In Situ Studies of the Primary Immune Response to (4-Hydroxy-3-Nitrophenyl)Acetyl. V. Affinity Maturation Develops in Two Stages of Clonal Selection

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Summary

To examine the role of germinal centers (GCs) in the generation and selection of high affinity antibody-forming cells (AFCs), we have analyzed the average affinity of (4-hydroxy-3-nitrophenyl)acetyl (NP)-specific AFCs and serum antibodies both during and after the GC phase of the immune response. In addition, the genetics of NP-binding AFCs were followed to monitor the generation and selection of high affinity AFCs at the clonal level. NP-binding AFCs gradually accumulate in bone marrow (BM) after immunization and BM becomes the predominant locale of specific AFCs in the late primary response. Although the average affinity of NP-specific BM AFCs rapidly increased while GCs were present (GC phase), the affinity of both BM AFCs and serum antibodies continued to increase even after GCs waned (post-GC phase). Affinity maturation in the post-GC phase was also reflected in a shift in the distribution of somatic mutations as well as in the CDR3 sequences of BM AFC antibody heavy chain genes. Disruption of GCs by injection of antibody specific for CD154 (CD40 ligand) decreased the average affinity of subsequent BM AFCs, suggesting that GCs generate the precursors of high affinity BM AFCs. Inhibition of CD154-dependent cellular interactions after the GC reaction was complete had no effect on high affinity BM AFCs. Interestingly, limited affinity maturation in the BM AFC compartment still occurs during the late primary response even after treatment with anti-CD154 antibody. Thus, GCs are necessary for the generation of high affinity AFC precursors but are not the only sites for the affinity-driven clonal selection responsible for the maturation of humoral immune responses.

Early in the course of infection, protection is achieved more effectively by preexisting neutralizing serum antibodies than by the later set of antibodies secreted upon re-stimulation of memory B cells (1). After infection or vaccination, neutralizing serum antibodies can be detected in humans for several decades (2, 3); immunized mice maintain neutralizing antibodies for more than one year. Particularly in situations of rapid and severe pathogenesis, these long-lasting antibodies can provide a powerful mechanism for protection against infection, morbidity, and mortality (1).

One of the characteristics of long-lasting serum antibody is a progressive increase in affinity for the immunogen over time, through a process called affinity maturation (4, 5). After the introduction of hybridoma technology, it was revealed that affinity maturation of serum antibody is achieved by two key events: the generation of antibody variants by V(D)J hypermutation and the subsequent selection of those variants that have high affinity for antigen (6, 7). Over time, these events lead to the preferential accumulation of antibody-forming cells (AFCs) that secrete antibodies with higher affinities and faster on-rates (8–10). It is widely believed that inter- and intraclonal competition for the antigen retained on the follicular dendritic cells of germinal centers (GCs; 11–13) is the basic mechanism that promotes the selective accumulation of high affinity memory B cells and AFCs over time (5). However, little is known about the cellular and molecular mechanisms underlying this selection.

After immunization with T cell–dependent antigens, antigen-responsive B cells in the spleen accumulate and proliferate in the margins of the T cell zones, or the periarteriolar lymphoid sheaths (PALS), and enter into two developmental pathways. B cells can either remain to form foci of AFCs
tion of high-affinity precursors of BM AFCs by somatic high affinity AFCs in BM. Here, we show that the genera-
tion of a nitrophenyl (NP) hapten (39–42) to investigate the antibody response of C57BL/6 mice to the (4-hydroxy-3-
titration; indeed, the majority of affinity maturation in se-
months after immunization the great majority of antigen-
specific AFCs are present in BM. Since serum antibodies have relatively short half-lives (22), it is now accepted that the long-lived BM AFCs are responsible for long-lasting antibody titerstes.

