In Situ Studies of the Primary Immune Response to (4-hydroxy-3-nitrophenyl)acetyl. III. The Kinetics of V Region Mutation and Selection in Germinal Center B Cells

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Summary

In the murine spleen, germinal centers are the anatomic sites for antigen-driven hypermutation and selection of immunoglobulin (Ig) genes. To detail the kinetics of Ig mutation and selection, 178 VDJ sequences from 16 antigen-induced germinal centers were analyzed. Although germinal centers appeared by day 4, mutation was not observed in germinal center B cells until day 8 postimmunization; thereafter, point mutations favoring asymmetrical transversions accumulated until day 14. During this period, strong phenotypic selection on the mutant B lymphocytes was inferred from progressively biased distributions of mutations within the Ig variable region, the loss of crippling mutations, decreased relative clonal diversity, and increasingly restricted use of canonical gene segments. The period of most intense selection on germinal center B cell populations preceded significant levels of mutation and may represent a physiologically determined restriction on B cells permitted to enter the memory pathway. Noncanonical Ig genes recovered from germinal centers were mostly unmutated although they probably came from antigen-reactive cells. Together, these observations demonstrate that the germinal center microenvironment is rich and temporally complex but may not be constitutive for somatic hypermutation.

After immunization with immunogenic conjugates of the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP), Ig hypermutation and selection take place in germinal centers (GC) (1), specialized histologic structures of secondary lymphoid tissues (2-4). Splenic GC support oligodonal B cell populations (2, 4, 5) that result from colonizing migration by antigen-activated (6) lymphocytes from the region of the periarteriolar lymphoid sheath (PALS) (4, 6, 7). Nonmigrating sister cells remain associated with the PALS, establishing large foci of antibody-forming cells (4, 6, 7). Interestingly, although focus and GC B cell populations arise from common founders, Ig hypermutation is not evident in foci (1, 6) and presumably is dependent upon the GC microenvironment.

Despite intensive study, our understanding of somatic Ig mutation and selection remains elementary. Mutations are introduced into rearranged V regions of transcriptionally active Ig k- and l loci (8, 9) at the rate of 10^{-3} mutations/bp per cell/generation (10). Mutations are predominately single nucleotide substitutions (90-95%) although small deletions and insertions also have been noted (11-15). These mutations are distributed asymmetrically within an ~2-kbp region whose 5’ boundary is defined by the V<sub>k</sub> promoter sequence (15, 16); the focus of this distribution is the rearranged V(D)J elements (15). Although mutation is believed to be approximately random (11-14), strand biases (17) and mutational hot spots (11, 18) have been reported. The mechanism of Ig hypermutation is unknown. These mitotic mutations differ significantly from those arising during meiosis (16), but all models for the process of somatic hypermutation (19-23) are largely speculative.

Considerable evidence suggests that mutant B cells are phenotypically selected by competition for antigen. For example, over time the relative frequency of mutations becomes highest in the CDRs that encode the antigen contact residues of antibody (12, 24, 25); ratios of replacement-to-silent (R/S) substitutions are biased in late antibody, favoring R mutations in the CDRs and S changes in the framework (FW) regions of V(D)J (10, 26-28); and, recurrent, or key, mutations in H and L chain V regions confer increased affinity for the antigen ligand (12, 28-30). Mutations that abrogate or significantly diminish the capacity of antibody to bind antigen are thought to result in apoptosis (2, 31). Presumably, cells that have accumulated (nearly) neutral mutations are simi-
larly lost as antigen concentrations fall below that required initially for clonal activation. However, the cellular events that underlie selection are poorly understood. For example, Foote and Milstein (32) recently provided unexpected evidence that fast on-rate constants \( k_{on} \) may also constitute an initial step for clonal activation. However, the cellular events that underlie selection are poorly understood.

