Gene Conversion and Hypermutation During Diversification of $V_H$ Sequences in Developing Splenic Germinal Centers of Immunized Rabbits

Enrico Schiaffella,1* Devinder Sehgal,1* Arthur O. Anderson, † and Rose G. Mage2*

The young rabbit appendix and the chicken bursa of Fabricius are primary lymphoid organs where the B cell Ab repertoire develops in germinal centers (GCs) mainly by a gene conversion-like process. In human and mouse, V-gene diversification by somatic hypermutation in GCs of secondary lymphoid organs leads to affinity maturation. We asked whether gene conversion, somatic hypermutation, or both occur in rabbit splenic GCs during responses to the hapten DNP. We determined DNA sequences of rearranged heavy and light chain V region genes in single cells from developing DNP-specific GCs after immunization with DNP-bovine $\gamma$-globulin and conclude that the changes at the DNA level that may lead to affinity maturation occur by both gene conversion and hypermutation. Selection was suggested by finding some recurrent amino acid replacements that may contribute increased affinity for antigen in the complementarity-determining region sequences of independently evolved clones, and a narrower range of complementarity-determining region 3 lengths at day 15. Some of the alterations of sequence may also lead to new members of the B cell repertoire in adult rabbits comparable with those produced in gut associated lymphoid tissues of young rabbits. The Journal of Immunology, 1999, 162: 3984–3995.

Germinal centers (GCs) in secondary lymphoid organs are specialized structures within which somatic diversification of rearranged V genes occurs that lead to affinity maturation of Abs in immune responses to T-dependent Ags. During some stages of GC development, a dark zone and a light zone (LZ) can be defined histologically. B cells in the dark zone (centroblasts) are characterized by intense proliferative activity (1, 2). The rearranged V region genes of the centroblasts undergo somatic hypermutation (3–7). Centroblasts then move to the LZ, become nondividing centrocytes, contact follicular dendritic cells (FDC) with the Ag in a native conformation on their surface, and undergo selection. B cells with high-affinity receptors for the Ag continue to differentiate toward Ab producing cells or memory B cells (8, 9). Cells that fail to bind Ag or that bind with a lower affinity die by apoptosis, or reexpress the recombination-activating genes 1 and 2 (RAG-1 and RAG-2) gene products and undergo receptor editing (10–20). The presence of RAG-1 and RAG-2 proteins in secondary follicles detected by immunohistochemistry (14), the re-expression of RAGs in activated B cells stimulated by LPS and IL-4 (10) and the presence of intermediate products of VJ recombination at the $\kappa$ locus, (15, 16) reflect GC B cells in which receptor editing or revision occurs.

Emerging evidence suggests that the description of GCs outlined above oversimplifies the actual events that occur. It was recently shown that proliferating cells are present in the LZ in contact with the FDC network at least until day 10 of the response of BALB/c mice to 2-phenyl oxazolone-BSA (21). Successive cycles of hypermutation and selection are thought to occur (22, 23) involving continued proliferation and selection of cells with surface B cell receptor (BCR) near FDC in the T cell-rich LZ (21) rather than by cells recycling between proliferative and selective compartments of the GC (24, 25). Studies in an adoptive transfer model suggest that Ag-specific CD4 T cells interact with dendritic cells in T cell areas. They subsequently participate in cognate T–B cell interactions at B cell follicular borders where lymphokines and signaling between CD40 and its ligand CD154 drive B cell proliferation and development of peanut agglutinin-positive GCs (26). Interactions of CD40 and its ligand remain crucial during much of the GC reaction. Other interactions such as between OX40 and OX40 ligand (27) and between B7–2 and CD28/CTLA-4 are also important. Interference with B7–2 function by administration of Ab interrupts hypermutation and generation of memory populations (28). Affinity-based selection and receptor editing may also be influenced by cognate T–B cell interactions involving CD40 and lymphokine signaling. B cells with low affinity for Ag may be signaled to up-regulate recombine expression whereas B cells with high affinity receptors may generate BCR-mediated signals that inhibit recombine reactivation (19). Very recently, the possibility has been raised that some repertoire development may occur in GCs of mice and humans (14, 17, 29) that resembles the primary repertoire development that occurs in tissues such as young rabbit appendix and the chicken bursa of Fabricius (30–34).

GCs are also present in primary lymphoid organs such as the young rabbit appendix (35) and the chicken bursa of Fabricius where they mainly function to produce a broad B cell repertoire rather than to generate a specific immune response (30–34, 36). Unlike mice and humans, rabbits rearrange only a few heavy chain
ulin (DNP-BGG) in adult rabbits, an Ag chosen because of its
rabbits received BGG at both time points.

V region (V<sub>H</sub>) genes, so that the diversity generated by combina-
torial mechanisms is limited (37, 38). Appendix GCs are seeded by
B cells that have already rearranged their Ig genes in sites such as
fetal liver, omentum, or bone marrow (36, 39). They colonize the
GCs of the appendix during the first weeks of life, proliferate in
response to Ags and superantigens (36) and their rearranged V<sub>H</sub>
genotypes diversify (30) by somatic hypermutation and a gene conver-
sion-like mechanism (38, 40, 41). We refer to the mechanism in
rabbits as gene conversion-like because nonreciprocal exchange
has not been formally shown in rabbits whereas studies in chickens
(42) showed that donor sequences were unaltered.

