Contrasting the In Situ Behavior of a Memory B Cell Clone During Primary and Secondary Immune Responses

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Whether memory B cells possess altered differentiative potentials and respond in a qualitatively distinct fashion to extrinsic signals as compared with their naïve precursors is a current subject of debate. We have investigated this issue by examining the participation of a predominant anti-arsionate clonotype in the primary and secondary responses in the spleens of A/J mice. While this clonotype gives rise to few Ab-forming cells (AFC) in the primary response, shortly after secondary immunization its memory cell progeny produce a massive splenic IgG AFC response, largely in the red pulp. Extensive clonal expansion and migration take place during the secondary AFC response but Ab V region somatic hypermutation is not reinduced. The primary and secondary germinal center (GC) responses of this clonotype are both characterized by ongoing V gene hypermutation and phenotypic selection, little or no inter-GC migration, and derivation of multiple, spatially distinct GCs from a single progenitor. However, the kinetics of these responses differ, with V genes containing a high frequency of total as well as affinity-enhancing mutations appearing rapidly in secondary GCs, suggesting either recruitment of memory cells into this response, or accelerated rates of hypermutation and selection. In contrast, the frequency of mutation observed per V gene does not increase monotonically during the primary GC response of this clonotype, suggesting ongoing emigration of B cells that have sustained affinity- and specificity-enhancing mutations.

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D uring a primary immune response to T cell-dependent Ags in mammals, B cells undergo extensive proliferation and differentiation in the microenvironment of the germinal center (GC) (1–6). This process ultimately results in the formation of the memory B cell compartment (7, 8). It is now well established that GCs are the primary locale in which Ab V gene somatic hypermutation takes place (9, 10). In addition, a number of previous studies have suggested that both positive selection based on increased affinity for Ag (9–14) and negative selection against autoreactive V regions (15–18) also occur in this microenvironment. GCs also form during secondary and higher order immune responses. These anamnestic GC responses appear to differ quantitatively from the primary GC reaction (19). However, whether qualitative differences in V gene hypermutation, phenotypic selection, and participation of memory B cell clones distinguish the secondary GC reaction from the primary GC reaction is a current area of controversy (20–26).

Before initiation of the primary GC reaction, Ag-specific B cells are thought to undergo activation via cognate interaction with helper T cells in the T cell-rich periarteriolar lymphoid sheath (PALS) of the spleen and the lymph node paracortex (6, 27–29). After this period, at least two differentiative pathways are available to a B cell, proliferation and differentiation to an Ab-forming cell (AFC) phenotype outside of follicles or migration to a follicle and participation in the GC/memory B cell pathway. In addition, recent data (30–33) have supported past assertions (34) that B cells participating in the primary immune response are capable of differentiation to long-lived AFCs that reside in the bone marrow. How these lineage and migratory decisions are made is currently unclear. It has been suggested that the nature of naïve B cells initially recruited into the response (35, 36), the affinity and specificity of the B cell Ag receptor (32, 33, 37, 38), the type of Ag driving the response (19), and levels of T cell help (19, 38, 39) can all influence these decisions. Moreover, whether all these pathways of differentiation are also available to memory B cells participating in anamnestic responses is a subject of debate (21–23, 34). Investigation of these issues is complicated by the fact that most immune responses to even simple epitopes are clonally diverse and often exhibit substantial response to response variation regarding the participation of individual clonotypes. In addition, in many immune responses clonotypes that dominate the primary immune response may not effectively seed the memory compartment, making the identification of B cells destined for this compartment during the primary response difficult (40–43).

We (44) and others (45, 46) have extensively characterized the T cell-dependent B cell response of A/J mice to the hapten arsonate (Ars). As assessed by serological and hydridoma studies, the primary response to Ars is clonally diverse, but during the latter stages of this response a single B cell clonotype reproducibly emerges and dominates subsequent anamnestic responses. This clonotype expresses a V region encoded by a single combination of V gene segments, with variation before hypermutation only at \( V_{\beta} \)-D and DJ\( _\beta \) junctions (47, 48). We term this V region and the clones that express it “canonical.” Canonical clonotypes undergo extensive V region somatic hypermutation and both positive and negative phenotypic selection during the primary response (16, 44). The secondary serum Ab response is largely composed of Abs.
derived from this clonotype that bear characteristic V<sub>H</sub> region amino acid substitutions due to somatic hypermutation. These recurrent mutations have been shown to independently and additively confer increased affinity for Ars (49). Thus, the canonical anti-Ars clonotype provides a paradigm for a true memory B cell clonotype, initially expressing a nearly invariant Ag receptor with known affinity and specificity, and reproducibly seeding the memory compartment after undergoing extensive V gene hypermutation and selection. Here, we have exploited this experimental system to determine how the primary and secondary AFC and GC responses of a single B cell clonotype may differ.

Materials and Methods

Mice

A/J mice 8–12 wk of age at the time of first immunization were purchased from The Jackson Laboratory (Bar Harbor, ME) and were housed in a specific pathogen-free rodent facility and given autoclaved food and water.

Immunizations

For primary GC analysis, A/J mice were immunized intraperitoneally (i.p.) with 100 μg Ars-keyhole limpet hemocyanin (KLH) precipitated in alum. Nine to 15 days after immunization, mice were sacrificed and their spleens were removed and prepared for cryosectioning as previously described (29, 37). For secondary GC and secondary AFC “cluster” analysis, mice were immunized i.p. with 100 μg Ars-KLH in alum or CFA, rested for at least 6 wk, and then boosted with 100 μg Ars-KLH in PBS i.p. After boosting, spleens were removed at various times and prepared for cryosectioning as described above.

