells are cycling and locally proliferate in long-lived GC in association with persisting antigen and therefore seem responsible for long-term maintenance of elevated antibody levels. These observations extend earlier studies with carrier hapten antigens in adjuvant depots or complexed with specific IgG; they are the first to show colocalization of antigen and specific memory B cells and to analyze a protective neutralizing antibody response against an acute viral infection.

B cell memory is characterized by (*a*) the presence of isotype-switched memory B cells and (*b*) elevated levels of specific antibody. After reexposure of the host to a pathogen, secondary B cell responses are generated more rapidly and produce higher antibody titers than primary B cell responses. Not the presence of memory B cells as such, but antibody titers available before secondary exposure are the most powerful strategy to cope with secondary infection, since it may allow the elimination of a given pathogen even before an actual infection occurs (1, 2). There is strong evidence that the maintenance of such memory Ig titers is dependent upon the presence of specific antigen persisting on follicular dendritic cells (FDC)[1] (3–6). However, it remains controversial whether or not the survival of memory B cells needs the continuous presence of persisting antigen. It has been shown that memory B cells die off rapidly upon adoptive transfer into antigen-free hosts (7, 8), supporting the concept of antigen dependence of memory B cells. On the other hand, phycoerythrin-specific memory B cells have been shown not to proliferate or proliferating only minimally, suggesting a more antigen-independent type of memory B cell (9). In addition, after local viral infections, no accumulation of specific memory B cells has been found at the sites of antigen persistence. On the contrary, after acute infection in the absence of adjuvants or preexisting IgG antibodies, memory B cells freely recirculate throughout the lymphatic system independently of the localization of persisting antigen, excluding a strict association between memory B cells and persisting antigen. This renders the demonstration of a strong antigen-dependence of memory B cells difficult and unlikely (6). In studies using hapten-antigen either in adjuvants or complexed with specific IgG, preferential accumulation of memory B cells in the local lymph node has been found (10–12). This is probably due to the continuous leaking of antigen in particular when adjuvant was used (11, 12). Also, the high fre-

[1] *Abbreviations used in this paper:* AFC, antibody forming cell; FDC, follicular dendritic cells; GC, germinal centers; TBS, Tris buffered saline; VSV, vesicular stomatitis virus; VSV-IND, VSV serotype Indiana; VSV-NJ, VSV serotype New Jersey.

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quency of hapten-specific B cells in mice in comparison to neutralizing specific ones is probably important to keep in mind (26). In addition, it is important to note that some of the difficulties in interpretation may be due to the fact that some studies did not distinguish between memory B cells and antibody forming cells. In contrast to these model situations, after short-term viral infection, memory antibody titers are maintained by a pool of plasma cells that are generated at the site of antigen persistence and subsequently migrate to the bone marrow (6). Since most of these plasma cells have a rather short life span (13) and have to be generated constantly from the pool of memory B cells, it is hard to envisage a mechanism constantly producing plasma cells in the absence of some memory B cell proliferation. This study therefore attempted to analyze the protective neutralizing memory B cell localization and proliferation after an acute viral infection.

We used vesicular stomatitis virus (VSV) which induces a potent antibody response early after immunization as a model antigen. Neutralizing glycoprotein (G)-specific IgM antibodies are induced in the absence of T help and peak around day 4. Neutralizing IgG antibodies become detectable around day 6 and peak titers are reached within a few days usually between day 8 and 12. This antibody response is critical for the survival of the host both in a primary and a secondary infection. Although the anti-VSV B cell response is very rapid, it represents a true primary B cell response and neither T nor B cells are preprimed by cross-reactive environmental antigens. Cross-reactive T help induced by a second serotype of VSV is short-lived and does not enhance the neutralizing IgG response for more than 2–3 wk (14). Accidentally cross-reactive T help is therefore unlikely to play a role in the VSV-specific B cell response. The presence of VSV-specific primed B cells in naive animals is also very unlikely since (*a*) nu/nu mice that do not have memory B cells due to the absence of T help produce normal IgM levels upon immunization with VSV, (*b*) neutralizing IgG antibodies are detectable 2–4 d after challenge infection in the presence of experimentally induced VSV-specific memory B cells (naive mice mount detectable IgG titers only by day 6–8 after immunization), and (*c*) VSV-neutralizing IgG antibodies from the early immune response are in germline configuration (15, and U. Kalinke, unpublished observation); since memory B cells exhibit hypermutated VDJ-regions (16, 17) the absence of hypermutation is strong evidence for an absence of memory B cells. In addition, sentinel mice kept together with VSV-infected mice do not produce detectable neutralizing antibodies; VSV, therefore, is not an antigen common in the mouse house facility, spreading from infected to non-infected mice.

VSV is a cytolytic virus that does not persist in an infectious form in mice. No infectious virus particles can be isolated from adult normal mice upon peripheral infection with usually 2×10^6 PFU (18, 19); viral RNA can only be detected for 24 h by sensitive PCR techniques detecting ~10–40 copies per spleen, liver, or lung (U. Hoffmann, unpublished observations).

We used VSV-virions as a specific amplification step for immunohistology and analyzed the VSV-specific B cell response in the spleen after primary immunization. This novel approach permitted following the VSV-specific antibody-producing B cells and antigen in parallel. GC containing VSV-specific B cells were present by day 6 after immunization and maximal numbers of GC were reached by day 8. GC specific for VSV were long-lived and could be detected more than 3 mo after immunization. These GC contained VSV-specific B cell blasts that proliferated in association with FDC and persisting antigen. These findings suggest that persisting antigen-associated memory B cells proliferate and differentiate into antibody forming cells to maintain elevated levels of memory IgG antibodies.

Materials and Methods

Mice and Viruses. C57BL/6 mice were obtained from the breeding colony of the Institut für Zuchthygiene (Zürich, Switzerland). VSV Indiana (VSV-IND; Mudd-Summers isolate) and VSV New Jersey (VSV-NJ; Pringle isolate) had been originally obtained from Professor D. Kolakofsky (University of Geneva, Switzerland). Both strains were grown on baby hamster kidney (BHK) 21 cells infected with low multiplicity of infection and plaqued on Vero cells (20). All experiments were performed using VSV-IND unless stated otherwise. The recombinant baculovirus expressing the glycoprotein of VSV-IND was a generous gift of Dr. D.H.L. Bishop (NERC Institute of Virology, Oxford, UK). It was derived from nuclear polyhedrosis virus and was grown at 28°C in Spodoptera frugiperda cells in spinner cultures in TC-100 medium (21).