The objective of this work was to investigate diversification of
rearranged V<sub>H</sub> and light chain V region sequences in splenic GCs
during T cell-dependent immune responses to DNP-bovine
-globulin (DNP-BGG) in adult rabbits, an Ag chosen because of its
previous use in documenting affinity maturation in the rabbit (43, 44).

The rearranged V<sub>H</sub>-gene conversion, somatic hypermutation or both also take place during the GC re-
action in response to a T cell-dependent Ag in GCs of the rabbit
spleen. We combined the techniques of microdissection of single
Ag-specific B cells with a PCR-based sequencing strategy that
avoids PCR artifacts. This approach is particularly successful in
Ag-specific B cells with a PCR-based sequencing strategy that
avoids PCR artifacts. This approach is particularly successful in
ag B cells that have already rearranged their Ig genes in sites such as
fetal liver, omentum, or bone marrow (36, 39). They colonize the
GCs of the appendix during the first weeks of life, proliferate in
response to Ags and superantigens (36) and their rearranged V<sub>H</sub>
genotypes diversify (30) by somatic hypermutation and a gene conver-
sion-like mechanism (38, 40, 41). We refer to the mechanism in
rabbits as gene conversion-like because nonreciprocal exchange
has not been formally shown in rabbits whereas studies in chickens
(42) showed that donor sequences were unaltered.

The objective of this work was to investigate diversification of
rearranged V<sub>H</sub> and light chain V region sequences in splenic GCs
during T cell-dependent immune responses to DNP-bovine γ-glob-
ulin (DNP-BGG) in adult rabbits, an Ag chosen because of its
previous use in documenting affinity maturation in the rabbit (43, 44).

The addressed the question of whether V<sub>H</sub>-gene conversion, somatic hypermutation or both also take place during the GC re-
action in response to a T cell-dependent Ag in GCs of the rabbit
spleen. We combined the techniques of microdissection of single
Ag-specific B cells with a PCR-based sequencing strategy that
avoids PCR artifacts. This approach is particularly successful in
rabbit because the B cells rearrange only a few VH genes and the
majority do not rearrange the second allele (45). Although it was
previously reported that V<sub>H</sub> sequences in adult rabbit B cells were
highly diversified (46) we found that cells collected during early
stages of the GC reaction (days 7 and 10) from adult rabbit spleen
had rearranged V<sub>H</sub> sequences that were identical or nearly identi-
cal with germline sequences (47). This report presents our obser-
vations, both at the histological and molecular levels, on the pro-
gression of the GC reaction in rabbit spleens during the response to
the T cell-dependent hapten DNP. We find changes that may
lead to affinity maturation occur by both gene conversion and so-
matic hypermutation.

**Materials and Methods**

**Ag and immunization**

DNP<sub>37</sub>-BGG was prepared as described (47, 48). Rabbits from 1 to 2 years
of age, homozygous for V<sub>λ</sub>μ2 (haplotype F-1) and C<sub>b</sub>57 allotypes, were
given a priming immunization of 500 μg of BGG in CFA injected s.c.
Between 7 and 10 days later, 500 μg of DNP-BGG were given i.v. Control
rabbits received BGG at both time points.

**Immunohistochemistry**

At 7, 10, and 15 days after the second injection, rabbits were killed, and the
spleens embedded in OCT and quickly immersed in liquid nitrogen. Serial

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>External primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSH1</td>
<td>GCTTCCTCTGGTCGTGCTSGTCCT</td>
<td>Leader (I&lt;sub&gt;κ&lt;/sub&gt;)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DSH7</td>
<td>GCAGCAGGGCCCTTCAAGATTG</td>
<td>3′ to J&lt;sub&gt;κ&lt;/sub&gt;2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DSH8</td>
<td>GCAGAAAAACTCGCTAGATTG</td>
<td>3′ to J&lt;sub&gt;κ&lt;/sub&gt;4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DSk1</td>
<td>TCTCCTGCTGCTTGGCTCCA</td>
<td>Leader (I&lt;sub&gt;κ&lt;/sub&gt;)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DSk7</td>
<td>CAGAAACCGTGAGAGAAGTCTG</td>
<td>3′ to J&lt;sub&gt;κ&lt;/sub&gt;2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Internal primers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DSH2</td>
<td>AGTGACAGTGGTC (C/T)GACAGTTG</td>
<td>L&lt;sub&gt;κ&lt;/sub&gt;′V&lt;sub&gt;H&lt;/sub&gt; intron&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DSH5</td>
<td>AGACTCAGCTGGTACGGACC</td>
<td>J&lt;sub&gt;κ&lt;/sub&gt;23/4&lt;sup&gt;α&lt;/sup&gt;</td>
</tr>
<tr>
<td>DSk2</td>
<td>ATGGTAGGGGTTGCTCTCTGTTCTC</td>
<td>L&lt;sub&gt;κ&lt;/sub&gt;′V&lt;sub&gt;H&lt;/sub&gt; intron&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DSk14</td>
<td>GAATCAGTGAGAGACGCCCAGCTAC</td>
<td>J&lt;sub&gt;κ&lt;/sub&gt;2-3′ intron junction&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> These primers were described in detail in Ref. 47.
<sup>b</sup> The primers for κ light chain were selected based on sequences bearing accession numbers D38376, K00751, K01360, K01361, K01363, K02131, K02438, K02439, K02440, M14067, M22541, X00032, X00231, X00977, X02336, X02337, X02338, X14364, X14365.