Immunohistochemistry

Frozen spleens were sectioned and three 5- to 6-μm thick parallel sections were placed on each slide. The slides were fixed in 100% cold acetone and stored at −80°C. The procedures used for immunohistochemical staining of the sections have been described in detail previously (37). Briefly, parallel sections were stained with biotinylated Ars-BSA or the anti-idiotypic Abs E4 or 5Ci followed by streptavidin coupled to alkaline phosphatase (Dako, Carpinteria, CA). All sections were also stained with peanut agglutinin (PNA) coupled to HRP (Sigma, St. Louis, MO) to elaborate GC. Some sections were also stained with HRP-conjugated donkey anti-mouse IgM or donkey anti-mouse IgG preparations (Jackson ImmunoResearch, West Grove, PA). Abs binding and idiotope-positive AFCs and GCs were microdissected from stained sections using a micromanipulator-controlled micropipette, and tissue was processed as previously described (10, 11).

PCR amplification of V<sub>H</sub> genes

V<sub>H</sub> genes in genomic DNA present in processed microdissected tissues was subjected to two rounds of PCR amplification using Ampli-Taq DNA polymerase (Perkin-Elmer, Foster City, CA) and various combinations of nested oligonucleotide primers. Each round of PCR consisted of 40 cycles of 95°C/1 min, 58°C/30 s, and 72°C/3 min ending with one cycle of 72°C/6 min. The 5′ primers were specific for sequences in the 5′ region of the canonical V<sub>H</sub>Id<sub>CR</sub> gene segment and were either hybridized in the promoter or just 5′ of the translation initiation codon, and the 3′ leader intron. The 5′ primers either hybridized between the J<sub>H</sub>3 and J<sub>H</sub>4 gene segments or to a region of common homology in the J<sub>H</sub>2, J<sub>H</sub>3 and J<sub>H</sub>4 gene segments. The second round amplification was performed on 1.5–2 l total). All of the primers contained re- 

Dendrogram construction and shape assignment

As detailed in Results, V<sub>H</sub> clones obtained from single spleens were judged to have been derived from a common precursor on the basis of sharing of their V<sub>H</sub>-D and D-J<sub>H</sub> functional nucleotides. The sequences of groups of related clones containing three or more members were used to construct genealogical dendrograms, essentially as has been described by us and others (10, 50–52). During this exercise, we assumed that a mutation shared by several clones had been sustained once in vivo, and then inherited by daughter cells that then each gave rise to a single PCR clone. Exceptions were sometimes made to this rule of “mutational parsimony” in the case of mutations in five V<sub>H</sub> codons: three (10, 31, and 105) that appear to be mutational “hotspots” (53) and overlap GRYW consensus hotspot sequences (54); and the CDR2 codon 58 and 59 affinity-enhancing mutations (see Results). Artificial base changes introduced via the PCR were assumed to be present at a frequency of one per clone or less. V<sub>H</sub> clones whose sequences could easily be explained by PCR recombination events (11, 55) were observed infrequently (<5% of clones) in our studies. However, when the locations of a clone in the dendrogram as defined by mutations in its 5′ half, as compared with mutations in its 3′ half, were discordant, it was assumed that the clone had been generated via PCR recombination and it was excluded from further analysis.

The dendrograms thus constructed could often be assigned one of three “shapes”: bush, tree, or trunk. A “bush” was defined by two or more sublinesages emanating from a single, hypothetical founder cell. A “tree” consists of a single genealogical “trunk” from a common founder cell, and extensive “branching” emanating from the top of this trunk. This “trunk” was defined by three or more mutations that were shared by all of the clones. A “trunk” was defined as described for a “tree,” except it lacked sublineage branches defined by two or more mutations. In some cases, the dendrograms did not fit into one of these three specific categories, and so were assigned mixed names.

Results

The pattern and extent of hypermutation in canonical V<sub>H</sub> genes derived from individual GCs at various stages of the primary anti-Ars response

We previously reported the results of an immunohistochemical analysis of the participation of canonical clonotypes in the primary anti-Ars response in the spleens of A/J mice (37). This study exploited the anti-idiotypic mAbs E4 and 5Ci, which are highly specific for canonical V<sub>H</sub> regions (56–58). We found that canonical B cells did not participate in the early PALS AFC response, but initially appeared infrequently and in a small number of GCs 6 wk, and then boosted with 100 μg Ars-KLH in PBS i.p. After boosting, spleens were removed at various times and prepared for cryosectioning as described above.

When genomic DNA from microdissected tissues was amplified by PCR, controls containing either no tissue or processed tissue microdissected from a region of the spleen that did not stain with E4 or 5Ci were always included. The no tissue controls never yielded a PCR product, but the unstained tissue controls sometimes did. This was not unexpected, as the primer combinations used would amplify any V<sub>H</sub> gene partially encoded by the VhId<sub>CR</sub> gene segment, irrespective of D or J<sub>H</sub> use. When these “control” PCR products were cloned and sequenced, they invariably were found to be noncanonical, being composed of VhId<sub>CR</sub> or a closely related germline V<sub>H</sub> gene segment, in combination with a noncanonical D or J<sub>H</sub> segment. These clones also were never found to contain somatic mutations, indicative of their derivation from the naive B cell compartment.

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To extend these in situ studies to an analysis of canonical V<sub>H</sub> region hypermutation and selection, an immunohistochemical-microdissection-PCR approach analogous to that pioneered by Kelsoe and colleagues (10, 11) was employed. A/J mice were immunized with Ars-KLH in alum, sacrificed at various times after immunization, and their spleens processed for histology. Expressed canonical VH region genes and their flanking sequences were recovered from genomic DNA via PCR from 17 microdissected E4, 5Ci<sup>1</sup> GCs representative of various stages of the primary anti-Ars response, and the PCR products were cloned. Microdissected regions within the GC (as defined by PNA staining) consisted of 50-100 cells. Care was taken to avoid the inclusion of PNA<sup>2</sup> cells in the surrounding mantle zone. Multiple plasmid clones obtained from each GC were then sequenced. Clones containing hypermutated versions of the canonical VH<sub>IDCR</sub> VH<sub>16.1</sub>-NNN-J<sub>H</sub><sup>2</sup> gene were easily distinguished from clones that might have been derived from other germline genes (obtained only rarely in this analysis) because most of these related J<sub>558</sub> family members have been characterized (59). Kelsoe and colleagues have previously measured the PCR error frequency under similar conditions, and have demonstrated that this frequency follows a Poisson distribution (11), making it extremely unlikely that when two or more mutations are observed per VH gene, that all of these mutations could have resulted from PCR error. Moreover, the results of the studies reported here suggest that, in our hands, the PCR error frequency is even lower, perhaps only 0.2 mutation(s) per VH gene, on average (see below).