The GC has been identified as a site for the generation of high affinity antibody variants through antigen-driven V(D)J hypermutation and clonal selection (24–27). Lymphocytes in the GC regain many characteristics of those present in primary lymphoid tissues (28–32), including high sensitivity to antigen receptor-mediated death (28–31), consistent with the idea that GCs are specialized sites for clonal selection. Previous reports also suggest that BM AFCs are derived from GC B cells (33–35), implying that GCs are sites for the generation and selection of high affinity BM AFCs. However, it remains unclear to what extent mutation and selection are mutually exclusive in GCs (36, 37). Recently, Smith et al. (35) showed that high affinity B cells migrate into the BM to become AFCs during the primary GC re-
action and concluded that the frequency of high affinity AFCs in BM is key elements in the affinity maturation of serum antibody and are crucial for protective immunity.

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action and concluded that the frequency of high affinity AFCs in BM is key elements in the affinity maturation of serum antibody and are crucial for protective immunity.

M⁻¹ (H33Lγ1/λ1) could be detected by both NP₂₅–BSA and NP₂₆–BSA. However, transfectomas with a Kᵦ = 10⁵ M⁻¹ could be detected by NP₂₆–BSA, but not by NP₂₅–BSA. Transfectomas with a Kᵦ = 2.3 × 10⁵ M⁻¹ could not be detected by either NP-BSA coat. Thus, AFCs secreting antibody with a Kᵦ > 2.3 × 10⁵ M⁻¹ can be detected with NP₂₅–BSA, and those with a Kᵦ ≥ 10⁶ M⁻¹ can be detected with NP₂₆–BSA.

Measurement of serum IgG₁ or λ1 antibody. IgG₁ or λ1 antibody specific for the NP hapten was detected by ELISA using two different coupling ratios of N-P-BSA as the coating antigens. In brief, 96-well flat bottom plates (Falcon; Becton Dickinson, Oxnard, CA) were coated with 50 μg/ml N-P-BSA or N-P₂₅–BSA in 0.1 M carbonate buffer (pH 9.0) at 4°C overnight, and blocked with 0.5% BSA in carbonate buffer. Serially diluted sera were then added to each well and incubated at 4°C overnight. On each plate, serially diluted H33Lγ1/λ1, a monoclonal antibody recognizing the NP hapten (Kᵦ = 2.0 × 10⁵ M⁻¹), was also included as a control. After washing with PBS containing 0.1% Tween 20, HRP-conjugated goat-anti mouse IgG₁ or biotinylated Ls136 was added and incubated at room temperature for 2 h. HRP-conjugated streptavidin was added to detect biotinylated Ls136, and those with a Kᵦ < 10⁻⁵ M⁻¹ could be detected by NP₂₆–BSA. The affinity threshold of antibody binding to each NP-BSA conjugate was determined by using several monoclonal antibodies with different affinities for NP. H33Lγ1/λ1 bound equally well to both NP-BSA conjugates, whereas a monoclonal antibody with a Kᵦ = 10⁵ M⁻¹ showed a 20-fold lower binding to NP₂₅–BSA than to NP₂₆–BSA. A monoclonal antibody with a Kᵦ > 2.3 × 10⁵ M⁻¹ had a 10-fold lower binding to NP₂₅–BSA than one with a Kᵦ = 1.0 × 10⁶ M⁻¹. Thus, antibody with a Kᵦ > 2.3 × 10⁵ M⁻¹ can be detected with NP₂₆–BSA, and one with a Kᵦ ≥ 10⁶ M⁻¹ can be detected with NP₂₆–BSA.

Flow Cytometry. Single cell suspensions of splenocytes and BM cells were prepared and RBCs were depleted by incubation in 0.83% NH₄Cl; cells were then washed with PBS (pH 7.4) containing 2% FCS and 0.08% sodium azide at 4°C for cytometric analysis, or washed with deficient RPMI 1640 (Irvine Scientific, Santa Ana, CA) containing 2% FCS for sorting. To estimate the prevalence of GC B cells, cells were stained with FITC-labeled Ls136, PE-conjugated anti-CD138, Tricolor-conjugated streptavidin (CalTag Laboratories, South San Francisco, CA), and 7-AAD. Finally, λ1, CD138⁺ cells within the Tricolor⁺ (IgM⁻, M-αc⁻, Gr-1⁻, Thy1.2⁺), 7-AAD⁻ fraction (routinely between 0.003 and 0.015% of cells) were sorted into Trizol (GIBCO BRL, Gaithersburg, MD) containing 50 μg/ml trNA (Esherichia coli; Boehringer Mannheim, Indianapolis, IN) at 50–200 cells per tube using a FACStar Plus® (Becton Dickinson). Sorting routinely yielded populations of ~90% purity.