Preparation of Recombinant Viral Proteins. To produce viral proteins, Spodoptera frugiperda cells at a density of 2×10^6 cells/ml were infected with recombinant baculoviruses expressing the glycoprotein of VSV with a multiplicity of infection of 10 for 24 h at 28°C. Infected cells were harvested, disrupted by sonication, and stored at −20°C.

The presence of antigen was confirmed by Western blot analysis and concentration of proteins was estimated by SDS gel analysis.

Serum Neutralization Test. Serum was collected from mice at specific time points after VSV infection. The sera were prediluted 40-fold in minimal essential medium containing 5% FCS, then heat-inactivated for 30 min at 56°C. Serial twofold dilutions were mixed with equal volumes of VSV diluted to contain 500 pfu/ml. The mixture was incubated for 90 min at 37°C in an atmosphere with 5% CO_2. 100 μl of the serum-virus mixture was transferred onto Vero cell monolayers in 96-well plates and incubated for 1 h at 37°C. The monolayers were then overlaid with 100 μl of DMEM containing 1% methyl cellulose. After incubation for 24 h at 37°C the overlay was flicked off and the monolayer was fixed and stained with 0.5% crystal violet. The highest dilution of serum that reduced the number of plaques by 50% was taken as titer. Because the addition of an equal volume of virus, the titer of serum was considered to be one step higher. To determine IgG titers, undiluted serum was pretreated with an equal volume of 0.1 mM β-mercaptoethanol in saline (22).

Enumeration of Antibody-forming Cells. Antibody-forming cells specific for VSV-IND were enumerated as described (23). In brief, 25-well plates (Greiner, Germany) were coated overnight with purified VSV-IND. After a blocking step, single cell suspensions of spleen cells were added and incubated for 5 h at 37°C. Plates were washed and incubated with goat anti–mouse IgG an-

tibodies, then washed and incubated with phosphatase-labeled rabbit anti–goat antibodies. Antibody spots were developed with alkaline buffer solution (Sigma Chemical Co., St. Louis, MO) containing 5-bromo-4-chloro-3-indolyl phosphate (100 mg/100 ml) mixed with 3% agarose in distilled water (4:1).

ELISA. 96-well plates (Petra Plastic, Switzerland) were incubated with purified VSV-specific monoclonal antibody VI10 (0.1 μg/well) or an irrelevant, isotype-matched control antibody (0.1 μg/ml) in 0.1 M NaH_2PO_4, pH 9.6, at 4°C. Plates were then blocked with 2% BSA in PBS for 2 h, washed, and then serial dilutions of VSV were added to the wells and then incubated for 1 h. Plates were washed and incubated with biotinylated VSV-specific monoclonal antibody VI22 for 1 h. Plates were washed and incubated with peroxidase-labeled Streptavidin (Tago, Burlingame, UK). After 1 h, plates were washed and developed with ABTS (5 mg 2,2′-azino-di-3-ethyl-benzthiazolinesulfonate and 20 μl H_2O_2 in 50 ml $NaHCO_3$, pH 4.0). Optical density was determined at 405 nm.

Immunohistology. Freshly removed organs were immersed in Hank's balanced salt solution and snap frozen in liquid nitrogen by submersing the specimens for a few minutes. Tissue sections of 5 μm thickness were cut in a cryostat, placed on siliconized glass slides, air dried, fixed with acetone for 10 min, and stored at −70°C.

Immunohistological Staining. If not stated otherwise, immunohistological reagents were purchased from DAKO (Glostrup, Denmark). Secondary affinity-purified polyclonal anti-immunoglobulin antisera were diluted in Tris-buffered saline (TBS) containing 5% normal mouse serum. All other dilutions were made in TBS. Incubations were performed at room temperature for 30 min; TBS was used for all washing steps. Sections were counterstained with hemalum and coverslips were mounted with glycerol/gelatin.

Detection of VSV-specific B Cells. Dehydrated tissue sections were overlaid with a solution of UV light-inactivated VSV (3×10^6 pfu/ml before inactivation) for 4 h. Specifically bound virus was detected by incubation with polyclonal rabbit anti-VSV-IND serum (diluted 1:1,500; the antiserum had a neutralizing titer of $>1{:}10^5$), followed by alkaline phosphatase-labeled goat antibodies to rabbit immunoglobulins and rabbit anti–goat immunoglobulin antibodies (diluted 1:80; Jackson ImmunoResearch Labs., Inc., West Grove, PA). Alkaline phosphatase was visualized using naphtol AS-BI phosphate and New Fuchsin as substrate, which yields a red precipitate. Endogenous alkaline phosphatase was blocked by levamisole.

Double Staining for VSV-specific B Cells and DNA-incorporated BrdU. The double staining was essentially performed as described (24). For the detection of VSV-specific B cells, the procedure as described above was used. The substrate used for alkaline phosphatase was naphtol AS-MX phosphate and Fast-Blue BB salt, yielding a blue precipitate. Sections were then treated with 1 M HCl for 20 min to denature the DNA and to terminate the immunoenzymatic reaction without displacing the blue reaction product. Slides were then incubated with the monoclonal rat anti-BrdU antibody BU1/75. This rat antibody was revealed by sequential incubation with rabbit antibodies against rat-immunoglobulins and rat APAAP-complex (diluted 1:50; applied three times). Alkaline phosphatase was visualized with naphtol AS-BI phosphate and New Fuchsin (red precipitation product).

PNA-binding and Staining of Other Markers. Sections were incubated with PNA (diluted 1:200) and bound PNA was detected by rabbit anti-PNA antibodies (diluted 1:300) followed by alkaline phosphatase-labeled antibodies to rabbit immunoglobulins (1:300; TAGO). Rabbit antisera against mouse IgM and IgG were used for the demonstration of mouse immunoglobulins. CD4 was detected by the rat monoclonal antibody YTS191 (ascites was kindly provided by Dr. H. Waldman, Oxford, UK; dilution 1:6,000). For follicular dendritic cell staining, sections were incubated with 1:300 diluted affinity-purified monoclonal rat antibody 4C11 (25). Primary antibodies were detected using a double sandwich staining technology. Alkaline phosphatase-labeled goat antibodies to rabbit immunoglobulins or rat immunoglobulins, respectively (Jackson ImmunoResearch Labs.), were diluted 1:80, applied for 30 min and followed by alkaline phosphatase-labeled donkey antibodies against goat immunoglobulins (TAGO) diluted 1:80 for another 30 min. Naphtol AS-BI phosphate and New Fuchsin were used for the color reaction.