As mentioned above, canonical VH<sub>16</sub> regions differ before hypermutation only at V<sub>H</sub>-D and D-J<sub>H</sub> junctions (47, 48). The consensus sequence in these regions is VH<sub>IDCR</sub>-TCN-NNN-D<sub>FL16.1</sub>-NNN-J<sub>H</sub><sup>2</sup>, where N indicates nucleotides often resulting from N region addition (the first “TC” nucleotides appear to result from P nucleotide addition). Thus, at least seven of the nine canonical VH junctional nucleotides are added in a template-independent fashion and therefore can be used as markers of common clonal origin. We and others have previously shown that it is improbable for canonical VH genes derived from different A/J mice to share all these junctional nucleotides (47, 60).

FIGURE 1. Somatic mutation in canonical VH<sub>16</sub> genes cloned from individual primary GCs. Data from individual GCs are shown on separate lines. Data from GCs microdissected from spleen sections obtained from different mice are separated by bold lines, and those obtained from different sections of the same spleen are separated by light lines. *, For VH<sub>16</sub> clones obtained from individual GCs in a single spleen, identity at junctional nucleotides is indicated by a “J” preceded by a number indicating a particular unique combination of these nucleotides. Also indicated is whether GCs were found in the same follicle (“F”) or whether they were present in adjacent regions (“A”) of the spleen. #, Average mutation frequency per V gene base pair was calculated by dividing the total number of base pairs sequenced in all VH<sub>16</sub> clones obtained from a given time interval (e.g., days 9 and 10) by the total number of somatic mutations observed in these same clones. The R/S mutation ratios in CDRs and FWRs were calculated in a similar manner, except only mutations in the mature VH<sub>region</sub> coding sequences were considered. S, Two of the clones obtained from this GC had apparent somatic mutations in the first two nucleotides of the VH<sub>D</sub> region junction. & A few of the VH<sub>16</sub> clones obtained were not completely “canonical” because they lacked D regions of the canonical length and derived from the DFL16.1 gene segment. Two of these had D regions derived from the Sp2 D segment family, and one had a 1-a D region characteristic of the CRI-D anti-Ars V region family (45). However, these three VH<sub>16</sub> genes did use the canonical J<sub>H</sub><sup>2</sup> gene segment. †, The number of unique somatic mutations per GC was evaluated by counting mutations shared by two or more VH<sub>16</sub> clones only once. NA, Not applicable.

To extend these in situ studies to an analysis of canonical V<sub>H</sub> region hypermutation and selection, an immunohistochemical-microdissection-PCR approach analogous to that pioneered by Kelsoe and colleagues (10, 11) was employed. A/J mice were immunized with Ars-KLH in alum, sacrificed at various times after immunization, and their spleens processed for histology. Expressed canonical VH<sub>H</sub> region genes and their flanking sequences were recovered from genomic DNA via PCR from 17 microdissected E4, 5Ci<sup>1</sup> GCs representative of various stages of the primary anti-Ars response, and the PCR products were cloned. Microdissected regions within the GC (as defined by PNA staining) consisted of 50–100 cells. Care was taken to avoid the inclusion of PNA<sup>2</sup> cells in the surrounding mantle zone. Multiple plasmid clones obtained from each GC were then sequenced. Clones containing hypermutated versions of the canonical VH<sub>IDCR</sub> VH<sub>16.1</sub>-NNN-D<sub>FL16.1</sub>-NNN-J<sub>H</sub><sup>2</sup> gene were easily distinguished from clones that might have been derived from other germline genes (obtained only rarely in this analysis) because most of these related J<sub>558</sub> family members have been characterized (59). Kelsoe and colleagues have previously measured the PCR error frequency under similar conditions, and have demonstrated that this frequency follows a Poisson distribution (11), making it extremely unlikely that when two or more mutations are observed per VH<sub>16</sub> gene, that all of these mutations could have resulted from PCR error. Moreover, the results of the studies reported here suggest that, in our hands, the PCR error frequency is even lower, perhaps only 0.2 mutation(s) per VH<sub>16</sub> gene, on average (see below).
PCR error. In addition, canonical V<sub>H</sub> clones obtained from individual GCs nearly always shared all junctional nucleotides. Attempts to reconstruct the hypermutation and clonal expansion events that took place in each GC were undertaken using the previously established procedure of building genealogical dendrograms (10, 50–52). During this exercise, we made the simplifying assumption that each plasmid clone represented a distinct GC B cell, which seems reasonable given that DNA from numerous E4, 5Ci<sup>+</sup> cells was amplified in each PCR reaction. These theoretical constructs rely on the doctrine of mutational parsimony, which assumes that if a mutation is shared by several clones, it took place only once in a precursor cell and was inherited by daughter cells.