To better understand the processes of antigen-driven Ig hypermutation and selection, we have studied the genetics of GC B cell populations generated by immunization with NP. The antibody response of Ig-h mice to NP is highly restricted, most primary anti-NP antibodies bear the \( \lambda \) L chain, and the predominant H chain is encoded by the \( v_\lambda \)V186.2, DFL16.1, and \( j_{\lambda 2} \) gene segments (24, 33, 34). GC containing these genetically restricted populations appear in the spleen 4 d after immunization and persist at least until day 16 of the response (6). Each GC represents a genetically isolated, pauciclonal population of antigen-reactive B cells (1, 2, 4–6, 35) that takes an independent path of somatic evolution as evidenced by distinct clonal genealogies (1, 6).

Here we analyze 178 VDJ sequences recovered from 16 \( \lambda_1^+ \) GC to detail the dynamics of mutation and selection in the primary antibody response. Surprisingly, Ig mutation is not coincident with the appearance of GC; GC B cell populations at 4 and 6 d postimmunization do not contain mutants. Nevertheless, these populations exhibit remarkable reductions in clonal diversity that suggest antigen-mediated purifying selection. Mutation is first seen 8 d postimmunization and the frequency of novel mutations increases monotonically until day 14 of the response. Strong selection on mutant GC B cells is inferred from shifts in the kinds and distribution of V region mutations in the course of the primary response. Remarkably, B cells expressing the V186.2 \( v_\lambda \) gene segment do not appear initially to dominate the antigen-induced, \( \lambda^+ \) GC populations. Instead, a majority of closely related \( v_\mu \) genes are recovered; their appearance and subsequent loss suggest early participation in the anti-NP response. However, with only a single exception, these V186.2-related, or analogue, \( v_\mu \) genes do not accumulate mutations.

### Materials and Methods

**Animals and Immunization.** Female C57BL/6 mice (viral antibody free; 5–7 wk old) were purchased from The Jackson Laboratory (Bar Harbor, ME), maintained in microisolator cages, and provided sterile bedding, food, and drinking water. Mice were immunized with a single intraperitoneal injection of 50 µg NP (Cambridge Research Biochemicals, Cambridge, UK) conjugated to chicken gamma globulin (CG; Accurate Chemical & Scientific Corp., Westbury, NY) and precipitated in alum (4). Two to four mice were killed by cervical dislocation at 4, 6, 8, 10, 14, and 16 d postimmunization; spleens were processed for histology and DNA amplification as described (1, 6). Serial, 6-µm-thick, frozen sections of spleen were cut in a cryostat microtome, thaw mounted onto poly-L-lysine-coated slides, fixed in ice-cold acetone for 10 min, and stored at \(-20^\circ C\) as described (4).

**Staining and Isolation of B Cells from Individual GC.** Frozen sections were stained in tandem with horseradish peroxidase (HRP)-conjugated anti-\( \lambda \) L chain antibody (Southern Biotechnology Associates, Birmingham, AL) and peanut agglutinin (PNA)-biotin (E-Y Laboratories, San Mateo, CA), followed by streptavidin–alkaline phosphatase (AP) (Southern Biotechnology Associates) as described (4). Bound HRP and AP activities were visualized using 3-aminophenyl carbazole and napthol AS-MX phosphate/Fast Blue BB (Sigma Chemical Co., St. Louis, MO), respectively (4). Cells (>100) were recovered from \( \lambda^+ \), PNA+ GC using a sharpened micropipette controlled by an electrically powered micromanipulator (Narishige, Tokyo, Japan) as described (1, 6).