Detection of Persisting Viral Antigen. Sections were incubated with a rabbit anti-VSV-G antiserum (diluted 1:1,000) for 30 min. Affinity-purified alkaline phosphatase-labeled goat antibodies to rabbit immunoglobulins were diluted 1:80, applied for 30 min, and followed by alkaline phosphatase-labeled donkey antibodies against goat immunoglobulins diluted 1:80 for another 30 min. Alkaline phosphatase was detected using Nitro Blue Tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolylphosphate (NBT) BCIP as a substrate. Sections were counterstained with Kernechtrot 0.1% for 3 min; the NBT/BCIP reaction is considerably more sensitive than the Napthol AS-BI phosphate reaction.

Results

VSV-specific B Cell Response: Neutralizing Antibodies and Antibody-forming Cells (AFC). Mice were immunized with VSV and the neutralizing B cell response was followed for 100 d and VSV-specific AFC frequencies were determined (Fig. 1). Neutralizing IgM antibody titers were maximal by around day 4 confirming earlier results and IgG antibodies appeared by day 6. Neutralizing IgG levels reached maximal levels by day 12–20 and stayed at a high titer for the time period measured (Fig. 1 *B*). Frequencies of VSV-specific IgG producing AFCs were maximal around day 8, declined relatively rapidly between day 8 and day 20, disappeared slowly thereafter but were still detectable 100 d after immunization (Fig. 1 *A*). The relatively more drastic decrease of the AFC frequencies compared to the antibody titer has been discussed in detail elsewhere and can be explained by the rapid proliferation of specific B cells during the early phase of the immune response and an increasing proportion of AFCs present in the bone marrow at the later time points (6, 26). Thus, VSV induces a long-lived neutralizing antibody response along with an increased frequency of VSV-specific antibody forming cells. Live virus is not necessary for this type of response, since recombinant VSV-glycoprotein induces a similar response (not shown).

Histological Detection of VSV-specific B Cells. In an attempt to analyze the VSV-specific B cell response in situ, a method to detect VSV-specific antibodies was developed in vitro. ELISA plates were coated with purified monoclonal VSV neutralizing antibody VI10 (15) and incubated with graded amounts of VSV particles. Bound VSV was detected with a biotinylated VSV-specific monoclonal antibody VI22. VSV was used either as purified virus or as non-purified stock virus; both virus preparations gave comparable results (Fig.

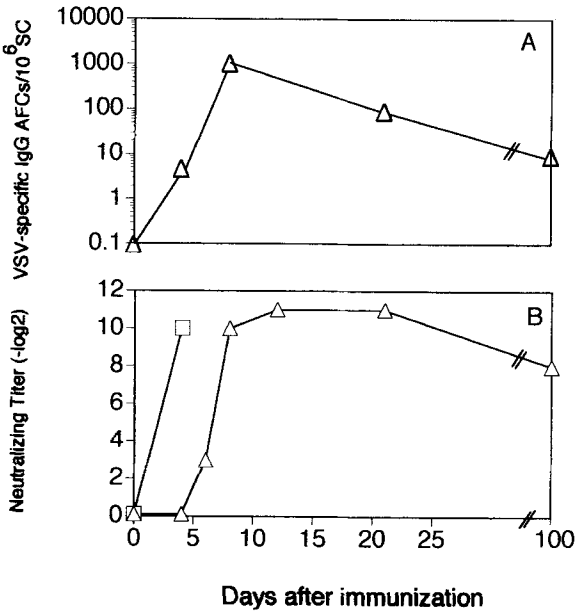

Figure 1. VSV-specific antibody response. Mice were immunized with VSV (2×10^6 PFU) and the number of specific IgG-producing AFCs was determined at the indicated time points (*A*). Neutralizing IgM (*squares*) and IgG (*triangles*) titers were determined from 40-fold prediluted sera (*B*). The mean of three mice is shown at all time points. Variations in *A* were smaller 30%; in *B*, variations were smaller than two dilution steps.

2). Thus, VSV virions could be used as a specific probe to detect VSV-specific antibodies. A similar method as this catcher ELISA was used to detect VSV-specific B cells expressing VSV-specific antibodies in the spleen of immunized mice (Fig. 3). Spleen cell sections were incubated with VSV used for specific amplification and bound virus was detected with a VSV-specific antibody. A polyclonal, VSV-specific rabbit serum was used and subsequently detected with phosphatase-labeled secondary antibodies.

4 d after i.v. immunization, spleen sections were stained for PNA-binding (Fig. 3 *A*) for FDCs (*B*), VSV-specific B cells (*C*), IgG (*D*), CD4 (*E*), and IgM (*F*). VSV-specific B cells were predominantly found in the red pulp and the marginal zone areas with some specific B cells scattered in the B cell follicles and very few B cell in T-areas (Fig. 3 *C* and see Fig. 6). Many of the VSV-specific cells were stained

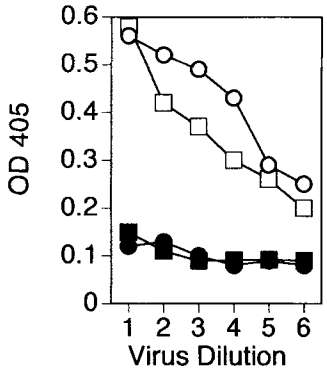

Figure 2. Detection of VSV-specific antibodies by ELISA. ELISA plates were coated with purified monoclonal VSV neutralizing antibody VI10 (*open symbols*) or an isotype matched, irrelevant antibody (*closed symbols*) and incubated with twofold serial dilutions of purified virus (*squares*) or non-purified stock virus (*circles*). Bound virus was detected with VSV-G-specific biotinylated monoclonal antibody VI22.

in the cytoplasm, suggesting that they were secreting antibody. No stained cells could be detected on spleen sections from non-immunized mice (not shown). At this early time point, no VSV-specific GC could be observed and no IgG-expressing B cells were found in IgM positive follicles. Since almost no VSV-specific IgG producing B cells can be detected on day 4 of the response, the stained B cells in Fig. 3 *C* apparently produce IgM. On day 2 after immunization, only very few VSV-specific B cells could be found (Fig. 4 *A*).