FIGURE 2. Representative examples of canonical V<sub>H</sub> sequence data obtained from GCs and AFC “clusters” at various stages of the anti-Ars response. The sequences of multiple V<sub>H</sub> PCR product clones obtained from the events indicated at the beginning of each sequence are shown compared with the consensus sequence of canonical VH genes. The consensus sequence is composed of the germline VhdCRI gene segment and 5' flanking sequences in the leader intron region, the AJ DFL16.1 and J<sub>H</sub>2 segments, and regions in the V-C intron flanking J<sub>H</sub>2. Within the D region, the highly variable nucleotides often resulting from N region addition are indicated by an “N.” The sequences of only the CDR1, 2, and D (CDR3) regions are shown completely, as well as codons or 10-bp sections of flanking sequence in which a somatic mutation was observed. The codon that encodes the mature amino terminus is indicated by a “1,” and the position of all other codons not in CDRs and some CDR codons relative to this codon are indicated by numbers. In the clone sequences, identity with the consensus sequence is indicated by a dash, and nucleotide differences are shown explicitly.
This assumption is subject to caveats due to the nonrandom nature of hypermutation (61) and the possibility that any given base change may have resulted from a PCR misincorporation or recombination event (11, 55). However, when appropriate caution is applied, the utility of these theoretical constructs for the analysis of data obtained from individual GCs is well established (10, 11).

One dendrogram form—the “trunk”—results when most or all mutations are shared by all clones. This indicates isolation of V gene clones during a period in which the rate of B cell division was much greater than the rate of V gene mutation per division. At the other extreme are dendrogram “bushes” in which many mutationally distinct clones emanate from a common, often unmutated precursor. This indicates a period in which the rate of V gene mutation was comparable or greater than the rate of B cell division. Between these extremes is the “tree,” which suggests the derivation of clones from a common, mutated precursor during a period in which the rate of mutation was similar or greater than that of B cell division. The criteria we used to assign dendrograms to these three categories are described in Materials and Methods.

As can be seen in Fig. 1, dendrograms of all of these types could be generated from the clones isolated from individual GCs during the 9–10, 11–12, and 13–15 day time frames. In addition, the average frequency of mutation per V region base pair at these time points was indistinguishable. The Ars affinity-enhancing mutation at CDR2 position 58 was reproducibly observed after days 9–10, but the analogous position 59 mutation was observed in only one GC from day 12. Another measure of phenotypic selection of V regions, the ratio of mutations causing amino acid substitutions (R, replacement) to those that do not (S, silent) in CDR vs framework regions (FWR) (25) was also evaluated. These ratios did not reveal...
a progressive pattern of change from days 9 to 15. Nonetheless, mutations resulting in translation termination codons, or replacement mutations in codons known to encode Ars contact residues (62) or highly conserved FWR residues (63), were only rarely observed (data not shown).

Migration and expansion of canonical clones before and during the primary GC response

The data obtained from GCs microdissected from the spleens of individual mice also provided information regarding clonal migration and expansion before and during the GC reaction. In the case of the spleens of four mice, three pairs (groups 1J, 2J, and 3J in Fig. 1) and one triplet (group 8J) of GCs gave rise to V gene clones that shared junctional nucleotides, indicating a common clonal origin. Thus, canonical clones must often undergo expansion before the nucleation of multiple GCs. Strikingly, however, in the case of each of these four groups of GCs, shared somatic mutations were rarely observed among clones derived from different GCs, save for the affinity-enhancing position 58 mutation. Two of the GCs from the triplet group were directly adjacent to one another (day 15) and a pair of GCs in another spleen was present in the same follicle (day 9). The adjacent pair of GCs that are members of the GC triplet isolated from the day 15 mouse is shown in Fig. 3, as well as a schematic diagram illustrating how we interpret migration, clonal expansion, and hypermutation to have taken place in this clone.

The early secondary anti-Ars response is characterized by a massive memory AFC response during which V region hypermutation does not take place

Approaches identical with those used for the analysis of the primary anti-Ars response were used to analyze the secondary response of canonical clonotypes in the spleen. Mice were primed with Ars-KLH in alum or CFA and rested for at least 6 wk. Spleens

FIGURE 4. Time course of the secondary canonical anti-Ars B cell response in the spleen. A/J mice were primed with Ars-KLH, rested, and boosted with Ars-KLH as described in Materials and Methods. Multiple mice were then sacrificed at 1- to 2-day intervals and their spleens processed for histology. Spleen sections were stained with PNA (red) and E4 (blue), also as described in Materials and Methods. Representative results of this analysis are shown. Just before boosting (day 0, time point indicated at the upper left of each panel), rare E4 cells were observed throughout the spleen with an apparent concentration in the marginal zones. At day 2, note the increased level of E4 cells throughout the follicles and red pulp, but not in GCs. At day 4, note the large and abundant clusters of intensely staining cells (most probably AFCs) in the red pulp and the follicular borders, but the few E4 cells in follicular areas, including GCs. At day 6, note the numerous E4 cells in GCs (in this panel, two of the GCs are of this type), with small numbers of intensely staining cells also in the red pulp, some adjacent to the red pulp sinuses. The day 8 panel illustrates the continuation of the E4 GC reaction, but at this time the E4 cells in the red pulp were infrequent. At day 12, note that E4 staining characteristic of surface Ig B cells or AFCs is not observed, but most GCs stain in a punctate, reticular pattern characteristic of immune complexes deposited on the surface of FDC. The distinction between the asymmetric, reticular staining characteristic of immune complexes on FDC and the individual, circular cell staining characteristic of Id-expressing B cells is illustrated in the insert panels. These GC images were taken at an original magnification of ×400 to maximize clarity. The original magnification of all other images was ×100.
from two mice that had been rested for 6 wk were processed for histology and stained for E4, 5Ci\textsuperscript{1} cells as described above. E4, 5Ci\textsuperscript{1} cells were observed at a low frequency (75–100 per section) scattered throughout the red and white pulp, with an apparent concentration in the follicular and marginal zone areas (Fig. 4). Because such cells are undetectable in the spleens of naive A/J mice (K.A.V. and K.T.B., unpublished observations), this indicates a substantial increase in canonical B cell precursor frequency in the spleen due to primary Ars immunization. The remaining mice were boosted with Ars-KLH in PBS and spleens taken at 1- to 2-day intervals up to day 12 and processed for histology. As illustrated in Fig. 4 with E4 staining, at day 2 canonical V region-expressing B cells were observed at a high frequency but were scattered throughout both the red and white pulp. By day 4, a massive E4,5Ci\textsuperscript{1} cellular response had developed, consisting largely of loose clusters of cells confined to the red pulp. The intensity and asymmetric pattern of staining of these cells was characteristic of AFCs. These cells also labeled intensely with Ars-BSA. Most of these AFCs were embedded in clusters of cells that stained intensely with anti-IgG. Only scattered IgM\textsuperscript{1} AFCs were found in these same locales (data not shown). At day 6, most of the AFCs were found surrounding the red pulp sinuses. This response gradually waned and was not detectable at day 10. At day 6, E4, 5Ci\textsuperscript{1} GCs were first observed, in greater abundance than seen at day 6.