Afinity Measurement of Serum Antibody by Fluorescence Quenching. In addition to differential ELISAs, the affinity of anti-NP serum antibody was estimated by fluorescence quenching (44). In brief, serum IgG was purified from cohorts of mice using protein G-Sepharose and adjusted to a concentration of 50 μg/ml in 2 ml of PBS containing 0.02% Tween 20. Titrations were carried out by adding monovalent N-P hapten (NP-caproate; Genoys, Woodlands, TX) over three-log molar range (10⁻²–10⁻⁵). For the correction of nonspecific quenching, quenching from an irrelevant antibody, a mouse anti-dextran IgG₁ (MOPC 21; Sigma Chemical Co.), was also determined.

Sequence Analysis of VDJ DNA from BM AFCs. Total RNA was extracted from sorted, Trizol-solubilized BM AFCs based on manufacturer’s protocol. First strand cDNA was synthesized from total RNA using a primer complementary to the C H1 region of Cγ₅₁ (Cγ₁₃/1 primer; 5'-GAGTTCCAGGTCACTGCTGGCTCAGGGA-3') and a Superscript Kit (GIBCO BRL). 5 μl of cDNA solution was used as a template for two rounds of nested PCR to amplify Vγ₄ genes combined to the Cγ₁ region. PCR amplification, cloning, and sequencing of Vγ₄ genes were performed as previously described (24, 30), except that the Cγ₁ primer was used as an antisense primer for the first round of PCR amplification. This PCR scheme minimizes any contribution of VDJ rearrangements from immature BM B cells to the data set.

Results

Primary Immunization with NP-CG Induces Long-lasting Serum Antibody and High Frequencies of Specific AFCs in BM. After primary immunization with 50 μg of NP-CG, the number of splenic AFCs secreting NP-specific, IgG₁ antibody peaked at day 8 after immunization and then rapidly declined (Fig. 1 A). Concomitantly, the number of BM AFCs slowly grew, such that by 4 mo after immunization the frequency of hapten-specific AFCs in the BM was >10-fold higher than in the spleen. The increased frequency and persistence of NP-specific AFCs in the BM correlated with persistent titers of NP-binding IgG₁ antibody in the serum (Fig. 1 B). Maximum levels of circulating antibody (900–1700 μg/ml) were observed at day 12 after immunization. Circulating antibody concentrations then declined to ~50% of this peak and remained stable from day 32 to day 119 after immunization. Combined with the relatively short half-life of circulating antibody (22) and the prominent role of BM in long-term antibody production (20, 21), persistent IgG₁ serum antibody appears to be the product of BM AFCs.

The near exclusive use of λ1 L chain in NP-binding antibody during the early primary response is diminished in secondary responses which support increased percentages of κ-bearing, NP-specific antibody (20–40%; references
To estimate the stability of $\lambda 1$ expression among persistent IgG1 antibody, both the $\lambda 1$ and the IgG1 antibody titer in each sera was calculated by ELISA using the same control antibody bearing IgG1/$\lambda 1$. As shown in Table 1, the percentage of $\lambda 1$/IgG1 remained above 80% from day 46 to day 119 after immunization. Thus, no significant replacement by NP-specific antibody-bearing $\kappa$ L chain was observed.

