In Situ Studies of the Primary Immune Response to (4-hydroxy-3-nitrophenyl)acetyl. II. A Common Clonal Origin for Periarteriolar Lymphoid Sheath-associated Foci and Germinal Centers

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Summary

In the genetically restricted response that follows immunization with (4-hydroxy-3-nitrophenyl)acetyl coupled to protein carriers, two distinct populations of B cells are observed in the spleens of C57BL/6 mice. By 48 h postimmunization, foci of antigen-binding B cells appear along the periphery of the periarteriolar lymphoid sheaths. These foci expand to contain large numbers of antibody-forming cells that neither bind the lectin, peanut agglutinin, nor mutate the rearranged immunoglobulin variable region loci. Germinal centers containing peanut agglutinin-positive B cells can be observed by 96-120 h after immunization. Although specific for the immunizing hapten, these B cells do not produce substantial amounts of antibody, but are the population that undergoes somatic hypermutation and affinity-driven selection. Both focus and germinal center populations are paucidonal, founded, on average, by three or fewer B lymphocytes. Despite the highly specialized roles of the focus (early antibody production) and germinal center (higher affinity memory cells) B cell populations, analysis of VH to D to J. joins in neighboring loci and germinal centers demonstrate that these B cell populations have a common clonal origin.

During the course of the primary immune response, participant B lymphocytes are segregated into distinct compartments, foci of antibody-forming cells (AFC) at the periphery of the periarteriolar lymphoid sheaths (PALS) and germinal centers (GC) in the lymphoid follicles (1-9). These cellular compartments are quite distinct and can be distinguished not only by their location within the spleen, but by a variety of other markers, including affinity for the plant lectin, peanut agglutinin (PNA) (for reviews see references 5 and 6).

We have studied this compartmentalization in the response of C57BL/6 mice to the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP) (8, 9) where the NP-specific antibodies produced bear the L chain and use the V186.2 segment of the V,58 gene family to encode the H chain V region (10-13). The PALS-associated foci and GC represent mono- to oligo-clonal populations that are similar in that both express the expected canonical V, and V, genes, exhibit heteroclitic binding of the NP analogue, (4-hydroxy-5-iodo-3-nitrophenyl)acetyl (NIP), and undergo IgM → IgG isotype switching (8). However, focus and GC B cell populations also exhibit differences that suggest specialization of their roles in the primary response. PALS-associated foci are the sites of considerable Ig synthesis and are thought to be the major source of early primary antibody (5, 14, 15). In contrast, throughout the primary response, GC B cells produce very little secreted Ig, but are the source for the affinity-selected mutant B cells which dominate the pool of memory lymphocytes (6, 9, 16-18).

The origin of GC B cells and their relationship to the extrafollicular focus populations remains problematic. The traditional view, based largely upon indirect evidence, is that GC B cells are recruited from antigen-activated lymphocytes in extra-follicular locations such as the PALS-associated foci (6). However, Linton et al. (19) have recently suggested that the AFC of foci and the GC cells that enter the memory pool are derived from different precursor cells which may represent independent cellular lineages. Although these hypotheses are not necessarily exclusive, if lineages of primary AFC and memory cell precursors are established substantially before their encounter with antigen, neighboring focus and GC populations would be unlikely to share clonal origins. On the other hand, if GC populations are founded by antigen-activated migrants from the foci, nearby foci and GC would be expected to be clonally related.
An established means of determining the genetic relatedness of B cells is comparison of the DNA encoding the third CDR (CDR3) of the H chain (20, 21). This region is created by the fusion of the Vn, D, and Jn gene segments and often contains imprecise joins and additional, untemplated nucleotides (22, 23). The complexity that results from these presumably random processes is sufficient to identify the members of a single rearrangement event (a clone) with a high degree of confidence (24).

Here we exploit the diversity of the H chain CDR3 to identify the clonal relatedness of focus and GC B cells. Using a novel technique of microdissecting single foci and GC from histological sections followed by the amplification and sequencing of genomic VDJ rearrangements (9), we have compared the constituent H chain CDR3 sequences found in adjacent PALS-associated foci and GC. Of four (λ*, PNA−) foci analyzed, two were associated with (λ*, PNA−) GC that contained cells with an identical CDR3 sequence. It is unlikely that this finding represents contamination during the process of microdissection or the retrograde migration from GC to foci, in that sequences recovered from GC show evidence of somatic hypermutation, whereas those taken from foci do not. Thus, we propose that a single B cell, activated by antigen along the border of the PALS, is capable of giving rise to both focus and GC populations.