**Amplification and Sequencing of VDJ DNA Recovered from Individual GC.** Cellular material microdissected from single \( \lambda^+ \), PNA+ GC was incubated with proteinase K for 16 h at 37°C (1). The protease was subsequently heat inactivated (96°C, 10 min) and the crude cellular extract subjected to two rounds of PCR amplification (1). Briefly, the initial round of 40 amplification cycles used primers complementary to the genomic DNA 5' of the transcriptional start site of the V186.2 \( v_\lambda \) gene segment and to a region in the \( j_{\lambda 2} \)-\( j_{\lambda 3} \) intron (1, 28). 2 µL aliquots of this reaction mixture were reamplified for an additional 40 cycles using a second set of nested primers complementary to the first 20 nucleotides of the V186.2 exon and to the terminal portion of the \( j_{\lambda 2} \) element (1). Amplified DNA was extracted in phenol/chloroform, precipitated in ethanol, digested with the BamHI and PstI restriction endonucleases (Boehringer-Mannheim Biochemicals, Indianapolis, IN), and ligated into pBluescript SK+ (pBSK+; Stratagene Cloning Systems, La Jolla, CA) as described (1, 6). Competent DH5α bacteria were transformed by electroporation and recombinant colonies screened with a 23P-labeled oligonucleotide corresponding to amino acid positions 70–74 of the V186.2 gene segment (1, 6, 28). DNA from positive clones was sequenced in both directions as in earlier studies (1, 6).

To ensure the validity of the PCR as a tool to sample heterogeneous populations, mixtures of a cloned myeloma line, TEPC-15, and hybridoma, HPCG-14 (the gift of Dr. P. J. Gearhart, Johns Hopkins University, Baltimore, MD), were centrifuged onto glass slides and fixed identically to splenic sections. Both cell lines express the V1 gene segment of the SI07 \( v_\lambda \) gene family in association with \( j_{\lambda 1} \) (36). HPCG-14 differs from TEPC-15 in the V region at positions 31 and 68 and in the CDR3 region (36). Rearranged V1-\( j_{\lambda 1} \) genomic DNA was amplified from these cells using a series of nested primers; the external primers were identical to those described by Feeney (37). Internal primers were made complementary to codons 6–15, 5'ACGGATCCGGTGACCGTGGTCCCTY (Kel-16) and to the terminal portion of the J1 element, 5'GGAGGAAGCTTGGTACAAGCCTC3' (I~1-8). Kel-16 and I~1-8 were digested with HindIII and BamHI and cloned into pBSK+ as described above.

**Analysis of DNA Sequences.** 178 VDJ fragments were sequenced from 16 (day 4, \( n = 1 \); day 6, \( n = 2 \); day 8, \( n = 4 \); day 10, \( n = 4 \); day 14, \( n = 2 \); day 16, \( n = 3 \)) histologically typical \( \lambda^+ \) GC. An average of 11.1 VDJ sequences (range, 8–23) were obtained from each GC. All VDJ DNA sequences were analyzed using the PC/GENE (Intelligenetics, Mountain View, CA) and GCG (Wisconsin Computer Group, Madison, WI) DNA analysis programs.

The clonal diversity of individual GC was estimated by the
number of unique CDR3 sequences recovered from each population (6). This value represents the lower bound of the numbers of clones within the GC and is virtually identical to phenotypic estimates of clonal diversity (2, 4, 5). However, CDR3 counts must underestimate the actual numbers of clones in GC and should be considered robust only for measures of relative clonal diversity.

Determination of Taq Polymerase Fidelity and the Frequency of Artificial Mutations. The Taq polymerase is error prone because it lacks proofreading activity (38, 39). To determine the average number of misincorporations resulting from polymerase error, we have determined the frequency of polymerase-induced mutations in VDJ DNA from a cloned hybridoma, B1-8 (V186.2, DFL16.1, J6.2), and from seven PALS-associated foci. The frequency of mutations in both populations was found to be virtually identical, from 1/338 (hybridoma) to 1/417 (focus B cells). These frequencies correspond to intrinsic error rates of 3.0–3.7 × 10^{-5}/bp per cycle, a value in excellent agreement with other reports (39). On average, each VDJ fragment recovered by 80 rounds of PCR amplification is expected to contain ~1 (0.8–0.9) mutation attributable to polymerase error; these artificial substitutions have a Poisson distribution (6). Mutations significantly in excess of this value were assumed to have resulted from in vivo processes.