Table 1. Continued Predominance of $\lambda 1^+$ Antibody in the Primary Anti-NP Response

<table>
<thead>
<tr>
<th>Days after immunization</th>
<th>NP-binding primary antibody</th>
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<tbody>
<tr>
<td></td>
<td>Average concentration $\mu g/ml$</td>
</tr>
<tr>
<td>22 (n = 4)</td>
<td>1,249</td>
</tr>
<tr>
<td>32 (n = 5)</td>
<td>753</td>
</tr>
<tr>
<td>46 (n = 4)</td>
<td>513</td>
</tr>
<tr>
<td>69 (n = 6)</td>
<td>296</td>
</tr>
<tr>
<td>119 (n = 4)</td>
<td>395</td>
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</tbody>
</table>

spleen (Fig. 2), and later continued a more gradual, but appreciable increase (68.4% at day 32, 85.4% at day 119; $P < 0.05$, Student's $t$ test) after the GC response became undetectable. The average affinity of NP-binding serum antibody was also determined in the same mice by ELISA (Fig. 4 A.) and fluorescence quenching (Fig. 4 B.). Mirroring affinity

Figure 2. Kinetics of the primary GC response. The percentage of GC B cells out of live lymphocytes was assessed by co-staining with anti-B220 and GL-7 antibodies. The data represent the average number and standard deviation from individual mice ($n = 3–9$). The background staining with these antibodies was determined from naive mice, and is indicated (along with SD) by the hatched area. *, **: Staining that is statistically different from background at $P < 0.001$ and $P < 0.005$, respectively, using the student’s $t$ test.
increases in BM AFCs, the average affinity of serum antibody also increased from day 12 to day 119 of the response (Fig. 4). Indeed, the majority of affinity maturation in the serum antibody took place after day 32, when GC B cells could no longer be detected in the spleen. The different pattern of increase in affinity between BM AFCs and serum antibody (Figs. 3 and 4) is likely to be due to the rate of decay (half-life) of serum IgG1 antibody. Together, these data suggest that antigen-driven clonal selection for high affinity BM AFCs or their precursors continues in the post GC environment.

Affinity Maturation of Serum Antibody Is Reflected in the Somatic Genetics of the BM AFC Population.

Initially, NP–CG activates a diverse population of splenic B cells that bear the λ1 L chain (39, 40) and H chain genes containing members of the V186.2 and V3 subfamilies of J558 VH gene segments C1H4, V23, CH10, V186.2, 24.8, or 165.1 (46, 47). With time, interclonal competition (47) leads to the predominance of higher affinity cells carrying V186.2-to-DFL16.1 rearrangements with a tyrosine-rich junctional motif, YYGS (42, 48) and an affinity-enhancing W → L mutation at position 33 (35, 49, 50).

To follow clonal competition and selection in BM AFCs, we enriched this population by depleting IgM, Gr-1, M ac-1, and Thy1.2 cells from BM and we then purified λ1 cells by fluorescence-activated cell sorting. Typically, >40% of this population secreted NP-binding, IgG1 antibody by ELISPOT. Enriched BM AFC populations obtained at days 12, 46, and 119 after immunization were subjected to a reverse transcriptase PCR that preferentially amplifies cDNA representing rearrangements of the V186.2 and V3 subfamilies of J558 VH gene segments joined to Cγ1. Cloned VDJ segments from each time point (n: day 12 = 18; day 46 = 31; day 119 = 22) were then sequenced to follow the somatic genetics of the BM AFC population over the course of the primary response.

Table 2 summarizes these data and shows that clonal dominance by IgG1 B cells expressing V186.2-to-DFL16.1 rearrangements takes place in the BM, albeit at a slower pace than reported for the splenic compartment. At day 12, some 78% of the VDJ rearrangements amplified from sorted λ1 BM AFCs contained the V186.2 gene segment; fewer than half of these (43%) were rearranged to DFL16.1 and none contained the YYGS motif in CDR3. However, with time the frequency of canonical V186.2 rearrangements increased; by day 119 only V186.2 rearrangements were recovered and the great majority of these (91%) were fused to DFL16.1. Almost half (41%) of the VDJ fragments from day 119 BM AFCs encoded YYGS at the V-to-D junction (Table 2).

Mutations in transcribed VDJ rearrangements accumulated in BM AFCs, reaching a maximum average of 4.6 mutations per VH gene segment by day 119 after immunization (Table 2). The apparent increase in VH mutations was most rapid in the earliest phase of the response. Indeed, the rate of accumulated mutations in the first 12 d of the
response is 11-fold greater than that observed late (days 47–119) in the response (0.17 versus 0.016 V<sub>H</sub> mutations per day). The frequency of silent mutations present in BM AFCs did not increase after day 46. Together, these observations suggest that most, if not all, V<sub>H</sub> mutations present in mature populations of IgG<sub>1</sub> BM AFCs were introduced during the first 30 d of the response.