Materials and Methods

Animals and Immunization. Female, viral antibody-free C57BL/6 mice (5–7 wk) were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in microisolator cages. Mice were immunized with a single intraperitoneal injection of 50 μg NP (Cambridge Research Biochemicals, Cambridge, UK) conjugated to chicken gamma globulin (Accurate Chemical & Scientific Corp., Westbury, NY) precipitated in alum (NP-CG/alum) (8). After immunization, mice were killed at 2-d intervals by cervical dislocation, and their spleens processed as described (8). Serial, 6-μm-thick frozen sections were thaw-mounted onto poly-l-lysine coated slides, fixed, and stored at −20°C as described (8).

Staining and Isolation of Individual GC and Foci. Frozen sections were stained in tandem with horseradish peroxidase (HRP)-conjugated anti-λ L chain antibody (Southern Biotechnology Associates, Birmingham, AL) and PNA-biotin (E-Y Laboratories, San Mateo, CA), followed by streptavidin-alkaline phosphatase (S-AP) (Southern Biotechnology) as described (8). HRP and AP activities were visualized using 3-aminoethyl carbazole (3-AEC) and napthol AS-MX phosphate/fast blue BB (Sigma Chemical Co., St. Louis, MO), respectively (8). GC (λ*, PNA−; red/blue) and foci (λ*, PNA−; red only) were identified by brightfield microscopy. Adjacent foci and GC were selected at random and microdissected from the stained sections of a single spleen using a micromanipulator (Narishige, Tokyo, Japan)-controlled micropipette. Approximately equal areas from each focus and GC were recovered for amplification to equalize the amount of recovered DNA. Each sample represented 50–100 cells (9).

DNA Amplification and Sequencing. The cellular material recovered by microdissection was introduced into a 0.5-ml microcentrifuge tube containing 15 μl H2O and 5 μl PBS. Afterwards, 5 μl protease K (2 mg/ml) (Boehringer-Mannheim Biochemicals, Indianapolis, IN) was added, and the suspension was incubated at 37°C for 16 h followed by heat inactivation of the protease (10 min, at 96°C). This crude lysate was then subjected to two rounds of PCR as described (9). Briefly, PCR reactions were carried out in a 50-μl volume of a reaction mixture composed of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM MgCl2, 0.01% gelatin (Sigma Chemical Co.), 200 μM dNTP (Pharmacia LKB Biotechnology, Piscataway, NJ), 20 pmol of each primer, and 2.5 U Taq polymerase (Bethesda Research Laboratories, Bethesda, MD). Two drops of mineral oil (Sigma Chemical Co.) were added to each tube to prevent evaporation. The first round of PCR consisted of 40 amplification cycles (96°C for 1.4 min, and 70°C for 3 min; Ericomp programmable cyclic reactor, Ericomp Inc., San Diego, CA) using the primer, 5′-CTTGACCCCATATCGCCCTAATCTCCAGCAGGGCAG-3′, which is complementary to the genomic DNA 5′ of the transcriptional start site of V186.2, and 5′-GGGTCTAGAGGTTGCCCACTGCAGCCCAGGGTG-3′, which is complementary to a region in the J2.2, j3 intron. 2 μl of this reaction mixture was reapplied for an additional 40 cycles (96°C for 1.4 min, and 70°C for 2 min) using a second set of nested primers. The 5′ internal primer is complementary to the first 20 nucleotides of the V186.2 gene segment, and in addition contains recognition sequences for the restriction enzymes XbaI and EcoR1 (5′-CTTAGATTCTAGGGTCCCACTGCAGCCCAGGGTG-3′). The 3′ internal primer binds to the J2 gene segment and contains an additional BamHI recognition sequence (5′-ACGGATCCAGGGTGCTAGGTTGCCCT-3′).