Microdissection of frozen tissue sections

The stained sections were incubated with 5 mg/ml collagenase H (Boehr-
inger Mannheim, Indianapolis, IN) as described (49). Single cells from
GCs obtained at days 7, 10, and 15 were collected from the sections using
an hydraulic micromanipulator (Narishige, Greenvale, NY) assembled on an
inverted microscope (Olympus, Lake Success, NY). From days 7 and 10
we collected cells from DNP<sup>+</sup> GCs that were either DNP<sup>+</sup> (day 7), Ki-67<sup>−</sup>
(day 10), or both (day 10) (47). From day 15 we collected proliferating
GCs that were either DNP<sup>+</sup> or Ki-67<sup>−</sup> and stained with DNP-AP and
staining of tissue sections were as described previously (47).

The stained sections were incubated with 5 mg/ml collagenase H (Boehr-
inger Mannheim, Indianapolis, IN) as described (49). Single cells from
GCs obtained at days 7, 10, and 15 were collected from the sections using
an inverted microscope (Olympus, Lake Success, NY). From days 7 and 10
we collected cells from DNP<sup>+</sup> GCs that were either DNP<sup>+</sup> (day 7), Ki-67<sup>−</sup>
(day 10), or both (day 10) (47). From day 15 we collected proliferating
cells based on positive staining with anti-Ki-67 from a GC that stained
positive for DNP-binding cells.

Individual cells were each transferred to 0.2-ml microfuge tubes con-
taining 5 μl of an alkaline lysing solution (200 mM KOH and 50 mM
DTT). The tubes were incubated at 65°C for 10 min before adding 5
μl of neutralizing solution (900 mM Tris-HCl (pH 8.5), 300 mM KCl, and 200
mM HCl).

DNA amplification and sequencing

We elected to use direct sequencing of PCR products from single cells
without a cloning step to avoid two potentially serious artifacts. First, with-
out a cloning step the effect of Taq DNA polymerase errors is minimized
(50–52) because Taq DNA polymerase-based errors are then detected
in sequences only if they occur in the first few cycles of amplification. Sec-
ond, by amplification of V<sub>H</sub>D<sub>H</sub>J<sub>H</sub> sequences from single cells we avoided
producing hybrid V<sub>H</sub>D<sub>H</sub>J<sub>H</sub> sequences that were found especially during the
somatic hypermutation stage of the GC reaction (6).

The rearranged V<sub>H</sub>D<sub>H</sub>J<sub>H</sub> and V<sub>κ</sub>L<sub>κ</sub> sequences were amplified from sin-
gle cells of DNP<sup>+</sup> GCs using a nested PCR strategy. The primers used are
shown in Table I. In the first round, the rearranged V<sub>H</sub>D<sub>H</sub>J<sub>H</sub> and V<sub>κ</sub>L<sub>κ</sub>
sequences were amplified simultaneously. Nested PCR was performed sepa-
ately for the rearranged heavy and κ light chain using internal primers
specific for the rearranged V<sub>H</sub>D<sub>H</sub>J<sub>H</sub> and V<sub>κ</sub>L<sub>κ</sub> sequences. The temperature
conditions for the first and second round of touchdown PCR were the same
(53). There was an initial 95°C 2-min denaturation step. This was followed

<sup>a</sup> These primers were described in detail in Ref. 47.
<sup>b</sup> The primers for κ light chain were selected based on sequences bearing accession numbers D38376, K00751, K01360, K01361, K01363, K02131, K02438, K02439, K02440, M14067, M22541, X00032, X00231, X00977, X02336, X02337, X02338, X14364, X14365.
by five cycles of denaturation at 94°C for 1 min; for the first cycle annealing was at 66°C for 30 s that dropped down to 56°C at the end of the fifth cycle at the rate of 2°C per cycle; and an extension step at 72°C for 1 min. The annealing temperature for the remaining 29 cycles was 56°C. The PCR ended with a 5-min extension at 72°C. The PCR was performed on a PTC-100 programmable thermal cycler (96-well model with hot bonnet) (MJ Research, Watertown, MA).