Induction of GC. On day 4 after immunization, GC were absent as seen by both lack of PNA binding and FDC accumulations (Fig. 3, *A* and *B*). By day 6 after immunization, GC became detectable (Fig. 4 *B*) and increased in numbers until day 8–12 (Fig. 5). On day 12, the GC were PNA positive (Fig. 3 *G*), exhibited grouped FDC (Fig. 3 *H*), VSV-specific B cells (Fig. 3 *I*), some $CD4^+$ T cells (Fig. 3 *L*) and lightly surface IgM-positive cells in GC clearly distinct from the IgM positive mantle zone B cells (while they were also B220 positive, not shown) and marginal zone B cells (Fig. 3 *M*). In addition, many IgG expressing B cells were present in GC (Fig. 3 *K*). The localization of FDC scattered on day 4 and well centered in GC after day 6 is very striking (Fig. 3, *B*, *H*, *O*). Almost all VSV-specific GC B cells only stained on the cell surface. In Fig. 4 *C*, a higher magnification of a day 12 spleen section is shown in which surface stained GC B cells (left side) can clearly be distinguished from more intensely, cytoplasmically stained non-GC B cells on the right side. The intensely stained B cells within aggregates in the red pulp outside of GC did not bind PNA as assessed on an adjacent section (not shown).

Great numbers of GC were still present 3 wk to 1 mo after immunization (Fig. 4 *D*) and some GC were detectable even 100 d after immunization (not shown and Fig. 5). These late GC were still well defined and associated with follicular dendritic cells (not shown).

Control spleens from non-immunized mice or from mice reconstituted with polyclonal anti-VSV-specific serum did not reveal any specific staining (not shown). Neutralizing antibodies do not cross-react by definition between VSV serotype Indiana and serotype New Jersey whereas non-neutralizing, binding antibodies cross-react to some extent (27, 28). A good specificity control was that spleen sections from mice immunized with VSV serotype New Jersey showed no significant staining distinct from non-immunized mice when VSV serotype Indiana was used for the amplification step, suggesting that this assay dominantly detects neutralizing antibodies (not shown).

VSV does not replicate measurably extraneuronally in normal adult mice and since it is cytolytic, it is unlikely that it persists latently in infected cells (19). We nevertheless tested whether the long-lived GC were dependent upon an infectious form of VSV-G or not. Mice were immunized intravenously with recombinant VSV-G protein (10 μg) and spleen sections were stained 1 mo later for the presence of VSV-specific B cells. As with live virus, spleens from mice immunized with recombinant VSV-G exhibited well-defined GC (not shown).

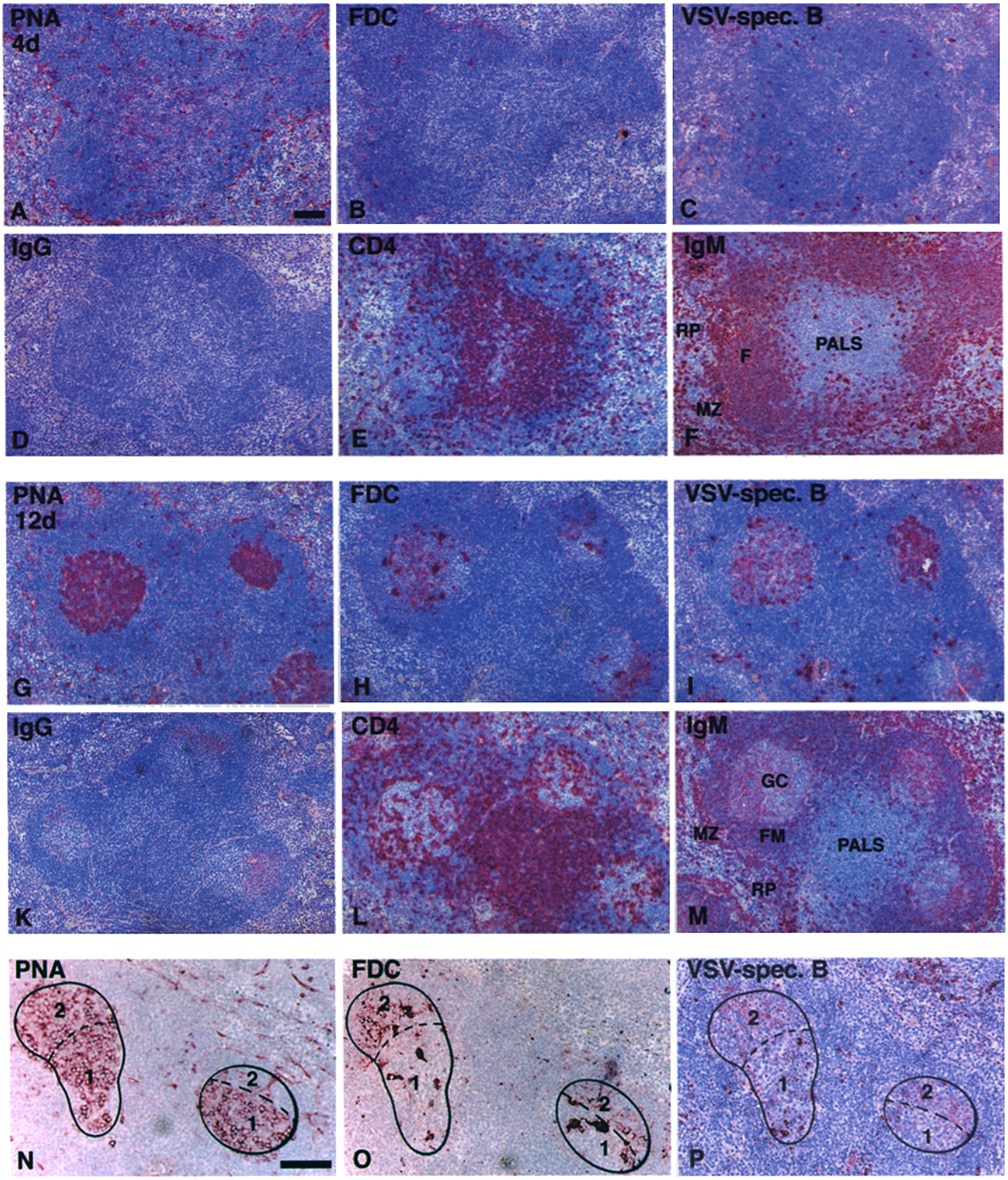

Figure 3. Localization of VSV-specific B cells upon immunization with VSV. Mice were immunized with VSV (2×10^6 PFU) and adjacent spleen sections were stained 4 d (*A–F*) or 12 d (*G–P*) later. The stained quality is indicated in each panel. *PNA*, peanut hemagglutitin binding; *FDC*, follicular dendritic cells; *VSV-spec. B*, VSV-binding B cells; *IgG*, IgG positive cells; *CD4*, CD4 positive cells; *IgM*, IgM positive cells; *PALS*, periarteriolar lymphatic sheath; *F*, follicle; *MZ*, marginal zone; RP, red pulp; *GC*, germinal center; *FM*, follicular mantle zone. *1* corresponding to dark zone and *2* corresponds to light zone of GC in *N–P* (see text). Bars: (*A–M* and *N–P*) 100 μm. Original magnification: *A–M*, ×110; *N–P*, ×140.