![FIGURE 5.](http://www.jimmunol.org/)

**FIGURE 5.** Somatic mutation in the canonical V\textsubscript{H} genes cloned from individual secondary AFC “clusters.” The data are presented as described in Fig. 1, except no dendrogram information is shown, as mutations shared by subsets of clones derived from single AFC “clusters” were never observed (see text). *, This cluster was adjacent to a day 6 GC (indicated by “AGC”) from which canonical V\textsubscript{H} clones were also obtained. This GC is indicated by “Acluster” in Fig. 6. However, the clones obtained from the AFC cluster and the GC did not have identical junctional sequences. #, A Lys\textsubscript{3} Thr mutation at codon 59 has been shown to increase affinity of canonical V\textsubscript{H} regions \~3-fold (49). Mutations to Ser, Asn, and several other amino acids are also recurrently observed at this position, but have not been individually tested for their affects of affinity for Ars. NA, Not applicable.

<table>
<thead>
<tr>
<th>Day (Junctions, Location)</th>
<th>Number of clones sequenced</th>
<th>Canonical?</th>
<th>Unique Mutations per GC</th>
<th>Affinity 58 Thr-&gt;Ile</th>
<th>Affinity 59 Lys-&gt;Thr</th>
<th>Hotspot 105</th>
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<td>Yes</td>
<td>9</td>
<td>Yes</td>
<td>No</td>
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![FIGURE 6.](http://www.jimmunol.org/)

**FIGURE 6.** Somatic mutation in canonical V\textsubscript{H} genes cloned from individual secondary GCs. The data are presented as described in Figs. 1 and 5. *, V\textsubscript{H} clones were obtained from an AFC cluster adjacent to this GC (indicated by “Acluster”). See Fig. 5 for information on this cluster. #, V\textsubscript{H} clones derived from different B cell precursors were isolated from a single GC (indicated by “GC”). Only one set of clones was canonical. @, Clones with canonical V\textsubscript{H} and J\textsubscript{H}2 segments but noncanonical D segments. NA, Not applicable.

<table>
<thead>
<tr>
<th>Day (Junctions, Location)</th>
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<th>Unique Mutations per GC</th>
<th>Affinity 58 Thr-&gt;Ile</th>
<th>Affinity 59 Lys-&gt;Thr</th>
<th>Hotspot 105</th>
<th>Dendrogram Shape</th>
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<tr>
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<td>Lys-&gt;Asn</td>
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<tr>
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<td>6</td>
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<td>14</td>
<td>No</td>
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FIGURE 7. During the secondary immune response, a single B cell clone can give rise to numerous GCs throughout the spleen in which hypermutation and clonal expansion take place rapidly and independently. The data obtained from the analysis of five GCs in the spleen of an AJ mouse boosted 6 days earlier is schematically illustrated, essentially as described in Fig. 2. However, in this figure, the individual mutations that define each dendrogram branch are not shown. The five GCs analyzed were present in three spatially distinct regions of the spleen. The left pair of GCs were located in adjacent regions of the white pulp (indicated by a solid line surrounding the GCs) but apparently in different follicles (indicated with a dotted line). The other three GCs were present in a different spleen section, ≈180 μM away from that containing the left pair of GCs. This large distance is illustrated with a thick solid line. The middle two GCs were present in adjacent regions of the white pulp, but apparently in different follicles. The right GC was present in the same section as these last two, but was located at the opposite end of the spleen. This distance is indicated by a thin solid line. The \( V_H \) clones obtained from these individual GCs all had identical \( V_H-D \) and \( D-J_H \) junctional nucleotides (shown in bold at the top of the figure), indicating derivation from a common precursor. \( V_H \) clones obtained from individual GCs also shared numerous somatic mutations among themselves, allowing the construction of the dendrograms illustrated in each GC. This suggests that hypermutation, phenotypic selection, and clonal expansion was confined to individual GCs after they were formed. Because the time course data illustrated in Fig. 3 indicate that GCs containing canonical B cell clones are absent from the spleen at day 4 after boosting, the data summarized here suggest an extremely rapid period of intra-GC hypermutation and clonal expansion between days 4 and 6 of the secondary response. \( V_H \) clones derived from three of the GCs also shared three mutations in common, indicating that a small amount of mutational diversification and clonal expansion took place before the nucleation of the five GCs. These mutations were: a G→C in codon 10 giving rise to a Glu→Asp mutation; and two G→A changes in codon 56 giving rise to a Gly→Asn mutation. None of these mutations are associated with increased affinity for Ars.

Early mutation period is illustrated by a shaded box, in which the hypothetical immediate GC precursor (“N1”) is shown. Also shown is the hypothetical unmutated parental (“P”) B cell, which apparently directly gave rise to the other GCs. While it cannot be determined whether this initial period of expansion and mutation took place at an anatomical site distinct from all of the GCs, this is illustrated for the sake of simplicity. One of the GCs gave rise to clones that appeared to have been derived from both “P” and “N1” precursors (see text).
hypermutation and affinity-based selection during the primary response.