The changing distribution and frequency of specific V<sub>H</sub> mutations in BM AFCs, like the eventual dominance of canonical V(D)J rearrangements, suggests affinity-driven selection in the BM compartment. On average, the ratio of replacement/silent mutations was suppressed in the framework regions of mutated V<sub>H</sub> genes. More significantly, the frequency of W<sup>fi</sup>L mutations in codon 33 increased from 0% at day 12 after immunization to a maximum of 25% by day 46 (Table 2).

### Table 2. Somatic Genetics of A1<sup>+</sup> AFC in BM of C57BL/6 Mice Immunized with NP-CG

<table>
<thead>
<tr>
<th>V186.2 (%)</th>
<th>d12</th>
<th>d46</th>
<th>d119</th>
</tr>
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<tbody>
<tr>
<td>Other (%)</td>
<td>22</td>
<td>10</td>
<td>0</td>
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</table>

| V186.2 mutation frequency | 2.0 (n = 14) | 3.4 (n = 28) | 4.6 (n = 22) |
| R/S ratio                | CDRA (14.0/1)* | CDRA (4.3/1) | CDRA (1.1/1) |
|                          | >1.0/1   | 1.2/1   | 9.0/1   |
|                          | >4.0/1   | 1.0/1   | 3.8/1   |
|                          | 2.3/1    | 2.1/1   | 2.6/1   |
| DFL16.1 (%)              | 43       | 39      | 91      |
| YYGS (%)                 | 0        | 0       | 41      |
| W→L 33 (%)               | 0        | 25      | 23      |

All sequence data are available from EmBL/GenBank/DDBJ under accession number AF028612-25 (day 12), AF028626-53 (day 46), and AF028654-75 (day 119).

*Expected R/S ratio of V<sub>H</sub> V186.2 given random mutagenesis.

The percentage of rearrangements using DFL16.1 gene segments in all rearrangements of V<sub>H</sub>V186.2.

The percentage of rearrangements encoding YYGS in CDR3 in all rearrangements of V<sub>H</sub>V186.2.

The percentage of all V<sub>H</sub> V186.2 rearrangements bearing a W→L mutation in 33.

### Figure 5. Disruption of GCs impairs affinity maturation of serum antibody due to the selective inhibition of high affinity BM AFCs. M R1 (open circles) or control antibody (filled circles) was intravenously administered at days 6, 8, and 10 (early), days 10, 12, and 14 (intermediate), or days 16, 18, and 20 (late). At day 69 after immunization, sera and BM cells were recovered. Numbers of NP<sub>5</sub>-binding and NP<sub>26</sub>-binding AFCs in BM were determined by ELISPOT (A) and the ratios of NP<sub>5</sub>-binding versus NP<sub>26</sub>-binding AFCs were plotted (B). The amount of NP<sub>5</sub>-binding or NP<sub>26</sub>-binding IgG<sub>1</sub> antibody in each serum was determined by ELISA (C) and the ratios of NP<sub>5</sub>-binding versus NP<sub>26</sub>-binding antibody were plotted (D).
Ratios of NP5- to NP26-binding AFCs were plotted; each point represented by ELISPOT assay on days 12, 22, and 69 of the response. (Fig. 5) without suppressing the titers of lower affinity antibody the production of high affinity serum antibody for NP

