Very Low Affinity B Cells Form Germinal Centers, Become Memory B Cells, and Participate in Secondary Immune Responses When Higher Affinity Competition Is Reduced

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Abstract

To understand the relationship between the affinity of the B cell antigen receptor (BCR) and the immune response to antigen, two lines of immunoglobulin H chain transgenic (Tg) mice were created. H50Gμa and T1(V23)μa mice express μ H chain transgenes that associate with the L chain to bind the (4-hydroxy-3-nitrophenyl)acetyl hapten with association constants (Kₐ) of only 1.2 × 10⁵ M⁻¹ and 3 × 10⁴ M⁻¹, respectively. Both lines mounted substantial antibody-forming cell (AFC) and germinal center (GC) responses. H50Gμa Tg mice also generated memory B cells. T1(V23)μa B cells formed AFC and GCs, but were largely replaced in late GCs by antigen-specific cells that express endogenous BCRs. Thus, B lymphocytes carrying BCRs with affinities previously thought to be irrelevant in specific immune responses are in fact capable of complete T cell–dependent immune responses when relieved of substantial competition from other B cells. The failure to observe such B cells normally in late primary responses and in memory B cell populations is the result of competition, rather than an intrinsic inability of low affinity B cells.

Key words: mutation • antibody affinity • plasma cell • variable region gene • λ1 immunoglobulin

Introduction

Early humoral responses are heterogeneous and mainly low affinity (1–4). As the response progresses, Ab diversity is reduced and affinity increases (5–7). It is possible that early low affinity Ab is biologically irrelevant and the result of nonspecific or bystander stimulation (8, 9). Alternatively, very low affinity B cells may respond specifically but are lost as the response progresses. For example, intrinsic affinity requirements could limit cell participation in humoral responses. Survival in germinal centers (GCs) or memory differentiation may require signaling above a threshold (10–12). Alternatively, the loss of low affinity B cells from immune responses could reflect competition among antigen-reactive B cells (13).

To distinguish these models for the antigen-driven selection of B cells, we generated the following two lines of transgenic (Tg) mice expressing Ig H chain Tgs that encode extremely low affinity anti-(4-hydroxy-3-nitrophenyl)acetyl (NP) antibodies when associated with the Ig H chain: 1.2 × 10⁵ M⁻¹ for H50Gμa and 3.0 × 10⁴ M⁻¹ for T1(V23)μa.

Materials and Methods

Mice. 6–8-wk-old C.B-17 and C.B-17-SCID mice were purchased from Taconic Farms, Inc. All mice were maintained in sterile microisolator cages.

Creation of H50Gma and T1(V23)ma Tg Mice. The VH186.2/DFL16.1 rearrangement, containing a single mutation at codon 50, designated H50G and V23 analogue VH rearrangement from an unmutated VDJ rearrangement of the V23 gene taken from a C57BL/6 mouse 10 d after immunization with NP, were subcloned into a vector that contains the 11.6-kb C/H9261 fragment that

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includes the membrane and secretory exons (14). The H50Gµα lineage had two copies of the Tg, whereas two separate T1(V23)µα lines with similar phenotypes were used to grow to three copies or a single copy of the Tg. Tg mice were back-crossed with C.B-17 mice for five or more generations.

Flow Cytometry. The following antibodies were used: phycoerythrin-conjugated anti-B220 (CD45RA-PE; BD PharMingen); fluorescein-conjugated anti-mouse IgMα (RS3.1-FITC); biotinylated Af6-7.8 (anti-IgM); fluorescein-conjugated or biotinylated L136 (anti-αL); and biotinylated goat anti-mouse κ L chain–specific and biotinylated goat anti-mouse Ig (Southern Biotechnology Associates, Inc.). Red 670-conjugated streptavidin (GIBCO BRL) was used to reveal biotin-coupled Ab staining.

Antigens and Immunizations. Mice were immunized intraperitoneally with 50 µg of NP16-chicken γ-globulin (CG), (4-hydroxy-5-iodo-3-nitrophenyl)acetyl (NIP)12-CG, or CG precipitated in alum. For secondary/memory responses and challenge of repopulated SCID mice, 20 µg of soluble NP16-CG in PBS was administered intravenously on the indicated days.

Immunohistology. Immunohistology was performed as previously described (3, 15). GCs were enumerated as peanut agglutination (PNA)α areas within lymphoid follicles (± SD from three separate sections). Endogenous IgMα-expressing B cells in the GC of H50Gµα or T1(V23)µα mice were enumerated using both Aff6-7.8-biotin and biotinylated goat anti–mouse IgG1, followed by streptavidin–alkaline phosphatase. To detect apoptotic cells, TUNEL assays were performed using in situ apoptosis detection kits (Oncor).