Induction of hypermutation and rapid phenotypic selection take place during the secondary GC response of canonical clones

A total of 13 GCs were analyzed, as described above, from mice that had been primed with Ars-KLH in alum or CFA at least 6 wk earlier and boosted 6 or 8 days before spleens were taken. The extensive apparent deposition of E4,5Ci− immune complexes on FDCs after day 8 (see Fig. 4) precluded the unambiguous identification of GCs that contained canonical V region-expressing B cells at later time points. Fig. 6 summarizes the data obtained, and Fig. 2 presents representative sequence data from individual day 6 and day 8 GCs. Eleven of the 13 GCs gave rise to only canonical V_{H} clones and two gave rise to clones representative of other anti-Ars V region families. One of these GCs also gave rise to canonical V_{H} clones—the only example of a “mixed clone” GC obtained in our entire analysis. At day 6, all GCs gave rise to clones whose sequences suggested ongoing hypermutation (resulting in dendrogram “trees” or “bushy trees”), but the average frequency of mutation in these GCs (1.3%) was similar to what had been observed in the primary response (1.1%). In addition, only two of the GCs produced clones with mutations at CDR2 positions 58 or 59 that might be affinity enhancing (see Fig. 5).

The data obtained from the analysis of day 8 secondary GCs differed somewhat, revealing a higher average mutation frequency per V_{H} clone bp (3.0%) and a greater prevalence of affinity-enhancing mutations at positions 58 and 59. In addition, the general shape of the dendrograms was somewhat different from day 6, now including “trees” with much longer “trunks.” While detailed comparison of the day 6 and day 8 secondary GC data are difficult due to the sample size, collectively they indicate that canonical B cells in the day 8 GCs had often undergone more extensive hypermutation as compared with that observed at day 6, as well as intense phenotypic selection. Nonetheless, analysis of the R/S ratio of mutations in CDRs and FWRs did not reveal obvious differences between these two time points (Fig. 6).

A single canonical clone that has undergone extensive expansion and migration can give rise to multiple GCs during the secondary response, followed by a period of clonal expansion and hypermutation that is confined to individual GCs

The most striking secondary GC data were obtained from a single mouse analyzed at day 6, from which six E4, 5Ci− GCs were analyzed. Of these GCs, five gave rise to canonical V_{H} clones, which all appeared to have been derived from a common precursor based on identity of V_{H} junctional nucleotides. All of the V_{H} clones obtained from these GCs were hypermutated, but only a few of the somatic mutations in clones isolated from different GCs were shared. Some of these GCs were adjacent to one another, while others were present in different locales of the spleen. As illustrated in Fig. 7, the data obtained from this analysis suggest that a common, unmutated precursor (labeled “P”), which initially underwent clonal expansion and extensive migration while sustaining only a few mutations (giving rise to the “N1” GC precursor cell), gave rise to these five GCs. After entering GCs, the progeny of this clone appear to have undergone extensive hypermutation and expansion with little or no inter-GC migration (see Fig. 7 for details).

Discussion

The previous studies of Liu et al. were the first to attempt to directly examine the participation of canonical anti-Ars B cell clonotypes in the primary GC response, using a FACS single-cell PCR approach (64). In general, the data we obtained from our immunohistochemistry/microdissection/PCR studies confirm and extend the conclusions of these investigators. We observed a low frequency of E4, 5Ci− GCs, consistent with their estimates that even at the height of the anti-Ars primary response only 40,000 GC B cells of this clonotype are present on average per spleen. Despite this minor participation, our data show that multiple GCs that are distinct with respect to somatic mutation are often derived from the offsprings of a single canonical precursor. This demonstrates that cell division and extensive migration in the absence of hypermutation usually take place in canonical clones before entry into the primary GC reaction. Because we have previously shown that PALs-associated “foci” do not form during the anti-Ars primary response (37), the site of this pre-GC phase of clonal expansion remains to be identified. Nonetheless, these data provide further evidence that extensive focal proliferation of Ag-activated B cells in the PALs is not a prerequisite for the generation of precursors to the GC/memory pathway (27, 37).

Liu et al. also documented a modest average frequency of V region hypermutation in canonical GC B cells isolated from days 10 and 12 of the primary anti-Ars response (0.5–1%), as compared with hybridomas expressing canonical V regions isolated from amnestic anti-Ars responses (2.5–3%; Ref. 24), yet 10 and 12 of the primary anti-Ars response (0.5–1%), as compared with hybridomas expressing canonical V regions isolated from amnestic anti-Ars responses (2.5–3%; Ref. 24), yet >80% of the E4−, PNA− B cells they analyzed at day 10 expressed hypermutated V genes (64). Our data corroborate these findings, and also indicate that this modest frequency per V region base pair remains rather constant through at least day 15 of the GC response. In addition, all of the primary GCs we analyzed showed evidence of ongoing hypermutation. Analogous studies using other hapten systems have indicated that clonal expansion in primary GCs can take place without induction of hypermutation, even during the intermediate stages of the response (14). Whether these differences in hypermutation frequency are reflective of changes in the nature of the GC response driven by different Ags, or variations in the potential of distinct B cell clonotypes to undergo hypermutation awaits further investigation.