Results

PCR Amplification Generates Representative Population Samples. For the analysis of lymphocyte populations, the distribution of products generated by PCR amplification must mirror the initial distribution of DNA templates recovered from tissue. Although PCR amplification requires (near) identity at the 5' and 3' primer sites (38), it was necessary to ascertain that internal sequence differences would not bias the relative composition of PCR end products. To address this issue, two cloned cell lines, TEPC-15 and HPCG-14, were mixed together in ratios varying from 7:3 to 3:7 (Fig. 1). These lines use identical Vκ and Jκ gene segments but differ from one another by two mutations and dissimilar CDR3s. Cell mixes were centrifuged onto glass slides, processed identically to splenic sections, and samples were taken from each slide by microdissection. Recovered VDJ DNA was amplified in two, 40-cycle rounds of PCR; amplified VDJ fragments were then cloned into bacteria. Randomly selected white colonies (n = 172–258) were transferred to grid plates and the relative proportions of colonies containing TEPC or HPCG V regions were determined by hybridization with CDR3-specific oligonucleotides. Fig. 1 illustrates that PCR amplification does generate samples that accurately reflect initial template populations.

Onset and Kinetics of Ig Hypermutation in GC Populations. The kinetics of Ig mutation in λ+, PNA+ GC after immunization with NP-CG are illustrated in Fig. 2. Although well-formed GC are present as early as 4 d postimmunization (6), mutation is not evident in VDJ DNA until day 8 of the response; the frequency of mutations recovered from GC B cells on days 4 and 6 does not exceed that expected for the Taq polymerase alone (Fig. 2). By day 8 of the response, significant increases in the mutation frequency are observed and the numbers of accumulated novel mutations rise monotonically at least until day 14 postimmunization. The linearity of the increase in mutations suggests a stepwise accumulation of mutations (10, 12, 30), and assuming a mitotic cycle of 10 h...
### DAY 10 λ+ GERMINAL CENTER

| CDR1 | CDR2 |
|------|------|
| 1    | GCT  |
| 2    | GAG  |
| 3    | CTT  |
| 4    | CTT  |
| 5    | CTT  |
| 6    | CTT  |
| 7    | CTT  |
| 8    | CTT  |
| 9    | CTT  |
| 10   | CTT  |
| 11   | CTT  |
| 12   | CTT  |
| 13   | CTT  |
| 14   | CTT  |
| 15   | CTT  |
| 16   | CTT  |
| 17   | CTT  |
| 18   | CTT  |
| 19   | CTT  |
| 20   | CTT  |
| 21   | CTT  |
| 22   | CTT  |
| 23   | CTT  |
| 24   | CTT  |
| 25   | CTT  |
| 26   | CTT  |
| 27   | CTT  |
| 28   | CTT  |
| 29   | CTT  |
| 30   | CTT  |
| 31   | CTT  |
| 32   | CTT  |
| 33   | CTT  |
| 34   | CTT  |
| 35   | CTT  |
| 36   | CTT  |
| 37   | CTT  |
| 38   | CTT  |
| 39   | CTT  |
| 40   | CTT  |
| 41   | CTT  |
| 42   | CTT  |
| 43   | CTT  |
| 44   | CTT  |
| 45   | CTT  |
| 46   | CTT  |
| 47   | CTT  |
| 48   | CTT  |
| 49   | CTT  |
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| 51   | CTT  |
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| 55   | CTT  |
| 56   | CTT  |
| 57   | CTT  |
| 58   | CTT  |
| 59   | CTT  |
| 60   | CTT  |

### D-Region

| GAA | TAG | AAG | GGT |
|-----|-----|-----|-----|
| 1   | GCT | TTA | TTA |
| 2   | GAC | TCC | TAC |
| 3   | TAG | TCT | TAC |
| 4   | AAG | GGT | TAC |
| 5   | GGT | CTA | TAC |