For the first round, MgCl₂, Tris-HCl (pH 8.3), Triton X-100, and external primers (DSH1, DSH7, and DSH8 for the heavy chain; and DŠx1 and DŠx7 for the κ light chain) (see Table I) were added to the tube containing the neutralized cell lysate such that the final concentration in a 40 µl volume were 3.75 mM, 10 mM, 0.1% (v/v), and 0.75 mM (of each primer), respectively. The DNA was denatured at 95°C for 10 min. Thereafter, 20 µl of a mix containing 10 nM Tris-HCl (pH 8.3), 0.1% (v/v) Triton X-100, 600 µM of each dNTP, and 5 units of AmpliTaq DNA polymerase (Perkin-Elmer, Branchburg, NJ) was added and the tube setup for PCR. From the first round PCR, 2.5 µl of the product served as the DNA template for the second round touchdown PCRs (53) that were done for the rearranged V₉D₅J₄ and V₉J₄ sequences separately. The internal primers used for amplifying the rearranged heavy chain were DSH2 and DSH5 (Table I). The corresponding set of internal primers used for amplifying the rearranged κ light chain were DŠx2 and DŠx14 (Table I). In addition to the template DNA, the other components in the 50 µl of PCR were 1× PCR buffer (containing MgCl₂) (Perkin-Elmer), 200 µM of each dNTP, 0.5 µM of each primer (internal to those used in the first round) (DSH2 and DSH5 for the heavy chain; DŠx2 and DŠx14 for the κ light chain) (see Table I) and 2 units of AmpliTaq DNA polymerase. The PCR parameters were the same as for the first round.

As a control we also conducted a blind experiment where we collected a group of 23 CD4⁺ T cells from the T cell area along with 69 Ki-67⁺ cells from within the GC. In the PCRs run at the same time, we amplified 48 of 69 V₉D₅J₄ sequences from the cells collected from the GC; none of the 23 T cells gave a PCR product.

Recovery of PCR products for sequencing was done as described (47). The Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) was used following the manufacturer’s instructions to sequence both strands using the second round primers system. Applied Biosystems model 377 automated sequencer (54). The sequences were analyzed using AUTOASSEMBLER version 1.3 (Applied Biosystems) and MACVECTOR versions 5.0 and 6.0 (Kodak Scientific Imaging Systems, Rochester, NY; Oxford Molecular Group, Campbell, CA).

Results and Discussion

Immunohistochemical characterization of splenic GCs in the rabbit

For histological characterization of GCs in the spleens of immunized rabbits, we stained frozen sections with the DNP-AP in combination with other Abs such as monoclonal mouse anti-human Ki-67 that crossreacts with rabbit, mouse anti-rabbit CD4, polyclonal goat anti-rabbit IgM and goat anti-rabbit IgG Abs. In rabbits that were carrier-primed 7–10 days before i.v. immunization with DNP-BGG, GCs were present at day 7 and their appearance and staining were similar at day 10. Fig. 1, A–F shows staining of tissue sections with polyclonal goat anti-rabbit IgM and goat anti-rabbit IgG Abs. DNP-BGG, GCs were present at day 7 and their appearance and staining were similar at day 10. Fig. 1, A–F shows staining of tissue sections with polyclonal goat anti-rabbit IgM and goat anti-rabbit IgG Abs. DNP-BGG, GCs were present at day 7 and their appearance and staining were similar at day 10. Fig. 1, A–F shows staining of tissue sections with polyclonal goat anti-rabbit IgM and goat anti-rabbit IgG Abs. DNP-BGG, GCs were present at day 7 and their appearance and staining were similar at day 10. Fig. 1, A–F, suggests that isotype switching had not yet occurred at day 7 because all the GCs stained positively for IgM, but little or no IgG staining was evident (Fig. 1, A and D). By day 15 (Fig. 1, B, C, E, and F) the cells in many GCs had undergone isotype switching. The GC shown in Fig. 1, B, E, and F, show evidence of recent isotype switching as adjacent sections stained positively with anti-IgM (B) and IgG (E). Fig. 1, C and F, show another GC at day 15 that lost most of its IgM expression (C) and stained with anti-IgG (F). Fig. 1, G–J, shows staining of adjacent sections at days 10 and 15 with anti-Ki-67 that identifies a nuclear Ag in proliferating cells (G and H) and of DNP-AP in combination with anti-CD4 (I and J). At day 15 (Fig. 1, H and J), proliferating cells were more concentrated in the areas of the GC where cells downregulated BCR expression, and staining with DNP-AP was less intense. CD4⁺ T cells constituted a major population in the periarteriolar lymphoid sheath area. Within the GC, they were found interspersed with DNP-binding cells.

Amplification and clonal relationships of rearranged Ig genes from GC B cells

Using PCR, we amplified the rearranged Ig genes in single cells obtained from several DNP-reacting splenic GCs of DNP-BGG immunized rabbits. Table II summarizes the numbers of cells collected from individual GCs at days 7, 10, and 15 and the numbers of sequences that we were able to amplify and analyze. In the seven different GCs studied at days 7 and 10, 55% and 63% of the sequences respectively, had unique complementarity-determining region 3 (CDR3) and were unrelated. At these time points we found one to four different clones per GC with only between two
we could also readily identify the germline V_H, D_H, and J_H genes sequences from cells in GC_N where we obtained no heavy chain clones. In addition, there was one group of light chain related light chain sequences showed correspondences with the into clones, 3 in GC_L and 6 in GC_N. Identification of clonally but 7 of the 85 successfully sequenced heavy chains were grouped occurring (58).

The data suggest that at the later time, more hypermutations and fewer gene conversions were oc-

markedly diminished by day 11. The data suggest that at the later time, more hypermutations and fewer gene conversions were occurring during clonal expansion.