B
tios of high affinity versus total NP-binding AFCs (Fig. 5) and led to decreased ratios of high affinity versus total NP-binding AFCs (Fig. 5 B). The MR1 antibody, but not hamster Ig, also inhibited the production of high affinity serum antibody for NP without suppressing the titers of lower affinity antibody (Fig. 5 C). Inhibition of high affinity antibody was most effective when MR1 was given on the early schedule; prolonging the GC reaction by delaying the injection of MR1 allowed proportionate recovery of the high affinity serum antibody (Fig. 5 D), and MR1 treatment after the GC reaction was complete (days 46, 48, and 50) had no effect on either high affinity BM AFCs or on serum antibody (see below). Thus, the average affinity of serum antibody for NP in immunized mice receiving the early schedule of passive MR1 antibody was about five times lower ($K_a = 6.1 \times 10^8 \text{ M}^{-1}$) than antibody from control mice ($K_a = 2.9 \times 10^5 \text{ M}^{-1}$). These data suggest that the major, if not sole, source of high affinity cells in the BM AFC compartment is the GC reaction. Lower affinity AFCs may represent GC emigrants that have not yet achieved high affinity by mutation and/or selection.

Disruption of GCs does not impair Affinity-driven Selection of BM AFCs. Disruption of nascent GCs by the administration of MR1 antibody at days 6, 8, and 10 after immunization suppresses the high affinity compartment of BM AFCs and the affinity maturation of serum antibody (Fig. 5). However, affinity maturation within the small residuum of higher affinity BM AFCs remained intact (Fig. 6). The affinities of BM AFCs were determined on days 12, 22, and 69 after immunization with NP–CG and early MR1 treatment. Consistent with results illustrated in Fig. 5, the frequency of BM AFCs capable of binding NP5-BSA on days 22 and 69 was suppressed. Nonetheless, the relative fraction of high affinity BM AFCs increased in MR1-treated mice in parallel with control animals. This increase occurred in the complete absence of detectable GCs or GC cells in the spleens of MR1-treated mice. Thus, while the GC reaction limits the extent and quality of the high affinity compartment of BM AFCs, antigen-driven selection in this compartment is independent of GCs.

This late phase of affinity maturation is relatively independent of CD154-mediated costimulation and thus is distinct from the earlier selection in GCs. MR1 treatment over days 46–50 after immunization did not suppress the number ($5.1 \times 10^8$ versus $5.3 \times 10^8$) or fraction of high affinity (81 versus 73%) BM AFCs, nor did it diminish high affinity serum antibody responses (219 $\mu$g/ml versus 140 $\mu$g/ml).

Discussion

Affinity maturation of serum antibody is a cardinal but poorly understood phenomenon of humoral immune responses to thymus-dependent antigens. Early studies attempted to explain affinity maturation in Darwinian terms: clonal competition for decreasing amounts of antigen selectively maintains proliferation in B cells bearing the most avid receptors (5). More recent work supports this view and demonstrates that clonal competition is coupled to a process of V(D)J hypermutation in GCs (24, 25). It is now widely believed that affinity maturation is accomplished during the GC reaction by inter- and intracellular competition (18, 25, 47, 54). Nonetheless, several incongruent observations suggest that some affinity-driven selection, perhaps even most, takes place outside of the GC microenvironment.

First, affinity maturation of serum antibody continues long after the usual termination of primary GC responses. As early as 1972, Davie and Paul noted a 25-fold increase in the affinity of serum antibody ($K_a = 2.5 \times 10^7 \text{ M}^{-1}$ to $6.3 \times 10^8 \text{ M}^{-1}$) for the 2,4-dinitrophenyl hapten from day 45 to 171 of a primary response (38), well after the end of most primary GC reactions (53). Another incongruent observation is that mature GCs often contain B cell populations that express receptor affinities below that of the serum
antibody (55).² Splenic GCs support oligoclonal lymphocyte populations that show no evidence for significant B cell trafficking between GCs and thus no homogenization of selected populations (17). Although B cells in the same GC bear receptors with similar affinities, GCs in the same spleen can hold cell populations with hapten-specific affinities that differ by 100-fold (55 and Shimoda, M., ., Dal Porto, and G. Kelsue, unpublished observations). Finally, limited affinity maturation has been observed in lymphotxin-rate of accumulation for VH mutation in the BM AFC population, 4.6 per VH gene segment, did not differ dramatically from that present in splenic GC B cells and rearrangements of the V3 and V186.2 VH subfamilies.