### DAY 14 λ+ GERMINAL CENTER

| CDR1 | CDR2 |
|------|------|
| 1    | GCT  |
| 2    | GAG  |
| 3    | CTT  |
| 4    | CTT  |
| 5    | CTT  |
| 6    | CTT  |
| 7    | CTT  |
| 8    | CTT  |
| 9    | CTT  |
| 10   | CTT  |
| 11   | CTT  |
| 12   | CTT  |
| 13   | CTT  |
| 14   | CTT  |
| 15   | CTT  |
| 16   | CTT  |
| 17   | CTT  |
| 18   | CTT  |
| 19   | CTT  |
| 20   | CTT  |
| 21   | CTT  |
| 22   | CTT  |
| 23   | CTT  |
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| 25   | CTT  |
| 26   | CTT  |
| 27   | CTT  |
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| 29   | CTT  |
| 30   | CTT  |
| 31   | CTT  |
| 32   | CTT  |
| 33   | CTT  |
| 34   | CTT  |
| 35   | CTT  |
| 36   | CTT  |
| 37   | CTT  |
| 38   | CTT  |
| 39   | CTT  |
| 40   | CTT  |
| 41   | CTT  |
| 42   | CTT  |
| 43   | CTT  |
| 44   | CTT  |
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| 58   | CTT  |
| 59   | CTT  |
| 60   | CTT  |

### D-Region

| GAA | TAG | AAG | GGT |
|-----|-----|-----|-----|
| 1   | GCT | TTA | TTA |
| 2   | GAC | TCC | TAC |
| 3   | TAG | TCT | TAC |
| 4   | AAG | GGT | TAC |
| 5   | GGT | CTA | TAC |

### DAY 16 λ+ GERMINAL CENTER

| CDR1 | CDR2 |
|------|------|
| 1    | GCT  |
| 2    | GAG  |
| 3    | CTT  |
| 4    | CTT  |
| 5    | CTT  |
| 6    | CTT  |
| 7    | CTT  |
| 8    | CTT  |
| 9    | CTT  |
| 10   | CTT  |
| 11   | CTT  |
| 12   | CTT  |
| 13   | CTT  |
| 14   | CTT  |
| 15   | CTT  |
| 16   | CTT  |
| 17   | CTT  |
| 18   | CTT  |
| 19   | CTT  |
| 20   | CTT  |
| 21   | CTT  |
| 22   | CTT  |
| 23   | CTT  |
| 24   | CTT  |
| 25   | CTT  |
| 26   | CTT  |
| 27   | CTT  |
| 28   | CTT  |
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| 31   | CTT  |
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| 39   | CTT  |
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| 56   | CTT  |
| 57   | CTT  |
| 58   | CTT  |
| 59   | CTT  |
| 60   | CTT  |

### D-Region

| GAA | TAG | AAG | GGT |
|-----|-----|-----|-----|
| 1   | GCT | TTA | TTA |
| 2   | GAC | TCC | TAC |
| 3   | TAG | TCT | TAC |
| 4   | AAG | GGT | TAC |
| 5   | GGT | CTA | TAC |

### D-Region

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(4), it is consistent with a mutation rate of ~10^{-3}/bp per cell division (10). Sequences of VDJ DNA from representative GC are presented in Fig. 3.

Accumulation of mutations within GC B cell populations allows the construction of clonal genealogies based upon shared and distinct mutations (1, 6). In Fig. 4, four representative genealogies are depicted, illustrating clonal development at 8, 10, 14, and 16 d after immunization. Each branch of these dendrograms represents inferred common descent. These genealogies confirm the stepwise accumulation of mutations implicit in Fig. 2 and illustrate the increasing genetic complexity that follows the repeated introduction of Ig V region mutations between days 8 and 16 of the response (Fig. 4). Note, however, that even while the genetic complexity of GC B cell populations increases with time, the arborization of genealogies decreases. For example, the genealogy representing the day 8 GC, M16, contains at least eight comparable yet distinct branches while later dendrograms (e.g., the day