Table II. Summary of PCR amplification, sequence analyses, and clones in splenic DNP⁺ GCs during immune responses of adult rabbits to DNP-BGG

<table>
<thead>
<tr>
<th>Days Post-immunization</th>
<th>No. of GCs Studied</th>
<th>Cells Collected (VDJs amplified)</th>
<th>Successful Sequences*</th>
<th>Independent Unique Sequences (%)</th>
<th>No. of clones</th>
<th>No. of members</th>
<th>Average no. of members/clone</th>
<th>Average no. of clones/GC</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>5</td>
<td>239 (97)</td>
<td>78</td>
<td>43 (55)</td>
<td>12</td>
<td>2–6</td>
<td>2.9</td>
<td>2.4</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>230 (79)</td>
<td>62</td>
<td>39 (63)</td>
<td>7</td>
<td>2–7</td>
<td>3.3</td>
<td>3.5</td>
</tr>
<tr>
<td>15</td>
<td>2</td>
<td>335 (134)</td>
<td>85</td>
<td>8 (9.5)</td>
<td>9</td>
<td>2–29</td>
<td>8.6</td>
<td>4.5</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>804 (310)</td>
<td>225</td>
<td>90</td>
<td>28</td>
<td>2–29</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Refers to the subset of amplified V_H D_H J_H sequences from which reliable nucleotide sequences were obtained. The GenBank accession numbers are AF058506–AF058645 (days 7 and 10; Ref. 47) and AF087712–AF087796 (day 15; this report).

and seven members (47). The proportions of independent unique sequences decreased from more than half (82/140) at days 7 and 10 to 9.5% (8/85) at day 15. Conversely, groups of clonally related sequences predominated at day 15 and on average contained larger numbers of members. Although it is generally accepted that GCs develop oligoclonally (55–57), we observed a predominance of unique clonally unrelated sequences at days 7 and 10, many with 10 or fewer changes from the germline sequence of the rearranged V_H gene. For the other small groups of clonally related sequences we could also readily identify the germline V_H, D_H, and J_H genes that rearranged in their precursors. At day 10, the population of proliferating cells still showed considerable heterogeneity (47). However, by day 15, most sequences were clonally related, the V region portion of every heavy chain sequence had nine or more differences from germline sequences and there were also on average more changes in the D_H and J_H sequences. In GCs from mice immunized with the hapten nitrophenyl (NP), clonal diversity also decreased with time but at 4 – 6 days postimmunization most GCs had only between three and six different CDR3 region sequences (55–57), we observed a predominance of diverse CDR3 region sequences with a high degree of sequence diversity and the corresponding
sequences one of which also yielded a heavy chain sequence. Thus GC_N had at least eight different clones and GC_L had at least three. The emergence of expanding, diversifying dominant clones by day 15 correlates with the switching from IgM to IgG (Fig. 1, A–F). Analyses of clonally related sequences from day 15 show that gene conversion as well as hypermutation was occurring during clonal expansion.

Gene conversion and hypermutation during clonal expansion of B cells in GCs

Fig. 1H shows GC_L stained for Ki-67 and Fig. 1J shows its adjacent section stained for DNP. Proliferating cells in GC_L were collected from two serial sections (La and Lb) and rearranged V_H D_H J_H and V_L J_L genes were amplified. GC_L had a predominant clone (L1) with 29 heavy chain members and an additional 13 cells from which we recovered only light chain sequences related to those in cells with heavy chains. A summary of this largest clone is shown in Fig. 2. Fig. 2A shows the approximate locations of the cells collected on a diagram of GC_L based on the stained sections shown in Fig. 1, H and J. Cells belonging to clones L1, L2, and L3 are shown as green, red, and black dots, respectively. Unique members are shown as black squares. We generated genealogical trees based on the changes that occurred in clonally related sequences. For L1, this is shown in Fig. 2B and the corresponding sequences in Fig. 2C. The cells of clone L1, recovered from the two serial sections appeared to share a common precursor sequence derived from rearrangement of V_H1a2, Df and J_H4 that had already undergone several sequence alterations involving blocks of sequence that are best explained as arising by gene conversion from known (V_H4, shaded orange) and unknown donors (UD, black boxes). The sequence of the rearranged V_H D_H J_H of cell La 55 appears closest to the precursor sequence from which the cells

Table III. Summary of PCR amplification, sequence analyses of rearranged V_H and V_L and clonally related groups of cells from DNP⁺ GCs at day 15 of the immune response of adult rabbits to DNP-BGG

<table>
<thead>
<tr>
<th>Days Post-immunization</th>
<th>GC</th>
<th>Cells Collected (VDJs, V_L J_L amplified)</th>
<th>Successful Sequences VDJ, V_L J_L</th>
<th>Independent Unique Sequences VDJ, V_L J_L</th>
<th>Groups of Clonally Related Sequences (no. of members)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>L</td>
<td>148 (71, 86)</td>
<td>43, 49</td>
<td>5*, 8</td>
<td>3 (29, 5, 4); (24, 3, 14)</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>187 (63, 69)</td>
<td>42, 28</td>
<td>2*, 5</td>
<td>8 (12, 11, 8*, 3, 3, 2, 1*, 0); (5, 2, 2, 4, 0, 0, 2, 8)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>335 (134, 155)</td>
<td>85, 77</td>
<td>7, 13</td>
<td></td>
</tr>
</tbody>
</table>