Amplified DNA was extracted in phenol/chloroform, precipitated in ethanol, digested with the BamHI and PstI restriction endonucleases (Boehringer-Mannheim Biochemicals), and ligated into pBluescript SK (9). Competent DH5α bacteria were transformed by electroporation (25 μF, 2.5 kV, 200 μΩ), and the recombinant colonies were screened with a 32P-labeled oligonucleotide corresponding to amino acid positions 70–74 of the V186.2 gene segment. This oligonucleotide probe imparts no bias for the CDR3 sequence (5′ACGGATCCAGGGTGCTAGGTTGCCCT-3′). This frequency corresponds to a misincorporation rate of 1/338. This frequency corresponds to a misincorporation rate of 4 × 10−5/bp amplification cycle, a value virtually identical to that reported by Weiss and Rajewsky (26) and actually below that found in early studies of Taq fidelity (27, 28). Amplification of VDJ DNA from seven foci (3 × 10−5/bp/cycle; [9] and this study) and three unmutated GC populations (3 × 10−5/bp/cycle; our unpublished results) indicate that polymerase fidelity is a constant. Thus, on average, each 320 bp VDJ fragment rescued from splenic tissue is expected to contain about one mutation because of polymerase error.

Results

Kinetics of Focus- and GC Formation. This study confirms our earlier report (8) on the order in which foci and GC ap-
Figure 1. Kinetics of the appearance and loss of \( \lambda^+ \) foci (hatched) and GC (solid) from the spleen after immunization with NP-CG. Foci (\( \lambda^+ \), PNA\(^-\)) are present 2 d postimmunization as loose clusters of 8–32 cells (8). By day 8–10, foci contain as many as 2 \( \times \) 10\(^3\) cells. After this peak, foci rapidly lose cells and are largely absent by day 16. GC (\( \lambda^+ \), PNA\(^+\)) first appear on day 4 of the response and are maintained in the spleen at least until day 16. By day 12 postimmunization, each GC contains \( \approx \) 10\(^3\) cells to occupy about 1% of the splenic volume (8).

Anatomical Relationship of Foci and GC. Fig. 2 illustrates the proximity of the PALS-associated foci and GC within a single region of splenic white pulp. Foci of \( \lambda^+ \) B cells (stained red) are located along the outer margins of the PALS which is dominated by Thy 1.2\(^+\) cells (Fig. 2). Lymphoid follicles containing \( \lambda^+ \) PNA\(^+\) GC B cells (stained red/blue) are located nearby in the B cell-rich region of the white pulp (Fig. 2). This approximation of foci and GC is typical (8) and permits the selection of associated focus and GC pairs. However, because of the considerable dimensions of both foci and GC (8), any single focus may be closely associated with multiple GC and vice versa.

Analysis of the Genetic Relatedness of Adjacent Foci and GC. Because both foci and GC are well developed in the spleen 10 d after challenge with NP-CG (Fig. 1 and reference 8), four typical sets of adjacent foci and GC were selected at random and microdissected from serial sections of a frozen spleen taken 10 d postimmunization. Two focus/GC pairs, focus 10.8/GC B8 and focus 10.15/GC B15, were taken from widely separated sites. Two other focus/GC pairs, focus 10.12/GC B12 and focus 10.17/GC B17, shared anatomic proximity. In all cases, the phenotype of the recovered foci and GC was (\( \lambda^+ \), PNA\(^-\)) and (\( \lambda^+ \), PNA\(^+\)), respectively.

Focus 10.8/GC B8. The CDR3 sequences of VDJ DNA recovered from focus 10.8 and GC B8 are shown in Fig. 3. Focus 10.8 was populated by at least three distinct B cell clones, as three unique CDR3 sequences were recovered. The 10.8 sequences did not show evidence of somatic hypermutation.
Focus 10.8

V gene segment

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<th>Vn</th>
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JH2

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<th>TAC</th>
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No. Isolates/ % Total

2 20%
7 70%
1 10%

Germinal Center B8

V gene segment

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JH2

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No. Isolates/ % Total

2 22%
2 22%
3 33%
1 11.5%

Focus 10.15/GC B15. CDR3 sequences recovered from focus 10.15 and GC B15 are given in Fig. 4. It is interesting that focus 10.15 appears to be populated by at least six B cell clones that do not make use of the V186.2 gene segment to encode the H chain. Instead, four other closely related members of the J558 Vn gene family, CH10, CH1H4, V23, and 24.8 (27), were amplified. None of the VDJ fragments obtained from focus 10.15 exhibited mutations in excess of that expected from Taq-related misincorporations (not shown; average frequency, 1/141), consistent with our earlier observations of GC 16 d after immunization (9).