To determine the extent and role of post-GC selection in affinity maturation, we measured the affinity of hapten-specific AFCs and serum antibody over a period of 4 mo (119 d) after primary immunization with NP–CG. Increased numbers of BM AFCs were correlated with persistent titers of serum antibody (Fig. 1) and both AFCs (Fig. 3) and antibody (Fig. 4) exhibited coordinated increases in affinity for the NP hapten. This affinity maturation took place over the entire period of study, even after the end of the GC response in the spleen (Fig. 2).

The clonal selection responsible for increased affinity of serum antibody and BM AFCs in the post-GC phase was reflected in the somatic genetics of the BM AFC population (Table 2). In concert with late affinity maturation, cells bearing VDJ rearrangements characteristic of high affinity anti-NP antibody, e.g., using the VHV186.2 and DFL16.1 gene segments and the YYGS motif in CDR3 (42, 48), accumulated in the BM AFC compartment. From day 46 to 119 of the response, the distribution of replacement mutations shifted dramatically from CDR2 to CDR1, without substantial increases in mutation frequencies. These changes in the BM AFC population are best explained by interclonal competition and selection after the GC reaction has ended. This sequence analysis (Table 2) also suggests that the GC reaction is the major, if not sole, source of VH mutagenesis present in BM AFCs. The rate of accumulation for VH mutations in BM AFCs was greatest during the active GC reaction and the maximum frequency of mutations achieved in the BM AFC population, 4.6 per VH gene segment, did not differ dramatically from that present in splenic GC B cells (3.8 per VH gene segment) recovered by fluorescence-activated cell sorting at day 16 of the response (56). Late affinity maturation acts upon the genetic diversity generated early in the response; BM AFCs, perhaps even those that are unmutated, appear to be derived from GC B cells.

This notion is supported by our use of the MR1 antibody to define a cell compartment responsible for the generation of high affinity BM AFCs and serum antibody (Fig. 5). Administration of this CD154-specific antibody after day 6 of the response to NP–CG has no effect on early primary antibody titers (52), the generation of T cell help (57), or T cell memory as determined by in vitro recall response (data not shown). However, the MR1 antibody does abrogate the GC reaction within hours of its injection (52) without the induction of significant apoptosis in B cells (30, 58). The MR1 treatment used in this study completely eliminated all splenic GCs as determined by histology and the enumeration of GL-7⁺ B220⁺ splenocytes (data not shown); continuing surveys found no evidence for the re-formation of GCs. Administration of MR1 antibody after the end of the GC reaction had no discernable effect on NP-specific BM AFCs or serum antibody level (data not shown). Thus, the progenitors of high affinity BM AFCs and GC B cells share sensitivity to suppression by the MR1 antibody during the second and third weeks of the response.

Despite the complete loss of GCs and splenic B cells bearing the GC phenotype, affinity maturation was present in all mice treated with MR1 antibody. Indeed, although disruption of CD40–CD154 interactions lowered the frequency of high affinity BM AFCs and suppressed the average affinity of serum antibody, antigen-driven selection was equally efficient in MR1-treated and control mice (Fig. 6). Even the administration of MR1 antibody during the late phase (days 46–50) of the primary response did not suppress affinity maturation in the BM AFC compartment. These results demonstrate that clonal selection among BM AFCs is independent of the GC microenvironment and resistant to blockade of CD154-mediated costimulation.

In contrast to a similar study by Smith et al. (35), we did not observe an early plateau in the affinity of BM AFCs. Nor did we observe preferential, early recruitment of AFCs carrying the affinity-enhancing W→L mutation at position 33 of the V186.2 VH gene segment (35, 49); instead, a maximum of only one-fourth of VHV186.2 rearrangements recovered from BM AFCs carried this exchange at days 46 and 119 after immunization (Table 2). These contradictions may be due to very different experimental approaches for the isolation of NP-binding B cells. Smith et al. determined the affinity and somatic genetics of BM AFCs only after antigen-based selection using a fluorescent NP-hapten (35), a process that may have biased the selected population for high affinity. Biased selection might be heightened in BM AFC populations, since surface Ig expression by these cells is low (23). Instead, we enriched NP-binding BM AFCs based on their expression of λl and CD138. Subsequently, cloned cDNAs were sequenced without further selection, such as colony hybridizations to detect CDR3 sequences typical of primary NP-specific B cells (49). Thus, although our selection criteria limited our study to λl⁺ cells and rearrangements of the V3 and V186.2 VH subfamilies, antibody affinity and CDR3 diversity were unconstrained.