* GenBank accession numbers AF087750–AF087754.
  # GenBank accession numbers AF087795–AF087796.
  GenBank accession numbers AF087778–AF087785.
  GenBank accession number AF087794.
FIGURE 2. Cells collected from GC L, the clonal relationships of members of the predominant clone L1 and their V_H D_H J_H sequences. Individual GCs were designated by capital letters, sections within a GC by lowercase letters, and cells were numbered. A, The approximate locations from which cells were collected from GC L for sequencing. Fig. 1, H and J, show sections serial to those used for collection. The members of clones L1, L2, and L3 that yielded V_H D_H J_H sequences are color coded as green, red, and black dots, respectively. Unique members are shown as black squares. The five cells that yielded identical V_H D_H J_H sequences have a plus (+) after the cell name. La and Lb in the cell names indicate cells collected from two serial sections. B, Diagrammatic representation of the clonal expansion and sequence changes (codon position number: amino acid new amino acid or silent change (lower case “s”)). Codon positions are numbered according to Kabat et al. (59). The hypothetical rearranged precursor is in a rectangular box. Df is a germline D_H gene described in Chen et al. (60). Another hypothetical precursor (P) is shown as a shaded circle. Lb 92+ indicates that in addition to Lb 92, four other cells (La 02, La 21, La 39, and La 58) had the same V_H D_H J_H sequence. Underlined cell names indicate that a V_K J_K sequence was obtained from the
aroise. A single point mutation in the sequence of cell La 01, replaced Thr with Asn at amino acid position 79 (Kabat numbering; Ref. 59) and the remaining sequences acquired a silent g in the Lys81 codon. Cells with identical sequences were found in serial sections and at some distance from each other in a given section (see Fig. 2A). Lb 92 and four other cells (La 02, La 21, La 39, and La 58) had the same VH1-DH-JH sequence (indicated by a plus after the cell name in Fig. 2A). The remaining cells diverged from this sequence by acquiring additional point mutations and gene conversions, but only two sequences (La 11 and Lb 22) had replacement changes in CDR3. The rearranged germline sequence of this CDR3 may have already been well suited for binding to DNP. Cell La 40 acquired a stretch of sequence in FR1 and CDR1 that matches a likely gene-conversion-donor gene sequence present in VH16 (boxed in dark blue); in La 04, a smaller tract only affected framework 1 (FR1). Both La 40 and Lb 83 underwent replacements of Gly86 with Asp that is found in many potential donor sequences including VH12, VH4, VH8, and VH9 (pink shading). Cells Lb 29 and Lb 30 exhibited the commonly observed replacements of Leu78 with Glu that are likely to have occurred through gene conversion from donor sequences present in both VH7 and VH9 (red and green box). This tract of changes extended at least through the silent g in Lys81. Lb 82 acquired a Ser codon TCT at position 50 that involved a two-base change plus acquisition of an adjacent silent base change. Another sequence, La 71 also acquired a Ser AGC at position 50 but it was via a single-base change from the Thr codon ACC. Ser81 was changed to Thr independently in several branches of the genealogical tree. This might reflect some selective advantage (e.g., for improved affinity). We also found two different Thr89 codons, ACA in Lb 17 and La 49 and ACC in five other sequences (Fig. 2C). Of 24 successful VHJH sequences, 11 were from the group of 29 cells with successfully sequenced VH1D1H1. This is indicated in Fig. 2B by underlining the cell name. As regards the Vκ sequences, deduced Vκ-Jκ protein sequences from four cells (La 55, La 64, Lb 22, and Lb 29) were identical; the others had between one and eight replacements. Definition of the complete database of germline Vκ sequences (unpublished manuscript) will allow us to reconstruct the events leading to the sequence changes with confidence. Now we can only conclude that the light chain sequences generally complement the heavy chain data (data not shown).

Although most of the VH1D1H1 sequences found in cells collected from GC L belonged to a single clone, two other smaller sets of clonally related sequences (clones L2 and L3; Fig. 3) and five independent unique sequences were found. Clone L2, with five members provided additional examples of gene conversions that appear to have occurred during expansion of the clone within the GC. The initial precursor appears to have rearranged VH1 to D1 and J5κA. A common precursor (P1) underwent a large gene conversion that extended from FR1 through the first codon of CDR2 with the likely donor being VH7, (green) and a change involving VH19 (red) extending from CDR2 through position 75 of FR3. Precursor P1 also acquired a silent change in Ile102 of the J region and two amino acid replacements in CDR3. Cells Lb 24 and Lb 31 then underwent additional changes that may have been due to point mutations. Cells Lb 12, La 20, and Lb 35 share a common precursor P3 that arose after a second gene conversion involving VH9 and a silent change in Ser30 that may have been due to VH6.