Among V_{H} clones obtained from primary day 11 through 15 GCs, we did observe a high frequency of the CDR2 position 58 affinity-enhancing mutation. However, we did not detect an increasing frequency of the CDR2 position 59 affinity-enhancing mutation. Moreover, dendrogram “trunks” or “trees” also did not increase with time. Either would have been indicative of preferential clonal expansion of hypermutated subclones that had acquired increased affinity for Ag. This discrepancy indicates that the position 58 mutation may be a “hot event,” resulting from both a high frequency of occurrence and phenotypic selection. Indeed, its frequency of observation was only slightly higher than a documented intrinsic hotspot mutation at a consensus RGYW motif (53), resulting in a translationally silent mutational change at position 105 in the D region (see Fig. 1). Although a much larger sample size would be required for unequivocal conclusions to be drawn, we also did not observe progressive increases in the R/S mutation ratio in CDRs, or a decrease in this ratio in FWRs. In contrast, we obtained only a few V_{H} clones with translation termination codons or replacement mutations in codons know to encode Ars contact residues in CDRs or highly conserved FWR amino acids (data not shown). Thus, these data suggest that there is a strong selection for maintenance of Ag binding capacity during the primary GC reaction, but not necessarily for iterative increases in affinity for Ag.

These findings appear inconsistent with previous conclusions that V gene mutations accumulate monotonically and that V region phenotypic selection occurs progressively during the primary GC response (5, 11). A means to reconcile our data with previous
We did isolate several groups of canonical V_H clones from indi-

primary GCs do not allow an unequivocal resolution of this issue.

including both position 58 and 59 affinity-enhancing mutations.

memory B cells to undergo further hypermutation ex vivo (21, 66).

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subject of controversy. Cerny and colleagues suggested that B cells

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increases in Ars affinity may be preferentially recruited into an

extrafollicular, and perhaps extrasplenic maturation pathway.

studies has recently been provided by Tarlinton, Kelsoe, and their

colleagues, analyzing affinity maturation during the the primary

response to (4-hydroxy-3-nitrophenyl)acetate (31, 32, 65). Their

findings suggest that while hypermutation may be restricted to

GCs, the phenotypic selection of mutant V regions takes place not

only in the GC, but in other microenvironmental locales as well.

During the anti-Ars primary GC response, B cells whose V regions

have sustained combinations of mutations that result in substantial

increases in Ars affinity may be preferentially recruited into an

extrafollicular, and perhaps extrasplenic maturation pathway.

Given this scenario, B cells that had recently entered the GC and

therefore had undergone only the initial stages of hypermutation

and phenotypic selection would be preferentially sampled by bulk

microdissection. Examination of the kinetics and extent to which

canonical B cell clones populate extrasplenic compartments such

as the bone marrow, as well as the patterns and frequency of so-

matic mutations in the V genes these canonical clones express, will

be required to further test this idea.

There have been limited studies of V gene hypermutation and

selection during the secondary GC reaction, and whether this re-

sponse differs qualitatively from the primary GC response is a

subject of controversy. Cerny and colleagues suggested that B cells

that have sustained V region hypermutation in the primary re-

sponse often undergo extensive expansion without hypermutation

in secondary GCs (39). In contrast, Milstein, Berek, and coworkers

have argued that memory B cells re-enter GCs during secondary

and higher order responses, where they sustain further rounds of

hypermutation and phenotypic selection (20, 26). Adding to the

confusion are conflicting results regarding the ability of purified

memory B cells to undergo further hypermutation ex vivo (21, 66).

The data we have obtained from the analysis of E4, 5Ci

secondary GCs do not allow an unequivocal resolution of this issue.

We did isolate several groups of canonical V_H clones from indi-

vidual day 8 secondary GCs that shared many somatic mutations,

including both position 58 and 59 affinity-enhancing mutations.

One interpretation is that these mutations took place in the primary

response in a memory cell precursor, whose progeny were subse-

quently recruited into the secondary GC reaction where they un-

derwent further hypermutation. This scenario is illustrated for a

group of V_H clones isolated from a day 8 secondary GC in den-

drogram form in Fig. 8B. However, similar data were sometimes

obtained from V_H clones isolated from primary GCs, also as il-

lustrated in dendrogram form for a day 12 primary GC in Fig. 8A.

Because all canonical V_HIdCR hypermutation takes place after Ars

immunization (54), such primary GC data reinforce the conclusion

that V gene mutation occurs at a very high rate in the GC micro-

environment (9–11, 13) and that majority representation of only a

few of the enormous number of V region variants generated by

mutation can also take place rapidly (11, 14). Thus, the shared

mutations observed among V_H clones derived from secondary GCs

might well have taken place during the early stages of the second-

ary GC reaction, in B cells directly recruited from the naive B cell

compartment. Indeed, if the rates of V gene hypermutation and

selection are accelerated in anamnestic GCs (see below), then na-

ive B cells recruited into this response could rapidly give rise to

progeny expressing V regions with frequencies and patterns of

mutation thought previously to be characteristic of only memory B

cells that had undergone multiple GC reactions (20, 26). In

this regard, we obtained no V_H clones containing known affinity-

enhancing 58 and 59 mutations from day 6 secondary GCs, while

such clones were often obtained from day 4 and 6 secondary AFC

“clusters.” Moreover, the scarcity of affinity-enhancing 58 and 59

mutations in day 6 secondary GCs indicates that even low-affinity

B cells can enter the secondary GC reaction, as previously shown

for the primary GC reaction (67). Clearly, to unambiguously ad-

dress the question of whether memory B cells can re-enter the

hypermutation-selection pathway in the secondary response will

require a reproducible method for tracking these cells in situ that

is independent of the structure and function of their Ag receptors.
It has often been suggested that hypermutation and phenotypic selection of V region structure take place in an iterative fashion (3, 4, 11, 25, 40, 51). Indeed, the temporal efficiency with which multiple affinity-enhancing mutations are fixed in a single B cell clone is inconsistent with one-step generation and subsequent outgrowth of a small number of cells that bear all these mutations (68, 69).