One might argue against the specificity of BM AFCs collected with regard to only λl and CD138 expression. However, we believe that the fraction of VDJ rearrangements amplified from nonspecific B cells was negligible, since nearly one-half of the selected cells secreted NP-binding IgG1 antibody and the majority of amplified VDJ rearrangements contained the V186.2 gene segment despite the ability of our PCR primers to amplify many other related VHV186.2 gene segments (47, 49). Furthermore, we did not detect λl⁺ CD138⁺ cells in BM after depletion of IgM⁺ M ac-⁺ Gr-I⁺ Thy1.2⁺ cells from 8–20-wk-old naive mice (data not shown). Thus, we propose that the relatively low
frequency of the W → L mutation in our study does not reflect contamination by large numbers of nonspecific B cells but represents the true pattern of clonal selection in vivo without the confounding effects of artificial enrichment.

In the primary response to NP–CG, affinity maturation of the serum antibody develops in two stages of clonal selection within and outside GCs. While GCs are present, antigen-driven selection progressed rapidly but could be halted by interrupting CD40–CD154 interactions with injections of the MR1 antibody. However, even after the GC reaction waned, affinity maturation and clonal selection continued, independent of GC structure and inhibition by MR1. Thus, the primary role for GCs in the affinity maturation of serum antibody seems to be the generation of high affinity variants by V(D)J hypermutation; afterwards, antigen-driven interclonal selection can occur outside of the GC microenvironment. Long-term clonal selection in the post-GC environment offers the opportunity to continue to improve the antigen-selected repertoire by allowing direct competition between the progeny of both high and lower affinity GC B cells. This process does not seem to take place between the isolated B cell populations present in different GCs, even when those populations are separated by only 20–50 μm (16).

The important question of where and how clonal selection takes place in the post-GC environment remains unanswered. The maintenance of memory B cells is thought to be dependent on the presence of persistent antigen (59) and ~10% of memory B cells are still in cell cycle at day 140 after primary immunization (60). These observations suggest that memory B cells are frequently restimulated by antigen held on follicular dendritic cells (11–13). One possible mechanism for continued selection might be competition among memory B cells for restimulation with persistent antigen. In support of this idea, it has been suggested that BM AFCs are generated by the migration of restimulated memory B cells from the spleen after secondary challenge (33, 61). In humans, B cells with memory phenotype have been shown to accumulate in BM with age, presumably as a consequence of a lifetime’s exposure to antigens (62). If this were the case in mice, clonal selection in the BM AFCs might represent the transition of selected, memory cells into AFCs (20, 34, 61).

However, we do not believe that restimulation of memory cells accounts for affinity maturation late in the primary response. Recent labeling studies (23) demonstrate that the BM AFC compartment is not sustained by the proliferation of (memory) B lymphocytes but represents an independent, long-lived population. Furthermore, BM AFC numbers, affinity, and antibody production are not diminished by administration of the MR1 antibody after the primary GC reaction is complete. In contrast, secondary B cell responses are highly dependent on CD154-mediated costimulation as they are dramatically suppressed by MR1 antibody (57). Indeed, BM AFCs may directly compete for activation by antigen; unlike the early AFCs present in the spleen, BM AFCs express mIg and exhibit low levels of proliferation (23). However, the question still remains as to where the antigen depot required for this competitive stimulation might be located.

Although many studies have focused on memory B cells to examine the protective role of humoral immune responses, the AFCs in the BM compartment have been relatively neglected despite their significant contribution to the maintenance and quality of serum antibody (1, 20, 23, 35, 61). Recent accumulating data suggest that BM AFCs are a unique population distinct from the terminally differentiated AFCs present in secondary lymphoid tissues. Further studies are required to determine the contribution of these cells to protective immunity and to understand the biology of their selection and longevity.

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References