Focus 10.12, 10.17/GC B12, B17. The association of loci 10.12 and 10.17 and the neighboring GC B12 and B17 is more complex than that for the previous focus and GC pairs. The anatomic relationship of the four populations was determined from multiple serial sections (8), and a diagrammatic representation of their association is illustrated in Fig. 5.

Sequenced V region DNA rescued from the loci 10.12 and 10.17 and GC B12 and B17 are shown in Fig. 6. Three different CDR3 sequences were recovered from focus 10.12. These sequences did not appear to contain excess mutations (average frequency, 1/141). This focus has an average mutation frequency of 1/402, which is compatible with the error rate expected for the Taq polymerase alone (1/338). Five distinct CDR3 sequences were amplified from GC B8 (Fig. 3). One of these, TACGACTACGGTAGT, is identical to a VDJ join isolated from the adjacent focus, 10.8. However, unlike the DNA recovered from focus 10.8, all sequences derived from GC B8 are somatically mutated (not shown; average mutation frequency 1/141), consistent with our earlier observations of GC 16 d after immunization (9).
Frequency, 1/353). Identical CDR3 sequences were not found in the adjacent focus 10.17 or in the neighboring GC B12 and B17 (see below). Sequence analysis of the focus 10.17 indicated that it may have been populated by a single dominant clone. The unique CDR3 recovered from this focus bears noteworthy complexity. The four terminal nucleotides of the V186.2 gene segment have been replaced with the N sequence, CCGT, and the last two codons of DFL16.1 are absent at the D/Jn border. This Vn/DJn join is rare. It is unique among the 22 VDJ fragments we have recovered from loci and GC (9 and our unpublished observations), and it has not been found in any of the considerable number (~260) of sequences published by other groups (26, 30–36). Like the other loci, the DNA from focus 10.17 is unmutated (average frequency, 1/419) beyond that expected for polymerase error.

Six of the nine sequenced VDJ inserts from GC B12 represent V186.2→DFL16.1 rearrangements (Fig. 6), whereas the remainder use the V186.2 analogue, C1H4, to encode the H chain V region (not shown). All of the six V186.2/DFL16.1 sequences of GC B12 have been derived from a single B cell clone that bears a CDR3 identical to that present in focus 10.17. However, unlike the 10.17 sequences, the VDJ DNA recovered from GC B12 is mutated well in excess of that expected for Taq-related errors (average frequency, 1/209). All VDJ sequences recovered from GC B17 share a CDR3 region that is identical to that found in focus 10.17 and the V186.2-containing rearrangements recovered from GC B12. Like GC B12, and in contrast to the 10.17 sequences, all GC B17 VDJ inserts show evidence of somatic hypermutation (average frequency, 1/190).

**Independent Genealogies of a Common B Cell Lineage in GC B12 and B17.** Based upon our analyses of CDR3 regions, GC B12 and B17 were colonized by a common B cell clone that was also present in focus 10.17. However, the accumulation of mutations in the Igh-V locus differs between the two GC (Fig. 6). For both B12 and B17, all of the relevant sequences may be fitted into single genealogies (Fig. 7), suggesting that the mutant lineages were founded by single cells, confirming our earlier observations (8, 9). Although GC B12 and B17 appear to have been established by identical sister cells, no mutation is shared between these two populations (Fig. 6). This observation suggests that mutation at distal anatomic sites has acted independently (Fig. 7).

**Discussion**

It is well established that GC form in secondary lymphoid tissues after challenge with thymus-dependent antigens (5–7, 29, 37). GC B cells are believed to produce little or no antibody during the early primary response (5, 6, 8, 29), although extensive immunization protocols can give rise to GC AFC (2–5). Indeed, abrogation of GC via irradiation or cytotoxic drugs causes little change in the levels of serum antibody, but severely depletes the spleen of memory B cell precursors (14, 15). Our results are compatible with these earlier observations. GC B cells contain relatively low amounts of Ig and H and L chain mRNA (8). Thus, it may be that the B cells within the PALS-associated foci and GC represent specializations for early antibody production and memory cell formation, respectively.