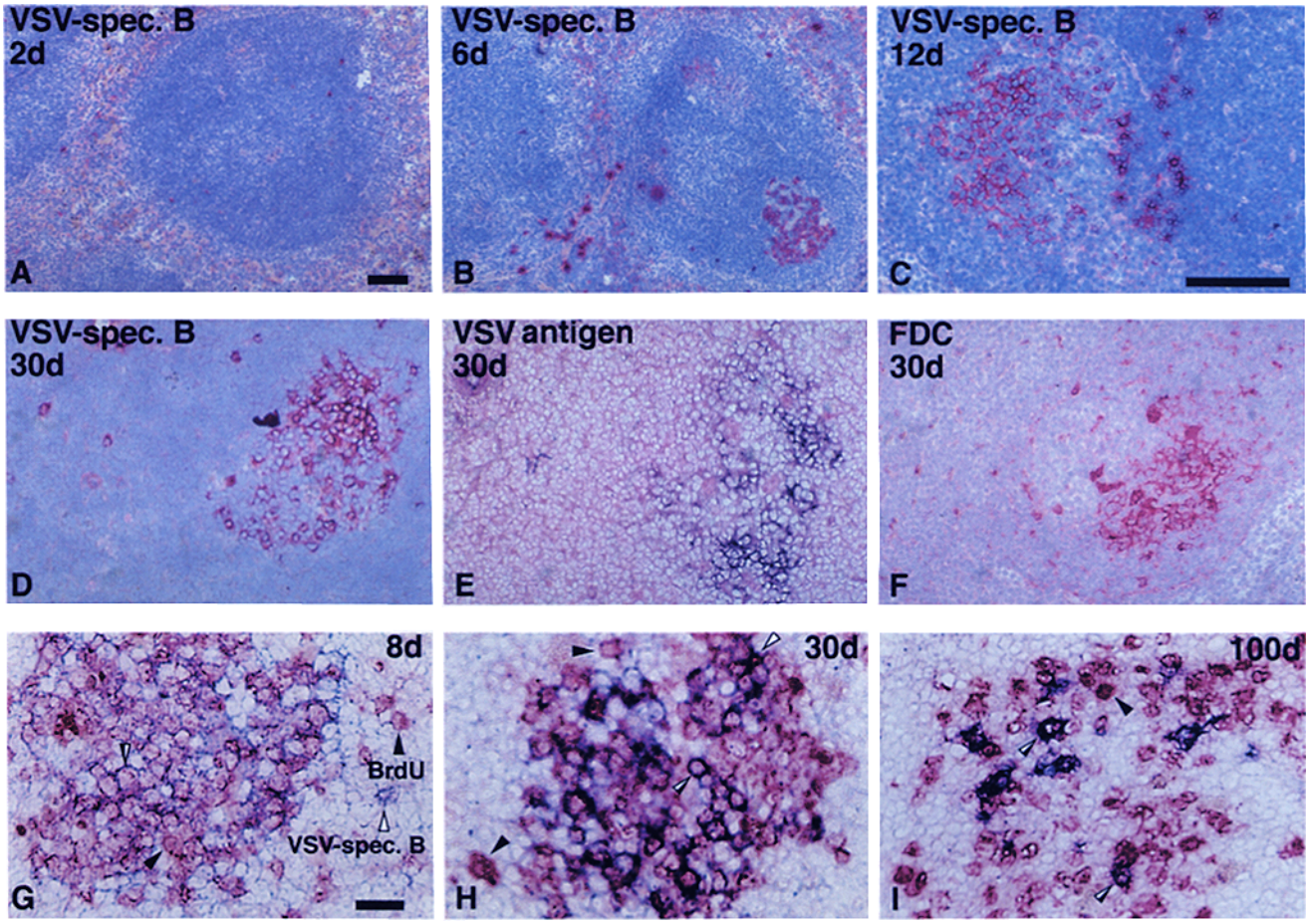

Figure 4. Histological analysis of VSV-specific B cell responses: Induction of long-lived GC associated with persisting antigen and containing proliferating B cells. Mice were immunized with VSV (2×10^6 PFU) and spleen sections were stained 2 d (*A*), 6 d (*B*), or 12 d (*C*) later for VSV-specific B cells. Spleen sections of mice immunized 30 d before with UV-inactivated lysates of infected cells (i.e., one dose of non-replicating antigen) were stained for VSV-specific B cells (*D*). Persisting VSV-antigens were detected on an adjacent section with a rabbit anti-VSV-G serum and a very sensitive alkaline phosphatase labeled goat anti-rabbit antibody plus a third stage antibody. Sections were developed with NBT/BCIP yielding a black reaction product (*E*). The same staining with a normal rabbit serum was negative. A second adjacent section was stained for FDC (*F*). Although *D*, *E*, and *F* are not quite identically oriented, the central arteriole and the FDC clumps seen in *F* can be used to compare the sections. In *G–I*, mice were immunized with VSV (2×10^6 PFU) and 16 h before analysis, 2 mg BrdU was injected intraperitoneally. Spleen cell sections were doubly stained (*half-open, half-closed triangle*) for VSV-specific B cells (*blue, open triangle*) and BrdU (*red, closed triangle*) 8 d (*G*), 30 d (*H*), or 100 d (*I*) after immunization. Even 100 d after immunization, some VSV-specific B cells had incorporated BrdU within 16 h. For abbreviations see Fig. 3 legend. Bars: (*A*, *B*, and *C–F*): 100 μm; *G–I*, 30 μm. Original magnifications: *A* and *B*, ×110; *C–F*, ×270; *G–I*, ×400.