Microdissection of Cells, DNA Amplification, and Sequencing. Microdissection of cells, DNA amplification, and sequencing was performed as previously described (14, 16). Cellular material (containing 10–20 cells) was microdissected from individual GCs (3, 15, 17). In H50Gµα mice, GCs sampled were also IgMα+ and devoid of endogenous IgMα-expressing (IgMαk+) or IgGk+ cells. DNA was amplified by Pfu polymerase (Stratagene) using nested primers specific for VA1 and κK1 elements (14).

Quantitation of Serum Ab. Ab titers were determined by endpoint dilution in ELISA using plates coated with NP16-BSA, NIP16-BSA, or CG (7). The endpoint was the last serial dilution that demonstrated a signal greater than twofold above the background. NP-specific ELISAs using differentially haptenated substrates were used to quantitate high and low affinity Ab (3).

Reduction of Serum Ab by 2-Mercaptoethanol (2-ME). Serum was assessed for NP reactivity by ELISA after treatment with 2-ME (3). For calculations of the percent of the IgMα titer resistant to 2-ME treatment, mock and 2-ME ELISA results were converted to relative Ab concentrations by comparison to a standard purified H50Gµα/κκ1 transfectoma Ab.

Adaptive Transfers of Splenocytes. 5 × 106 splenocytes recovered from H50Gµα and C.B-17 mice 30 d after immunization with 50 µg of NP16-CG precipitated in alum (18) were injected intravenously into recipient SCID mice. Recipients were challenged with 20 µg of NP16-CG in PBS or PBS alone 24 h later. 9 d after immunization the recipient sera were collected.

Online Supplemental Material. The supplementary material shows original VA sequence data as well as summary tables that analyze the distribution of mutations in the sequences. Fig. S1 shows all codons that contained mutations in VA1 sequences derived from either C.B-17 or H50Gµα mice, as described in the Materials and Methods and Results sections. Table S1 is a comparison of the two datasets, showing the frequency of overall mutations as well as replacement and silent mutations. Table S2 shows the ratios of replacement and silent ratios in each subregion of VA. Online supplemental material available at http://www.jem.org/cgi/content/full/jem.20011550/CD1.

Results

Characterization of H50Gµα and T1(V23)µα Tg Mice. Splenic B cells from naive H50Gµα and T1(V23)µα mice demonstrated efficient allelic exclusion, with ≤2% expressing endogenous IgMα protein (unpublished data). The ratio of αL:κ L chain–bearing B cells were comparable in both Tg and control mice, and all Tg lines displayed total surface Ig densities comparable to normal, naive B cells (unpublished data).

Humoral Immune Responses in H50Gµα Tg Mice. Naive H50Gµα Tg mice had low titers (≤1:60) of NP-specific serum Ab that sharply increased after immunization with NP-CG (Fig. 1 A). At day 12, the titers of NP-binding Tg (IgMα) Ab increased 1,000-fold in H50Gµα mice. Only in 3 out of 31 H50Gµα mice were substantial levels of IgG1 anti-NP detected (Fig. 1 A), indicating that typically most anti-NP was Tg derived. Levels for anti-NP αL and total Ig at day four were similar for Tg and control animals. However, C.B-17 mice later achieved 5–10-fold higher titers. To confirm that H50Gµα mice expressed only low affinity Tg-encoded antibodies in response to immunization, sera of H50Gµα and C.B-17 mice were mildly reduced with 2-ME (3). The binding of very low affinity IgG Ab is avidity dependent and lost upon 2-ME reduction (5), whereas higher affinity IgM or IgG Abs are unaffected. The reduction of Tg sera led to a near complete loss (≥97%) of NP reactivity (Fig. 1 B). This loss was comparable to that observed for purified H50Gµα/κκ1 transfectoma Ab (3). In contrast, C.B-17 serum titers were unaffected.

Immune Response of Very Low Affinity B Cells. To directly test whether B cells with very low affinities were capable of initiating and sustaining specific humoral responses, H50Gµα Tg mice were immunized with NIP-CG and T1(V23)µα Tg mice were challenged with NP-CG and NIP-CG. H50Gµα/κκ1 Ab has a lower affinity for NIP (6.3 × 105 M−1) than for NP (3). T1(V23)µα/κκ1 Ab has a Ks = 5.0 × 104 M−1 for NIP and an affinity for NP that is too low for reliable measurement by fluorescence quenching (5).