Little is known about the B cells that found GC (5, 7). However, Linton et al. (19) have made the observation that primary AFC and memory B cells may originate from distinct cell populations that can be discriminated by the mAb J11d (19). These investigators have concluded that J11dB B cells appear to be the founders of the memory response and are enriched for cells capable of producing GC, whereas J11dH cells predominantly give rise to the primary AFC (19, 38). Unfortunately, in our hands the pattern of J11d staining in splenic sections is uninformative because of a substantial background of reactivity (data not shown). In contrast, others (6) have proposed that B cells, activated by antigen in the T-dependent areas of the spleen, migrate into the lymphoid follicles and proliferate to produce GC. Although these hy-
Figure 6. Comparison of the VDJ DNA sequences recovered from foci 10.12 and 10.17, and GC B12 and B17. All mutations present in the recovered VDJ fragments are shown in comparison to V186.2, DFL16.1, and Jk2 germline sequences (boxed). (Boxed) Shared CDR3 sequences. Focus 10.12 contained three different CDR3 sequences that were not observed in any of the other associated populations. However, the single CDR3 sequence recovered from focus 10.17 was also recovered from GC B12 and B17. Note that the number of mutations is substantially higher in the GC sequences compared with those recovered from focus (see text). GC sequences contain multiple shared mutations indicating clonal diversification. These mutations are not found in the focus sequences.
protheses are not mutually exclusive, Linton suggests that the developmental program of mature peripheral B lymphocytes is fixed before encounter with antigen. The more traditional view proposes that the developmental potential for B cells to enter the AFC or memory cell pathways is unrestricted. Although a mechanism for the canalization of the developmental potential of peripheral B cells before antigen triggering has not been described (stochastic?), decisions by developmentally unrestricted B cells to become AFC in foci or to colonize the lymphoid follicle would presumably be determined by the local microenvironment.

We have attempted to test this hypothesis by determining the genetic relatedness of neighboring foci and GC by searching for CDR3 sequences shared between focus and GC pairs. The CDR3 region is generated by rearrangement of Vα, D, and Jα gene segments, and the complexity that results from this rearrangement process is sufficiently great to identify members of a single rearrangement event (a clone) with a high degree of confidence. This notion is strongly supported by earlier studies assessing clonal relationships within hybridoma cohorts (20, 21), and the elegant statistical analysis of Litwin and Shlomchik (24), who have demonstrated that (at a 95% confidence limit) the probability of independent identity at VDJ junctions to be no greater than 0.06/event.

The pauciclonal nature of foci and GC (8, 9, 37) makes the incidental sharing of rare CDR3 sequences between discrete B cell populations even less likely. Of the eight foci and GC sampled in this study, six contained three or fewer distinct VDJ junctions and two, GC B8 and focus 10.15, contained five and six, respectively (Figs. 3, 4, and 6). The average number of different CDR3 sequences recovered from each sample population was 2.9 (± 0.7), in full agreement with our earlier estimates based upon phenotypic diversity (8).

Two foci, 10.8 and 10.17, out of the four we analyzed, contained B cells that shared identical VDJ junctions with cells in neighboring GC (Figs. 3 and 6). Shared CDR3 sequences were observed only in adjacent populations even though all samples were obtained from a single spleen. Especially noteworthy is the CDR3 sequence shared between foci 10.17 and the GC B12 and B17 (Fig. 6). This VDJ junction is rare in that it is not reproduced in any of the 44,415 reported sequences obtained from NP-specific hybridomas (21, 31-34) or among some 200 V186.2 rearrangements recovered by PCR (26, 35, 36). It is also unique among our sample of 222 VDJ fragments recovered from 19 foci and GC (Figs. 3, 4, and 6; reference 9 and our unpublished observations). Thus, we believe that the probability that the sharing of this unusual CDR3 among associated focus and GC populations represents coincident independent events is vanishingly small. The focus 10.17 and GC B12 and B17 share a common clonal origin.