Figure 3. VDJ DNA sequences recovered from GC representative of days 4, 6, 8, 10, 14, and 16 of the primary response to NP-CG are shown in comparison to the canonical V186.2 and J2 germline sequences. Only those codons that differed from the reference sequences are shown. Dashes indicate identity with the reference sequence. Some recovered VDJ fragments contained noncanonical, V186.2-related analogue V\textsubscript{\textalpha} gene segments. To distinguish these from mutants, codons identical to analogue V\textsubscript{\textalpha} segments are indicated by: * CH4, * V23, * CH10, and 5 V102. (A) VDJ sequences recovered from the day 4 \lambda\textsuperscript{+} GC, H4. Clones 1, 7, and 10 are V\textsubscript{\textalpha} V102; 2, 4, 8, and 9 are V\textsubscript{\textalpha} CH14; and 6 is V\textsubscript{\textalpha} CH10. (B) VDJ sequences from the day 6 \lambda\textsuperscript{+} GC, J5. Clones 3, 8, 9, and 10 are V\textsubscript{\textalpha} V186.2; 1, 2, 4, and 6 are V\textsubscript{\textalpha} V23; 5 is V\textsubscript{\textalpha} CH14; and 7 is V\textsubscript{\textalpha} CH10. (C) VDJ DNA sequences from the day 8 \lambda\textsuperscript{+} GC, M16. All sequences use the V186.2 V\textsubscript{\textalpha} gene segment. (D) Sequences from day 10 \lambda\textsuperscript{+} GC, B8. Clones 1, 2, 4, and 10 are V\textsubscript{\textalpha} V186.2; 3, 5, 8, and 9 are V\textsubscript{\textalpha} V23; and 7 is V\textsubscript{\textalpha} CH10. (E) VDJ sequences from the day 14 \lambda\textsuperscript{+} GC, D2. All sequences contain the V186.2 gene segment. (F) VDJ fragments recovered from the day 16 GC, 61AA02. All sequences contain the V186.2 gene segment. 61AA02d clone contains a cluster of six nucleotide exchanges identical to the V23 sequence. It is unknown if this insertion reflects an in vivo or in vitro process. The DNA sequences for the VDJ fragments recovered in this study are available from EMBL/GenBank/DDBJ under accession numbers DS13953 and X67341-X67391.

Figure 4. Genealogical relationship of VDJ DNA sequences derived from GC. Representative genealogies are shown, each typical of that time point (A, day 8; B, day 10; C, day 14; D day 16) in the response. (g/) Germline; and (broken lines) hypothetical intermediates. Mutations are indicated by the codon number of the affected site. Numbers in branches indicate the number of mutations to the next node.

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16 GC, 61AA02) are comprised of progressively fewer major lines of descent (Fig. 4). We believe that the extensive arborization of early GC genealogies represents the recent introduction of random mutations into a large population of unmaturated sister B lymphocytes. Only a small fraction of these early mutants prove successful, restricting later genealogies to one or a few major branches (Fig. 4).

Analysis of Mutations in V_{11.186.2} Recovered from GC Cell Populations. The 238 unique mutations observed in V186.2 gene segments recovered from mutating GC (days 8–16) are detailed in Table 1. These data are presented in reference to the DNA sense strand, therefore, an A → G substitution means that an A:T nucleotide pair has been replaced by a G:C pair; the original misincorporation, A → G (sense strand) or T → C (antisense strand), cannot be known. These reciprocal transitions and transversions are paired in Table 1.

Patterns of Ig mutations have been used to infer characteristics of the mechanism(s) for somatic hypermutation, e.g., stand bias (17). Thus, we compared the mutations we observed to an expected number based upon a study of 587 spontaneous meiotic point mutations in eight pseudogenes (40). Importantly, not all meiotic point mutations are equally likely, even in the virtual absence of selection (40). For example, on average, the numbers of meiotic transitions are greater or equal to the numbers of transversions, even though twice as many transversions are possible (16 and Table 1).