Although clone L3 had only four heavy chain members, we obtained rearranged heavy and light chain sequences from all four cells and there were 10 additional light chain members. The light chain data complemented the heavy chain data and strongly favored the clonal tree we show compared with an alternative tree (data not shown). The presence of a replacement of Ser17 by Gly on two branches of the tree may indicate a selective advantage for improved affinity.

Based on heavy chain sequences, GC N yielded six groups of clonally related cells. One group of eight cells (N3) all had a frameshift mutation at the VH to DH junction and will not be considered in detail (sequences deposited in GenBank and given accession numbers AF087778–AF087785). Analyses of the other sequences allowed construction of clonal trees shown in Figs. 4 and 5. In clone N1 (Fig. 4), rearrangement of VH1-2-D2a-JH4 then led to a common precursor (P1) with a gene conversion that was likely due to VH4, four replacement changes in CDR3, and a silent change in J4. Cell Na 73 branched off and 11 other cells shared a set of gene conversions that could have involved VH9 (red box) and either VH2, VH5, or VH8 (pink shading). Na 16, Nb 49, and Nb 56 from two serial sections had the same heavy chain sequence and two additional cells Nb 37 and Nb 72 had only a silent change in Ser11. Nb 09 acquired replacements in FR1, CDR1, and CDR3. The change of Val34 to Met and the reversions of Tyr32, Gly33, and Ile35 to Asn, Ala, and Ser can all be accounted for by a block derived from VH6 (dark blue box). Similarly, the loss of the silent base change in Ser17 (red box) in cells Na 11 and Nb 58 can be explained by a gene conversion block from VH2 or VH7 (green box). The remaining few silent and replacement changes may have been due to point mutations. Fig. 4 also shows diagrammatically the three small clones N4, N5, and N6. Clones N5 and N6 both had precursors with changes in CDR1 that can be explained by two different gene conversions from the VH4 sequence. All three sequences from clone N5 also had the frequently observed replacements of Leu 75 with Glu that can be explained by conversion involving either VH17 or VH19 (red and green box). In N6 this replacement occurred in the sequence of Na 65 after divergence from Na 77.

The sequences and clonal tree derived for clone N2 are shown in Fig. 5. During expansion of clone N2 from precursor P1, several additional gene conversion events occurred involving a donor that could have been VH6 (dark blue boxes), and an unknown donor (UD, boxed in black) to generate the 10 functional heavy chain sequences that we recovered. An eleventh sequence (Nb 69) acquired a frameshift at position 6. The common precursor, P1, already showed evidence for gene conversions involving VH3 (orange), VH5 (green), VH2, VH5, or VH19 (pink), and VH7 or VH9 (red and green). Although studies of VH4 and/or Vκ sequences in B cells of splenic GCs from chickens immunized with NP-BSA (61) or FITC-BSA (58) also found somatic gene conversion had occurred during clonal expansion in spleen, the precursor cells with between

![FIGURE 2. (continued)](https://example.com/figure2 continuation)
FIGURE 3. The sequences and clonal relationships of two additional clones from GC L (see also Fig. 2). Underlined cell names indicate that a V\textsubscript{k}J\textsubscript{k} sequence was obtained from the same cell. The approximate locations from which cells were collected for sequencing from GC L were shown in Fig. 2A and Fig. 1, H and J, show sections serial to those used for collection. The V\textsubscript{H}D\textsubscript{H}J\textsubscript{H} sequences of members of clones L2 and L3 have been assigned GenBank accession numbers AF087741–AF087745 and AF087746–AF087749, respectively.
FIGURE 4. The sequences and clonal relationships of clone N1 from GC N and diagrammatic summaries of three smaller clones from the same GC (see also Fig. 2). Underlined cell names indicate that a $V_J$ sequence was obtained from the same cell. Nb 16$\pm$ indicates that two other cells, Nb 49 and Na 16, yielded identical sequences. Nb 37$\pm$ indicates that Nb 72 yielded an identical sequence. The $V_H D_H J_H$ sequences of members of clones N1, N4, N5, and N6 have been assigned GenBank accession numbers AF087755–AF087766, AF087786–AF087788, AF087789–AF087791, and AF087792–AF087793, respectively.
three and five gene conversion blocks identified 7 days after immunization, were presumed to have initiated the clones in splenic GCs and to have been bursal derived (61). In the rabbit, we cannot presume that they were derived from cells that developed the primary repertoire in appendix or other gut-associated lymphoid tissue (30, 31) because we found splenic precursors with germline or

FIGURE 5. The sequences and clonal relationships of clone N2 from GC N (see also Fig. 2). Underlined cell names indicate that a $V_J_k$ sequence was obtained from the same cell. UD, unknown donor. The $V_H D_H J_H$ sequences of members of clone N2 have been assigned GenBank accession numbers AF087767–AF087777.
to analyze because many changes appear to have resulted from gene conversion events. The donors that are the proposed sources of the altered sequences themselves contain base changes that mainly result in replacements. Thus the strikingly low proportions of silent changes in the V regions may reflect both the highly efficient gene conversion mechanism for introducing templated replacements (64) and selection for favorable replacements during the clonal expansion and selection stages of the GC reaction.