T1(V23)µα mice had 8–10-fold increases in hapten-specific IgMα Ab between 4 and 16 d after immunization with either immunogen, with some mice showing 20–30-fold increases in Ab titer (Fig. 1, C and D; ▲, Δ). IgMα responses by T1(V23)µα mice to NP-CG or NIP-CG were lower (5–10-fold) than those observed in H50Gµα mice for the same immunogen (Fig. 1, C and D; ▲, Δ vs. ○). IgG1 responses, presumably encoded by the endogenous IgH loci, were higher in T1(V23)µα mice compared with H50Gµα mice. The higher levels of endogenously derived anti-NP antibodies in T1(V23)µα mice may reflect the extraordinarily poor affinity of these Tg B cells, which confer little advantage in competition with rare B cells that express endogenous VH genes. B cells expressing endogenously encoded VH genes comprise 1–3% of the initial repertoire,
of which only a small fraction of B cells would have NP/NIP specificity. Thus, the emergence of these B cells in response to NP/NIP reflects the competition between rare, presumably higher affinity B cells and the much more common low affinity Tg B cells.

At the height of the response, H50Gμα mice challenged with NIP had a 100-fold increase in hapten-binding IgMα Ab titers compared with controls (Fig. 1 D), which is 2–10-fold lower than those after challenge with NP-CG (Fig. 1, C and D). This observation establishes a relationship between B cell antigen receptor (BCR) affinity/avidity and the magnitude of the Ab response in a single Tg mouse line.

Low Affinity Tg B Cells Can Initiate Primary GCs. PNA+ GCs in H50Gμα mice 12 d after immunization (Fig. 2 A) were observed in numbers (~70–80 per section) equivalent to normal mice (6, 15). 65 ± 5.8% of the GCs in H50Gμα mice were stained with both anti-IgMα and anti-Î1 Ab, indicating that they contained NP-specific B cells expressing the H50Gμα Tg (Fig. 2, A and C). The percentage of Î1+ GCs was only slightly higher than that seen in non-Tg C.B-17 mice for the same day, which was 54 ± 3.4%. Many B cells in the splenic red pulp exhibited strong cytoplasmic staining with Î1- and IgMα-specific Ab (unpublished data), the characteristic phenotype of plasma blasts and antibody-forming cell (AFC) (18).

H50Gμα Tg B cells also formed Tg+ GCs when their BCR affinity was less than 10^-5 M^1, i.e., after immunization with NIP. Such mice had PNA+ and IgMα GCs in numbers equivalent to C.B-17 controls, although many of these GCs were smaller than those in control animals (unpublished data). We also investigated the capacity of very low affinity B cells to form GC in T1(V23)μα mice. Surprisingly, there was no difference in GC number and size in T1(V23)μα mice challenged with NP or NIP compared with C.B-17 controls. However, ~80% of GCs in T1(V23)μα mice contained both Tg IgMα- and endogenous Ig-expressing B cells (unpublished data). When present, endogenous Ig-expressing B cells (demonstrated with anti-IgMα Ab and anti-IgG1 Ab) constituted as much as 50% of the PNA+ cells in each GC. Presumably these endogenously derived B cells reflect the competitive success of rare, higher affinity B cells. To confirm this, we microdissected and sequenced several clusters of Î1+ B cells.
containing for IgM\textsuperscript{b}/IgG\textsuperscript{1} and sequenced their VH regions. In fact, >75% of recovered sequences were V\textsubscript{iH}186.2, the canonical VH of anti-NP responses, which established that the endogenously derived B cells are higher affinity anti-NP B cells. It is likely that the population of B cells expressing endogenous VDJ rearrangements accounts for the normal size and frequencies of GCs in immunized T1(V23)\textsubscript{1/2} animals.

**Late GCs of H50\textsuperscript{G\mu} Mice Show Evidence of Increased Apoptosis.** 12 d after immunization with NP-CG, B cells bearing BCRs encoded by the endogenous Igh\textsuperscript{b} loci are infrequent in the GC of H50\textsuperscript{G\mu} Tg mice (Fig. 3 A). However, at day 16 of the primary response, endogenous Ig−expressing B cells (IgM\textsuperscript{a} or IgG\textsuperscript{1}) begin to appear in the GCs, increasing to 5–10% of all PNA\textsuperscript{+} GC cells by day 20 (Fig. 3 B and C). Late GCs (16–20 d after immunization) of H50\textsuperscript{G\mu} mice had two- to threefold more apoptotic B cells compared with C.B-17 controls, as indicated by TUNEL labeling (Fig. 3, D and E). GCs (n = 30) sampled from six H50\textsuperscript{G\mu} mice at 16 or 20 d after immunization had an average 32% (±10%) TUNEL\textsuperscript{+}/PNA\textsuperscript{+}/IgD\textsuperscript{−} cells. This was in contrast to GCs (n = 20) from four C.B-17 mice at similar days, which contained only 12% (±7%) TUNEL\textsuperscript{+} GC cells.