Table 1 suggests that Ig hypermutation makes use of processes distinct from those responsible for meiotic mutation rather than their catalysis. While observed V_{11} mutations consisted of approximately twice as many transitions (n = 162) as transversions (n = 76), the pattern of Ig exchanges differed significantly ($\chi^2 = 77.2; p > 0.05$) from that expected for meiotic substitutions. Undoubtedly, much of this difference results from the PCR. The largest discrepancy between observed and expected mutations is an excess of V_{11}A → G transitions, the predominant error of the Taq polymerase (39). Indeed, VDJ DNA amplified from the antigen-activated but nonmutating cell populations of $\lambda^+$ PALS-associated foci (6) also showed a large excess of A → G substitutions (data not shown). However, even in the absence of likely polymerase error, the character of somatic mutations remained distinct. For example, biased transversions were observed at template A:T nucleotide pairs; the frequency of A → N exchange was always double that of the reciprocal

| Transitions | Observed* | Expected† | Residuals‡ |
|-------------|-----------|-----------|------------|
| A→G        | 56        | 22.4 (19.3–25.5) | 50.4       |
| T→C        | 28        | 19.5 (16.4–22.6) | 3.7        |
| G→A        | 43        | 49.9 (44.1–54.5) | 1.0        |
| C→T        | 35        | 49.9 (44.9–55.0) | 4.4        |
| Transversions |         |           |            |
| A→T        | 19        | 11.2 (8.1–14.3)  | 5.4        |
| T→A        | 9         | 10.4 (8.1–13.1)  | 0.2        |
| A→C        | 8         | 11.8 (10.2–13.6) | 1.2        |
| T→G        | 5         | 7.9 (5.0–10.7)   | 1.1        |
| C→A        | 5         | 15.4 (12.8–18.1) | 7.0        |
| G→T        | 11        | 17.2 (14.5–19.7) | 2.2        |
| G→C        | 11        | 12.5 (10.2–14.9) | 0.2        |
| C→G        | 8         | 9.9 (8.9–11.2)   | 0.4        |
| **Total**  | 238       | 238.0      | 77.2†      |

* Mutations in rearranged V186.2 gene segments recovered from GC cell populations at days 8–16 postimmunization.
† Calculated from references 16 and 40, based upon the frequency of 587 transitions and transversions observed in eight pseudogenes. Numbers in parentheses represent range of expected values.
‡ Calculated as $(0 − E)^2/E$.
§ $x^2 = 77.2$; d.f. = 11; $p > 0.05$. 
Figure 5. Distribution of somatic mutations in the GC Ig V regions of mice immunized 8–16 d earlier with 50 μg NP-CG/alum. The frequencies of mutations that fall in FW 1–3 and CDR1 and -2 were determined. Data are presented as the mean frequency of somatic mutation in FW 1–3 (filled bars) and CDR1 and -2 (crosshatched bars) at days 8–16 postimmunization.

Figure 6. Estimated clonal diversity of individual GC decreases with time. (A) Clones within individual GC were identified as unique CDR3 sequences; the mean number of distinct CDR3 regions/GC is plotted against time postimmunization. (B) Dominance of single CDR3s (clones) within individual GC occurs with time. The frequency of clones that share CDR3 within GC was determined and the mean frequencies are plotted against days postimmunization. Primary refers to the frequency of the predominant clone, secondary refers to the second most common clone, and so on.
T → N event (Table 1). The pattern could indicate a mutational mechanism that acts asymmetrically on the DNA template (16, 17, 19).

Selection of B Cell Mutants in GC. About 79% of the V186.2 Vh gene segment corresponds to FW 1–3, while the remaining 21% accounts for CDR1 and -2. In the absence of selection, mutations randomly introduced into the V186.2 gene segment should follow the same distribution (41). In fact, although variability exists, immediately after the start of Ig hypermutation (days 8 and 10; Fig. 2), 60–70% of all mutations are located within FW 1–3 and 30–40% lie in CDR1 and -2 (Fig. 5). However, as the response progresses, the frequency of mutations in the CDRs increases such that by days 14 and 16 postimmunization mutations are approximately evenly distributed between the FW and CDR nucleotides (Fig. 5). This biased distribution is consistent with antigen-mediated selection (41).