In an extensive analysis of serum anti-DNP Abs produced by rabbits in our National Institutes of Health allotype-defined breeding colony (44), anti-DNP affinities and concentrations were found to increase most between days 14 and 28. Analyses of affinities of serum Abs indicated that some molecules of the highest affinity species were present in all rabbits by day 42. It is likely that in rabbits as in mice, high-affinity precursors of anti-hapten Ab-producing cells exit GCs and undergo further selection for high-affinity Ab forming cells in the bone marrow (65, 66). The selection that may occur in bone marrow would involve competition between independently evolved cells with high affinity. In addition to narrowing of the HCDR3 lengths in the dominant clones found at day 15, comparisons of the CDR sequences in the separately evolved clones revealed certain recurrent amino acid replacements in the dominant clones that may contribute to favorable increases in affinity for Ag, or on rates of binding to hapten (67). The recurrences shown in Table V are particularly striking in view of the variations occurring in parallel in the light chain CDRs and the likelihood that many CDR structures can yield high affinity anti-DNP Abs in rabbits. Although only seen in 2 of 29 sequences from L1 (Lb 81 and Lb 84; Fig. 2) the replacement of Asn58 by Tyr was seen in 30 of the 31 sequences from clones L2, L3, N1, N2, and N5. The largest clone from day 10, M1 (47), had two sequences with this replacement (Ma 28 and Ma 34). None of the five sequences in the other two small clones N4 and N6 had this change. In CDR2, there were recurrent replacements of Ala50 by Tyr (or Phe, Ile, and Val) and of Tyr58 by Asp (or Asn in clone N2). Interestingly, the smaller clones (N4, N5, and N6) did not have the replacements in CDR2 seen in the larger clones. In CDR3 we observed a recurrence of Tyr-Ile at positions 99 and 100 (or 100A).

**Evidence for selection of CDR amino acid replacements and heavy chain CDR3 (HCDR3) lengths**

In the clonal anti-DNP populations present on day 15 we observed slightly shorter CDR3 lengths. Although differences in the mean lengths were not statistically significant, there appeared to be selective narrowing of the range of CDR3 lengths compared with the earlier time points. These results are summarized in Table IV. By day 15 the range of HCDR3 lengths narrowed to 8–14 codons from 4–18 and 4–19 codons at days 7 and 10, respectively. Our results are consonant with those of Brezinschek et al. (62) who recently reported that human memory B lymphocytes have slightly shorter (11.9 ± 3.4 codons vs 14.3 ± 4.3 codons) and a more limited range of HCDR3 lengths than naïve B cells and suggested this may facilitate more effective Ag binding. Significantly shorter HCDR3 were also found in sequences that also had affinity-increasing mutations at day 12 of the response to NP in C57BL/6 mice (63).

By day 15, some D<sub>H</sub> regions had and were continuing to accumulate mutations. Because in rabbit there are no known donor sequences for D<sub>H</sub> regions, it is likely that the replacements in the D<sub>H</sub> regions were the result of point mutations and selection. Replacement changes in the body of the V region are more complex

---

Table V. **Recurrent amino acids in the HCDRs of different clones**

<table>
<thead>
<tr>
<th>Clone Name&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HCDR1</th>
<th>HCDR2</th>
<th>HCDR3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Asn&lt;sup&gt;52&lt;/sup&gt; to Tyr</td>
<td>Ala&lt;sup&gt;50&lt;/sup&gt; to Tyr (Phe, Ile)</td>
<td>Tyr&lt;sup&gt;58&lt;/sup&gt; to Asp (Asn)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1</td>
<td>2/29</td>
<td>0/29</td>
<td>28/29</td>
</tr>
<tr>
<td>L2</td>
<td>5/5</td>
<td>3/5</td>
<td>3/5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L3</td>
<td>4/4</td>
<td>1/4</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N1</td>
<td>8/9</td>
<td>8/9</td>
<td>8/9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td>10/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N4</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N5</td>
<td>3/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N6</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>2/7</td>
<td>0/7</td>
<td>0/7</td>
</tr>
</tbody>
</table>

<sup>a</sup> These are from GCs at day 15 of the anti-DNP immune response with the exception of M1, which is from day 10 (Ref. 47).

<sup>b</sup> Maintained from germline.

<sup>c</sup> One nonfunctional sequence was not included.
and 100A) in all L1 and three of four L3 sequences. Whereas in L1, Tyr-Ile was retained from the germline sequence, L3 acquired Tyr-Ile from the germline sequence and had a two-base change to yield Trp at position 100. Clone N1 maintained germline Tyr100 and replaced Ala110A with Ser (8/9) or Phe (1/9).

The overall pattern that is documented by this and the previous study (Figs. 2–5 and Ref. 47) is one of splenic precursor cells whose germline or near germline sequences changed both by gene conversion and point mutations during early divisions and largely by point mutations during later divisions. Because our preliminary data show that within the same expanding clonal populations, considerable diversification of light chain sequences occurred in parallel with the changes in the VH sequences, these events may produce the diverse sequences that serve as substrates for further affinity maturation by selection either within GCs or later among mature plasma cells. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl: IV. Affinity-dependent, antigen-driven selection in rapidly cycling small B cells. Science 278:298.