**Somatic Mutation in H50\textsuperscript{G\mu} Mice.** Somatic hypermutation was assayed in the endogenous \lambda L chain gene of H50\textsuperscript{G\mu} mice, as ectopic heavy chain Tg generally does not mutate. IgM\textsuperscript{a}/\lambda L, GC cells from three H50\textsuperscript{G\mu} mice, and three C.B-17 mice were microdissected and their \lambda regions amplified and sequenced (Fig. S1 and Tables S1 and S2). Of 28 H50\textsuperscript{G\mu} VA1/\lambda L rearrangements examined, 22 were mutated with an average of 5.1 (±3.7) mutations per rearrangement.

The distribution and type of somatic mutations observed in VA1 genes present in Tg and control GC B cells is summarized in Tables SI and SII. 57 of 68 H50\textsuperscript{G\mu} VA1 mutations encoded amino acid replacements. The 68 unique mutations were a striking contrast to the 11 unique mutations found in C.B-17 GCs. Mutations in the H50\textsuperscript{G\mu} VA1/J\lambda L rearrangements had an overall replacement/silent (R/S) ratio three times greater than C.B-17 rearrangements. In addition, the R/S ratio of mutations in CDR1 and CDR2 of H50\textsuperscript{G\mu} VA1 genes was 10- and 6-fold higher, respectively, than the predicted ratios based on codon usage. These data demonstrate enhanced mutation and selection in the endogenous VA1 gene rearrangements of H50\textsuperscript{G\mu} GC B cells.

**Low Affinity Primary and Memory Responses in H50\textsuperscript{G\mu} Mice.** To determine the degree of affinity maturation in the primary and memory responses of H50\textsuperscript{G\mu} mice, Tg and C.B-17 mice were immunized with NP-CG and 60 d later were rechallenged intravenously with soluble NP-CG. Fig. 4 shows the change in the NP-specific IgM\textsuperscript{a}-Tg Ab titers. Upon secondary immunization, a prompt and significant increase in NP-specific IgM\textsuperscript{a} Ab was observed. By day 9 of the secondary response, Tg-IgM\textsuperscript{a} endpoint titers increased from 1:4,500 to 1:72,000 (Fig. 4, C). No significant Ab response (>1 out of 200) was observed in unprimed H50\textsuperscript{G\mu} mice given the same soluble antigen (Fig. 4, D).

Affinity maturation, presumably through the mutation of VA1, did enhance the avidity of H50\textsuperscript{G\mu} Ab, as re-
flected by the increased portion that bound NP$_5$-BSA (Fig. 4; Δ, bars). However, this affinity maturation was modest, as 85–90% of secondary Ab generated by H50Gµ mice remained sensitive to 2-ME treatment (unpublished data). Interestingly, the rechallenge of primed H50Gµ mice with soluble NP-CG elicited a rapid increase in low affinity Ab, as inferred from the precipitous decrease in the NP$_5$/NP$_{22}$ binding ratio of the serum Ab (Fig. 4, bars). Only 30% of the NP-specific IgM titer at day 9 of the secondary response showed the ability to bind NP$_5$-BSA. This is a contrast to the 70% observed in the late primary response (Fig. 4) and consistent with the reactivation of low affinity memory B cells and their differentiation into AFC (7).

To exclude that T cell priming alone was responsible for the memory response of H50Gµ mice, Tg and control mice were primed with the carrier protein CG, and 60 d later challenged intravenously with soluble NP-CG. Average NP-specific λ1$^+$ Ab titers from CG-primed H50Gµ mice were 15-fold lower than cohorts primed with NP-CG (Table I; 1,2,200 vs. 1,33,400). On challenge with soluble antigen, NP-CG–primed H50Gµ mice exhibited a 38% absolute increase in the serum Ab titer capable of binding NP$_5$-BSA, whereas there was no appreciable increase in the NP$_5$-BSA–binding titer in CG-primed H50Gµ mice. Thus, T cell priming alone cannot account for the extent of low affinity Ab elicited in the memory responses of H50Gµ mice.