Long-lived GC Are Associated with Persisting Antigen. As previously described (6), persisting antigen can optimally be detected by immunohistology after immunization of mice with VSV-infected cell lysates. Mice were immunized i.p. with 10^7 VSV infected, sonicated and UV-light inactivated cell lysates and 1 mo later, persisting VSV-G was detected using a specific rabbit anti-VSV-G serum (Fig. 4 *E*). As expected, sites of antigen persistence colocalized with FDCs, as revealed by FDC-specific staining of an adjacent section (Fig. 4 *F*). On a second adjacent section, VSV-specific B cells were stained, demonstrating that the same GC contained persisting VSV antigens and VSV-specific B cells (Fig. 4 *D*). For this staining, the following practical problem had to be solved: VSV-specific B cells are stained by detecting viral particles bound to specific B cells with a polyclonal rabbit anti-VSV serum. Persisting antigen, on the other hand, is detected similarly with a rabbit anti-VSV-G serum. Thus, we had to be able to distinguish between viral particles bound to specific B cells and viral antigens persisting on FDCs. VSV particles bound to specific B cells represent a rather sizable concentration of viral antigens considerably greater in amounts than VSV antigens persisting on FDCs. We therefore were able to selectively use staining conditions that were only sensitive enough to reveal viral particles bound to specific B cells that were, however, not sufficient to stain FDC-associated persisting antigen. This was done by (*a*) using a very high titered rabbit serum for detection of persisting antigen and a different, lower titered one for detection of B cell–bound viral particles and (*b*) using two different color reactions, the greater sensitivity of the NBT/BCIP for detection of persisting antigen versus the less sensitive AS-BI phosphate and New

Fuchsin reaction for the added and bound VSV. To ensure that in Fig. 4 *D* indeed VSV-specific B cells were detected and not persisting viral antigens, a control section was stained as in Fig. 4 *D*, except that no viral particles were added (not shown). As expected, no specific staining was revealed, confirming that VSV-specific B cells were detected in Fig. 4 *D*. Thus, GC containing VSV-specific B cells colocalize with persisting antigen bound on FDCs.

Characterization of GC. Mouse GC B cells bind PNA. In addition, they colocalize with FDCs, which present native antigen to specific B cells (for review see reference 29). In Fig. 3 *P*, two GC containing VSV-specific B cells are shown 12 d after immunization; in addition, there is a cluster of specific B cells in the red pulp (*top*, to the right side). In Fig. 3, *N* and *O* adjacent sections were stained for PNA binding and FDCs, respectively. As expected, the VSV-specific B cells in GC bound PNA (Fig. 3 *N*) but did not express IgD (not shown) and colocalized with FDCs (Fig. 3 *O*). In contrast, the B cells of the cluster in the red pulp (Fig. 3 *P*) did not bind PNA.

The fine architecture of GC as has been described for hapten-specific immune responses and human GC (29). Although less clear, they were also found here for VSV-specific GC. Accordingly, a zone 1 (dark zone) containing centroblasts that do bind only little antigen (Fig. 3 *P*) but bind PNA (Fig. 3 *N*) and do not colocalize with the fine mesh-forming FDCs (Fig. 3 *O*) and a zone 2 (*light zone*), containing centrocytes that do bind the antigen and colocalize with FDCs was observed. Occasionally, few B cells binding VSV on the surface and also intracellularly could be observed in the zone 1 (Fig. 3 *P*). The relevance or function of these B cells is not known; it is conceivable that these cells are freshly induced plasma cells leaving the GC.

VSV-specific B cells in GC of spleens from mice immunized 100 d previously did also bind PNA and colocalized with FDCs (not shown). Interestingly, the distribution of FDCs was not uniform, but FDCs predominated on one side of the GC (Figs. 3 *O* and 4 *F*). It is also noteworthy that although the network of FDCs was dominant on one side of the GC, some areas of densely packed FDCs could be seen on the border where dark and light zone meet and also within the dark zone on day 12 after immunization (Fig. 3, *H* and *O*). The same was true for the late GC (Fig. 4 *F*).

For all sections shown in Figs. 3 and 4, adjacent sections were stained for PNA and FDCs (only few are shown); all GC were PNA positive and colocalized with FDCs. Interestingly, some GC B cells of the later time points exhibited intracellular staining for antibody (e.g., Fig. 4, *D* and *I*); this was hardly observed in day 6 (Fig. 4 *B*) or day 12 (Fig. 3, *I* and *P* and Fig. 4 *C*) GC. Apparently, the late GC B cells appeared to have matured more towards the plasma cell phenotype.

GC are sites of intensive B cell proliferation. We confirmed this for VSV-specific B cells in GC of spleens from mice immunized 8 d previously. Mice immunized with VSV were treated with BrdU 7 d later (2 mg in PBS, i.p.). The next day, spleens were harvested and double-stained for VSV-specific B cells (*blue*) and BrdU incorporation (*red*); (note that the red color here denotes BrdU-positive cells and blue indicates VSV-specific B cells in Fig. 4, *G–I*). Nearly all VSV-specific B cells incorporated BrdU indicating that they were proliferating (Fig. 4 *G*). Interestingly, 30 and even 100 d after immunization, VSV-specific B cells (*blue*) of GC still incorporated BrdU (*red*) during the 16 h before fixation (Fig. 4, *H* and *I*). As already mentioned, the GC B cells of the late time points (Fig. 4, *H* and *I*) exhibited a more intensive staining (blue, that in some cells is intracellular) than the only surface-stained B cells of the day 8 GC (Fig. 4 *G*).

The number of GC was analyzed at the different time points after immunization (Fig. 5). The first GC appeared by day 6 after immunization and some of these early GC were heterogeneous in size. At later time points, GC were much more numerous and also larger with some exhibiting diameters of up to 500 μm. By day 100, the number of GC had decreased considerably and very large GC were not observed.

Localization of Specific B Cells Outside GC. On day 2 (Fig. 4 *A*) or day 4 (Fig. 3 *C*) after immunization almost no VSV-specific B cells were found in the T cell areas; they were only present in the red pulp/marginal zone or as single cells in B cell follicles (Fig. 6). Thus, VSV-specific B cells seem not to be induced in the T cell areas but rather in the primary B cell follicles or in the red pulp/marginal zone. This contrasts with hapten-specific B cell responses, since hapten-specific B cells have been shown to be induced in the T cell areas where they can be observed as plasma cell foci at the edge of the T cell areas (24, 30). Between day 6 and day 12, most VSV-specific B cells outside GC were present in the red pulp. Interestingly, this distribution had changed by 3 wk after immunization, since an increasing proportion of specific B cells was present as single cells within the follicles. At these later time points, no large B cell aggregates were present in the red pulp and B cells outside follicles were often associated with the marginal zone. These B cells probably represent marginal zone memory B cells (31). The proportional increase of VSV-