The order in which foci and GC appear after primary immunization (Fig. 1) suggests that GC are established by migration of antigen-activated B cells from the periphery of the PALS into the lymphoid follicles. This supposition is supported by our sequence analyses of VDJ DNA recovered from foci and GC (Figs. 3, 4, and 6). In every case, the V-region sequences recovered from focus B cell populations were not mutated in excess of that expected from the error rate of the Taq polymerase alone (9), whereas sequences derived from GC cells contained an excess of accumulated mutations. For example, of the 19 sequences analyzed from the foci 10.12 and 10.17 (Fig. 6), 9 (47%) have no mutations whatsoever. The average number of misincorporations/VDJ fragment among both foci is 0.8 ± 0.2 (X ± SEM), actually below that expected for polymerase errors. In contrast, none of the 15 sequences derived from GC B12 and B17 are unmutated, and the average frequency of mutations/VDJ fragment is 2.9 ± 0.3, threefold above that seen in the related foci. This difference, though small, is consistent with the onset of mutagenesis in this system (day 8, data not shown) and is unlikely to have been produced in vitro. The Taq error rate is identical for both focus- and GC-derived DNA, and roughly equal amounts of DNA were recovered from all samples. Thus, we do not believe that the apparent common origin of the 10.17, B12, and B17 populations is an artifact of sample contamination during micropreparation or retrograde migration from GC into foci. Both processes would tend to homogenize the frequency of mutared (or unmutated) sequences in focus and GC populations. Sample contamination could not possibly account for the presence of the 10.17 CDR3 sequence in GC B12 as these samples were recovered from different histologic sections (Fig. 5). It is significant that none of the

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**Figure 7.** Genealogical relationship of the VDJ sequences recovered from GC B12 and B17. The common progenitor is identified by the shared CDR3 sequence, CCGT. (Broken circles) Hypothetical intermediates. Mutations are indicated by specifying the number of the affected codon (Fig. 6). Numbers in the dendrograms indicate the number of substitutions to the next node. All sequences recovered from GC B12 and B17 could be fitted into two distinct genealogies indicating random mutation and selection at distal anatomic sites.
mutations seen in the 10.17 sequences is compatible with the clonal genealogies of GC B12 or B17 (Figs. 6 and 7).

In agreement with our earlier observations (8, 9), there appears to be little or no migration of B cells between GC. Thus the evolutionary pathways of the B cell populations in GC B12 and B17 are distinct (Fig. 7), suggesting that mutation and selection at distal sites are independent even when operating on identical V(D)J substrates. The analysis of a significant number of GC founded by identical progenitor cells may provide a reasonable test of hypothetical mutational processes that depend on the fine genetic structure of the Ig V-region (39), as well insight into the selection forces that drive the somatic evolution of antibody (40).

Our observations provide very good evidence that MacLennan's proposal (6) that GC B cells represent the progeny of antigen-activated migrants from nonfollicular regions of the spleen is correct. Some fraction of the activated B cells found in newly formed foci leave the PALS, perhaps guided by the products of the classical C pathway (41, 42), to establish GC. It is interesting that our earlier studies of focus and GC architecture (8) indicated that the number of splenic GC was two to three times greater than the number of foci. This suggests that the establishment of two GC by focus 10.17 (Fig. 6) is not an uncommon event. In contrast, although these findings do not disprove Linton's hypothesis of separate precursor cell populations for primary AFC and memory B cells (19, 36), they do suggest that early in the response, antigen-activated splenic lymphocytes are unrestricted with regard to either developmental pathway. However, the import of regulated expression of the heat-stable antigen recognized by the J11d antibody (43) has been recently underscored by the finding that this molecule may serve as a costimulator of T cell growth (44).

Finally, it will be interesting to determine if mutated, memory B lymphocytes have the potential to form PALS-associated foci in early secondary responses. If this were indeed the case, it would demonstrate that the differentiation pathways leading to the formation and specialization of foci and GC are accessible to both naive and memory peripheral B lymphocytes, and that their choice is likely determined by the cellular environment.

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Note added in proof: The DNA sequences illustrated in Figures 3, 4, and 6 are available from EMBL/GenBank/DDBJ under accession number X67341-X67391.

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