To minimize the influence of preformed Ab on secondary responses, splenocytes from naive or NP-CG–primed (day 30) H50Gµ and C.B-17 mice were adoptively transferred into SCID recipients, which were then challenged intravenously with soluble NP-CG. SCID mice that received primed H50Gµ splenocytes generated Ab titers that were more than 100-fold higher than SCIDs that were re-populated with naive H50Gµ splenocytes. The magnitude of the increase was similar in control mice receiving C.B-17 donor cells (unpublished data). These differences in serum Ab were not due to the transfer of residual AFC, as SCID mice that received primed H50Gµ splenocytes but were not challenged with antigen had no appreciable anti-NP serum Ab (titer of 1:60).

Table I. Memory Response to Soluble Antigen from NP-CG Primed and Carrier Primed Mice

<table>
<thead>
<tr>
<th>Immunization Protocola</th>
<th>Serum Responseb (end-point dilution)</th>
<th>Percent NPS/NP22 Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>α-NP22 λ1 titer (1/X)</td>
<td></td>
</tr>
<tr>
<td>CB-17</td>
<td>NP-CG</td>
<td>135,826 ± 69,607</td>
</tr>
<tr>
<td>H50Gµ</td>
<td>NP-CG</td>
<td>33,417 ± 10,919</td>
</tr>
<tr>
<td>C.B-17</td>
<td>CG</td>
<td>4,797 ± 2,010</td>
</tr>
<tr>
<td>H50Gµ</td>
<td>CG</td>
<td>2,218 ± 1,581</td>
</tr>
<tr>
<td>C.B-17</td>
<td>none</td>
<td>130 ± 60</td>
</tr>
<tr>
<td>H50Gµ</td>
<td>none</td>
<td>60 ± 20</td>
</tr>
</tbody>
</table>

aSerum data were obtained from mice 9 d after secondary challenge with soluble antigen.
bPrimary immunizations were by intraperitoneal administration of 50 µg NP-CG or CG alone in alum. Secondary/memory responses were elicited through the intravenous injection of 20 µg soluble NP-CG 60 d after primary challenge.

The NP-specific 1A$^+$ end-point serum titer was used as a measure of the secondary immune response to allow direct comparison between H50Gµ and C.B-17 mice.
tent H50G\mu\alpha mice, as demonstrated by the presence of non-Tg Ab (Fig. 1) and histology (Fig. 3). Presumably, the extremely low affinity of T1(V23)\mu\alpha B cells for the NP and NIP haptens allows them to be overwhelmed by rare B lymphocytes that escape allelic exclusion and respond to NP/NIP, as indicated by their frequent expression of \( V_{H186.2} \), a hallmark of high affinity NP Ab. Thus, higher affinity B cells expressing endogenous genes outcompete a numerous but very low affinity Tg population.

As V(D)J hypermutation creates L chain diversity in GCs (Figure S1), intraclonal competition also exists. R/S ratios in VA CDR1 and VA CDR2 suggest strong selection for GC B cell mutants. Although VA mutations can improve BCR affinity in H50G\mu\alpha mice, the low affinity of secondary Ab in these mice (Fig. 4) suggests that this pathway for affinity maturation is limited. Nonetheless, the high frequency (1.4%) of point mutations in the VA1 rearrangements of H50G\mu\alpha GC B cells is almost twice that found in a similar mouse with a germline affinity, VH 186.2 Tg (14), and much higher than wild-type mice. The high frequency and intense selection of VA1 mutations in H50G\mu\alpha mice could reflect the difficulty in improving BCR affinity through L chain mutations alone. However, mutation rates may be intrinsically higher in low affinity B cells.

Our data do not rule out the role(s) for differential signaling governed by affinity/avidity in determining B cell fates (11, 12). Aside from memory development, other aspects of B cell differentiation like isotype switching could be governed by BCR signal strength.

The ability of very low affinity B cells to establish GCs and humoral memory has implications for understanding natural B cell responses. Low affinity BCRs can acquire higher affinities with a single point mutation (19, 21), and the many low affinity B cells that reach GCs are the potential precursors of effective memory B cells. This view is also consistent with direct estimates of clonal diversity in nascent GCs (6) and computer models, which suggest that GCs are seeded by 40–50 precursors (22). Finally, the latent genetic potential of low affinity clones could underlie “clonal shifts” in secondary responses whereby dominant, primary clonotypes are replaced by mutated, dissimilar clones (2, 23).

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