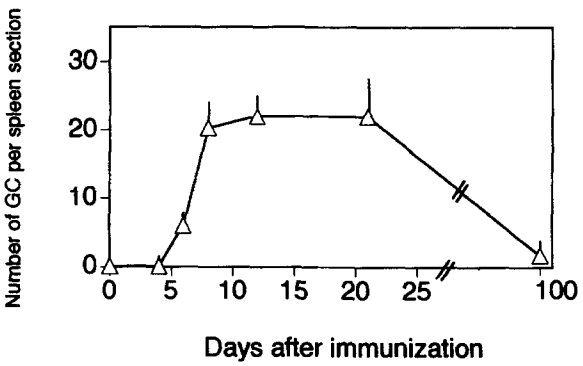

Figure 5. Number of VSV-specific GC per spleen section. Mice were immunized with VSV (2×10^6 PFU) and spleen sections were analyzed for the presence of VSV-specific GC. At least three sections from two different mice were counted. Bars indicate the standard error of the mean.

specific follicular B cells at these late time points does not reflect an increase of absolute numbers, since the total number of specific B cells decreases (Fig. 6).

Discussion

VSV induces a potent antibody response that is protective in vivo (26, 32). The early IgM response is independent of the presence of T helper cells due to the repetitive pattern of the VSV-G in the envelope (33), whereas the ensuing IgG response strictly requires T help (34). VSV induces a strong memory B cell response with life-long high IgG titers. VSV differs in this respect from most other normally studied antigens, since it is both a T cell–independent antigen for IgM and a T cell–dependent antigen for IgG. This, however, appears to be a common characteristic of many pathogens (35–37).

Although the sites of B cell induction and proliferation upon immunization have been described in great detail for haptenated proteins alone, in adjuvants, or given as antibody-complexes and for haptenated LPS (9–13, 30, 31, 38–40), no histological analysis of a virus-specific neutralizing and protective B cell response has been performed so far, most probably due to technical limitations. In this study we analyzed the VSV-specific B cell response using the virus itself as a specific amplification step on immunohistological sections.

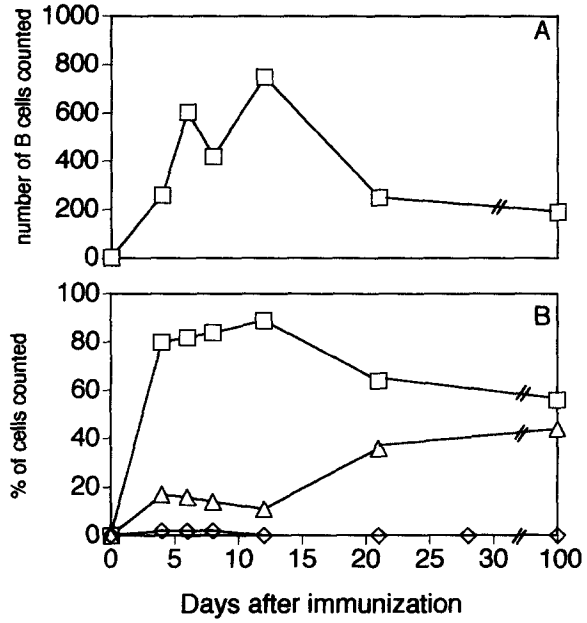

Figure 6. Number of VSV-specific B cells outside GC. Mice were immunized with VSV (2×10^6 PFU) and spleen sections were analyzed for the presence of VSV-specific B cells. The total number of non-GC B cells within 20 mm^2 is indicated in *A* for each time point. In *B*, the percentage of B cells in the red pulp/marginal zone (*squares*), B cell follicles (*triangles*), and T cells areas (*diamonds*) is indicated. Note the small variability in percentages in B within the early (day 4 to day 12) and within the late (day 21 to day 100) time points indicating the reliability of the assessment.

T-dependent B cell responses are exclusively induced in the T regions of the spleen and lymph nodes and first foci of antibody-producing B cells were usually found at the contact sites of the T region and B cell follicles when hapten carrier responses were studied (24, 30, 31). In contrast to these results, no significant numbers of VSV-specific B cells were found within the T area on day 2 and day 4 during the early phase of the B cell response, but specific B cells were found mostly in the red pulp and in B cell follicles. As specific B cells in the red pulp by far outnumbered specific B cells in the follicles and since all of the viral antigen is found in the red pulp/marginal zone of the spleen or the subcapsular sinus in the lymph nodes early after immunization (6, 41), it is likely that B cells were directly activated in the red pulp or the marginal zone. Although we cannot formally exclude that few early B cells are induced in the follicles and subsequently migrate to the red pulp and proliferate, it appears that in the early response most VSV-specific B cells are not induced in the T area. This may not appear surprising since VSV is a strong T-independent type I antigen (37) without being a polyclonal activator (42) and apparently induces B cells directly and in the absence of T help (33, 37). This notion is supported by the fact that on day 4 after immunization, VSV-specific B cells are distributed similarly in mice depleted of $CD4^+$ T cells (not shown). However, these findings seem to differ from results obtained with haptens coupled to LPS, another type I T-independent antigen (38), where B cell activation was found to predominantly occur in T areas. The difference between the two analyses may reflect different localization of antigens after immunization and/or are related to the large difference in specific B cell frequencies of $\sim 10^{-2}$–10^{-3} for some haptens versus 10^{-5} for VSV-neutralizing B cells (26).

Throughout the early B cell response, many VSV-specific B cells were found in the red pulp and around terminal arterioles. These B cells exhibited intensive intracellular staining and therefore were antibody-secreting cells. This is consistent with the finding of very high frequencies of AFCs at these early time points (10^{-3} spleen cells). 3 wk after immunization and later, the proportion of specific B cells in the red pulp decreased substantially and the proportion of specific follicular B cells, that were not associated with GC, increased. This suggests that memory B cells are not only found in the marginal zone (31) but are also present as follicular B cells at least for late anti-VSV responses. The reduced number of specific B cells in the red pulp is consistent with the finding of overall decreasing frequencies of AFCs at these later time points.