One possible mechanism whereby hypermutation and selection could take place in an iterative fashion is via the sequential formation of GCs (and, thus, discrete periods of hypermutation and selection) by the progeny of a single B cell clone. In this scenario, if the stringency of selection was based on the affinity or specificity of the V region expressed by the GC founder B cell (70), then sequential GC reactions would lead to progressive intraclonal increases in affinity or specificity. Testing this idea was one of the reasons that we chose to microdissect E4,5Ci+ GCs present in the same splenic locales. The data we and others (10, 28) have obtained do not provide support for this hypothesis. None of the clonally related groups of VH genes recovered from distinct GCs that we analyzed revealed evidence of a mutational precursor-product relationship (for example, see Fig. 3). The data derived from the five clonally related secondary GCs illustrated in Fig. 7 did suggest a brief period of mutation in a single clone, before the nucleation of multiple GCs, but none of the few mutations apparently sustained during this initial period are associated with increased affinity for Ars (see Fig. 7). In total, these data further reinforce the previous suggestions by us (70) and others (28, 29) that primary GCs are “independent islands of clonal hypermutation and phenotypic selection,” and extend this conclusion to secondary GCs. Testing models that propose that iterative cycles of hypermutation and selection take place in single GCs, perhaps via “cyclical re-entry” of phenotypically selected B cells into a hypermutating pool (69), must await methods that will allow dissection of the kinetics of B cell differentiation in various subcompartments of individual GCs.

Somewhat surprisingly, the primary and secondary GC reactions were qualitatively similar, suggesting a lack of substantial mechanistic differences in the nature of these responses. Nonetheless, comparison of the data obtained from them revealed some distinctions. As expected from previous studies (19), the secondary GC response developed more rapidly, and while V gene mutation frequencies similar to those observed throughout the primary GC response were also observed at day 6, by day 8 we observed 2- to 3-fold higher frequencies, although substantial GC to GC variation was apparent, as was the case among primary GCs. Moreover, several day 8 secondary GCs gave rise to clones containing both CDR2 58 and 59 affinity-enhancing mutations. Taken together with the primary GC data, these data indicate that phenotypic selection may take place more rapidly in secondary GCs, consistent with the accelerated rate of this response overall.

Unfortunately, whether affinity maturation beyond the fixation of the 58 and 59 mutations or emigration of these high-affinity clones from the GC, as appears to take place in the primary response, took place at later times could not be determined due to the extensive apparent deposition of E4, 5Ci+ immune complexes in all GCs after day 8. The extent of canonical clone expansion, migration, and GC nucleation also appears more robust during the secondary response, as exemplified by comparing the data summarized in Figs. 3 and 7. Whether these quantitative and kinetic differences result from an increased precursor frequency of canonical clones, increased levels of carrier specific T cell help (19, 39), increased levels of Ag deposited on FDCs (34), or a combination of these factors will require further investigation.

The most remarkable difference between the behavior of canonical clonotypes in the primary and secondary responses occurs in the AFC pathway. Canonical AFCs are infrequent and appear as isolated cells during the primary response in the spleen. In marked contrast, the early secondary response is characterized by a massive canonical IgG AFC response in which memory cells recruited into this pathway undergo extensive migration and proliferation and are ultimately organized in loose clusters, predominantly in the red pulp. During this response, V region hypermutation does not take place, analogous to the situation in PALS “foci” characteristic of the primary anti-(4-hydroxy-3-nitrophenyl)acetyl response of C57BL/6 mice (28). Interestingly, our previous hybridoma analysis of the early stages of the “original antigenic sin” response of canonical memory clones to a cross-reactive hapten indicated that during the “sin” response these clones underwent extensive expansion without hypermutation (71). In total, our data demonstrate that canonical clonotypes are incompetent to give rise to PALS AFC foci during the primary response, but during the primary GC reaction they acquire the potential to subsequently undergo vigorous bursts of hypermutation-free expansion and Ab production during secondary responses. However, apparently only a minor fraction of all canonical B cells produced during the primary GC response acquire this potential, as revealed by the mutational homogeneity of the VH genes expressed by secondary AFC foci derived from single progenitors. This oligoclonal nature of the early secondary response has been extensively documented by hybridoma technology (50, 71, 72). We have previously suggested that this extreme “repertoire narrowing” is due to “specificity maturation”—a combination of stringent negative selection of V regions with cross-reactivity for self-Ags and positive selection for V regions with increased affinity for the driving foreign Ag (16).

Whether the hypermutation-free growth that takes place during the secondary AFC response results in maintenance or regeneration of a canonical memory AFC precursor population or leads only to terminal differentiation remains to be determined. However, in this regard several previous studies of the memory B cell response using adoptive transfers and hybridoma technology have indicated that memory cells can undergo extensive clonal expansion and persist for prolonged periods without induction of V gene hypermutation (73, 74). The data we present here suggest that these past studies preferentially sampled B cells in an anamnestic AFC pathway, rather than in an anamnestic GC pathway.

Unfortunately, the near lack of overlap of the secondary canonical AFC and GC responses thwarted attempts to determine whether a precursor-product relationship might exist between canonical AFC clusters and canonical GCs. By day 6, when such GCs are first detected, the size of canonical AFC clusters has decreased substantially from what is observed at day 4, and most day 6 clusters surround the red pulp sinuses (Fig. 4), suggesting migration of the cells in these clusters from their site of origin. These observations are consistent with previous observations that most splenic AFCs have a very short half life (75). Due to this situation, E4, 5Ci+ AFC clusters are usually not observed adjacent to E4, 5Ci+ GCs at this time. We did obtain PCR VH clones from a single day 6 E4, 5Ci+ GC/AFC “cluster” pair (see Figs. 5 and 6). Sequencing of these clones revealed canonical VH genes with distinct junctional nucleotides and no shared somatic mutations between the two groups of clones, strongly suggesting derivation from distinct progenitors. We are now pursuing alternative approaches to address this important issue. Indeed, current data regarding the relationship of B cells capable of giving rise to primary and anamnestic AFC and GC responses have not allowed agreement on whether the precursors to these pathways of differentiation are members of distinct developmentally committed lineages or not (28, 35, 36).
Acknowledgments

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References