GC appeared as early as 6 d after immunization with similar kinetics as T help and IgG antibodies are generated (14). $CD4^+$ T cells could be identified in VSV-specific GC but at this stage of the analysis we cannot define the specificity of the $CD4^+$ T cells. GC increased rapidly both in size and number. At the peak of the response, almost all GC present in the spleen contained B cells specific for VSV. Many GC exhibited a typical architecture and the previously described dark and light zones (29) could be identified (Fig. 3). This architecture is thought to be cru-

cially involved in the affinity maturation of the antibody response. It is therefore interesting to note that despite the occurrence of GC with a normal structure and containing $CD4^+$ T cells during the anti-VSV response, the overall affinity of the antibodies does not increase after day 6 of the response (15). It may be that for antigens inducing a high affinity response early, as is the case for VSV, the chances are high that hypermutation (which is occurring and is being analyzed; U. Kalinke, H. Hengartner, and R.M. Zinkernagel, unpublished observation) causes a reduction rather than a further increase of affinity. Somatic selection in the GC therefore may maintain high affinity in an equilibrated fashion by eliminating hypermutated clones that lowered or lost their specificity. Therefore, no overall increase of the already high average affinity is seen.

GC did not disappear by 3 wk after immunization but remained present at high numbers; they were still detectable 100 d after immunization although fewer and no large GC were observed. GC were still associated with persisting antigen on FDC and GC B cells were PNA^{high} and IgD^{low} (not shown), and B cells proliferated also at the late time points (Fig. 4, *H* and *I*). These late GC were clearly different from the very few specific B cell blasts found scattered in B cell follicles 6–7 wk after immunization with haptens (24) in that specific B cells were clustered and associated with persisting antigens on FDC. Many of the late GC B cells found 100 d after an acute VSV infection had a different phenotype as compared to GC B cells of day 8–12. Many B cells were not only surface positive for Ig expression but also the cytoplasm stained for presence of specific antibody (Fig. 4, *D* and *I*). It therefore appeared that GC B cells at the later time points had differentiated towards the plasma cell phenotype. This suggests that the GC may be involved in antibody production and the maintenance of memory IgG titers (see below). GC B cells apparently differentiate to the memory cell phenotype in the presence of activated T helper cells (i.e., via CD40− ligand) and to the plasma cell phenotype in the absence of activated T helper cells (43). Since the memory T helper cell response after VSV− infection seems to decline rapidly (14), it is likely that GC B cells differentiate at the later time points in the presence of few or even in absence of activated T helper cells and differentiate to the plasma cell phenotype (see also reference 44).

VSV, similar to many vaccines, induces a memory B cell response in the absence of a long-term depot in adjuvants (also in the absence of detectable viral RNA; U. Hoffmann, unpublished observation) and viral antigen persists only in its natural form in association with FDCs. Despite the fact that VSV infects few cells in an abortive way after infection and therefore behaves like a "non-infectious" antigen, our findings overall contrast with kinetics of hapten-specific B cell responses that are considerably more short-lived, despite the use of adjuvants or of preformed antigen-antibody complexes. Nevertheless, to overcome the potential problem that undetected VSV genetic information may maintain antigen supply, experiments were repeated with recombinant VSV-G protein, injected i.v. or subcutaneously in the absence of adjuvants with comparable results. Thus the induction of long-lived GC was independent of the use of a replicating agent but most probably dependent on the presence of persisting FDC-associated antigen. This is supported by the findings (*a*) long-lived GC are associated with persisting antigen, (*b*) that VSV-specific AFC are only found in lymphoid organs containing persisting antigen, and (*c*) that memory anti-VSV IgG levels can be increased by antigen-antibody complexes that bind to FDC (5, 6). These results on the role of antigen complexes in local lymphoid organs persisting bound to FDCs extend those obtained earlier with carrier hapten antigens injected in adjuvants or complexed with antibodies (3, 4, 10, 11, 24). Since viral antigens seem to maintain such long-term memory without adjuvants our results suggest that viral antigens may have evolved to persist for a long time possibly by being relatively resistant to degradation, when compared to immunological model antigens.

It has been shown previously that memory B cells recirculate throughout the lymphatic tissue whereas antibody forming cells are restricted to the sites of antigen persistence (and the bone marrow) (6, 10, 29). Memory AFC induction may therefore reflect: (*a*) recirculating B cells migrate through the lymphatic tissue and differentiate to AFC whenever they encounter persisting antigen. (*b*) There are two distinct sets of memory B cells, one population of recirculating B cells that do not proliferate and that are detected in classical adoptive transfer experiments and a second population of memory B cells that remain associated with persisting antigen, proliferate and differentiate to AFC. These AFC then either migrate to the red pulp/marginal zone or eventually to the bone marrow.

The finding of long-lived GC associated with persisting antigen on FDC presented here supports the latter model. If recirculating memory B cells differentiated to AFC whenever they encounter persisting antigen, one would not expect GC like structures with a high concentration of specific B cells as found here with high rates of proliferation, but rather fewer specific B cells as found in the study by Liu et al. for hapten specific B cells 7 wk after immunization (24). Our data are probably also compatible with the previously published observation that most memory B cells apparently do not proliferate (9). In this particular study, phycoerythrin was used as a hapten to induce memory B cells. Since haptens induced short-lived GC and since no long-lived antibody titers are usually measurable in the absence of an antigen depot in adjuvants, this report probably analyzed predominantly the population of non-cycling but recirculating memory B cells.

It should be pointed out that antibody-mediated protective immunity not only depends on the presence of memory B cells, but requires preexisting specific antibody at sufficiently high titers. In many cases, preexisting antibody prevents a secondary infection completely, whereas memory B cells may only enhance kinetics of pathogen elimination without alone being protective (1, 2). As discussed previously for hapten-carrier in adjuvants (3, 4, 10, 12) and here for viral antigens without adjuvant, the maintenance

of memory antibody titers may strictly depend upon the presence of persisting antigen, whereas resting memory B cells appear to be less antigen-dependent. This suggests that only memory B cells activated by persisting antigen differentiate to AFC and produce and maintain protective antibody levels. These protective antibody responses may therefore result from coevolution of the critical neutralizing epitopes to persist in the host so that high levels of antibodies are maintained. These antibodies are also essential for transmission of passive protection by the mother to immunodeficient offspring during maturation of their T cell immunity (45).

Overall, there is a parallel to memory cytotoxic T cells where physical presence of memory cytotoxic T cells seems to be independent of persisting antigen (46, 47). These memory cytotoxic T cells may circulate in the blood; however, they only recirculate through tissue and mediate protective immunity peripherally if activated by persisting antigen (48).

In summary, immunization with VSV leads to long-lived GC associated with FDC that most probably are responsible for the maintenance of the memory IgG titer. This demonstrates that subpopulations of memory B cells may not be static (non-proliferating) but may be highly dynamic and actively proliferating locally in GC.

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