The kinds of mutations found early and later in the response also imply phenotypic selection. For example, 33% of the V186.2 sequences recovered from the day 8 GC, GC M16 (Fig. 3), contained mutations that generated translational stop codons or resulted in the loss of invariant Cys (C92) residues. In contrast, later GC B cell populations contained few (obvious) crippling mutations. In the 69 VDJ sequences recovered from GC at days 14 and 16 of the response, only two mutations resulted in a translational terminator (Fig. 3). Interestingly, one of the three day 16 GC, 61AA02, expressed a key mutation (Trp → Leu, position 33) known to confer increased affinity for NP (29, 30).

Selection was also inferred from the decreasing clonal diversity of GC populations as estimated by the number (Fig. 6 A) and proportions (Fig. 6 B) of unique H chain CDR3 sequences recovered from individual GC. Early in the response, clonal diversity in GC cell populations actually increased, from an average of about three distinct CDR3 sequences/GC on day 4 to six CDR3s on day 6 (Fig. 6 A). We believe this increase represents continued immigration of B cells from nonfollicular sites (6). At the initiation of somatic hypermutation (Fig. 2), the numbers of unique CDR3/GC abruptly decline, such that for days 8–16 the average number of dis-

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**Table 2.** D Segment Use in VDJ Fragments Amplified from λ⁺ GC

| Frequency in recovered VDJ (percent of clones) | All Vₜ | V186.2 |
|---|---|---|
| **Day** | DFL16.1 | DSP2 | DQ52 | Unidentified | DFL16.1 |
| 4 | 33 | - | - | 67 | - |
| 6 | 25 | 17 | - | 58 | 17 |
| 8 | 100 | - | - | - | 100 |
| 10 | 50 | 30 | 10 | 10 | 50 |
| 14 | 67 | - | - | 33 | 67 |
| 16 | 67 | - | - | 33 | 67 |
| **Totals** | 57 | 8 | 2 | 33 |

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**Table 3.** Frequencies of Vₜ Gene Segments Recovered from λ⁺ GC

| Vₜ | Frequency in GC* | Vₜ frequency† | Percent total Vₜ | Percent analogue Vₜ |
|---|---|---|---|---|
| V186.2 | 15/16 | 154/178 | 87 | - |
| CH14 | 3/16 | 8/178 | 5 | 32 |
| V23 | 2/16 | 8/178 | 5 | 32 |
| CH10 | 4/16 | 4/178 | 2 | 16 |
| V102 | 1/16 | 3/178 | 1 | 12 |
| 24.8 | 1/16 | 1/178 | <1 | 4 |
| V165.1 | 1/16 | 1/178 | <1 |

* Represents numbers of GC containing specific Vₜ/all GC studied.
† Represents numbers of VDJ fragments containing specific Vₜ/all VDJ fragments studied.
distinct clones/GC is only 2.2 (Fig. 6 A). The relative proportions of distinct CDR3 sequences recovered from individual GC similarly indicate the progressive dominance of few clones (Fig. 6 B). On days 4 and 6 of the response, the frequency of each recovered CDR3 sequence is roughly comparable (Fig. 6 B). However, by day 8, concomitant with the dramatic reduction in the number of clones/GC (Fig. 6 A), clonal diversity is further diminished by the predominance of single CDR3s within each GC (Fig. 6 B). This progression towards the domination of GC B cell populations by single clones is what is expected for a process of local, antigen-driven selection.

Early Unrestricted Use of D<sub>n</sub> Families in GC Populations. In contrast to the dominant use of the DFL16.1 element in hybridoma collections (14, 24, 29, 42), VDJ fragments recovered from X + GC cell populations used several D families, although DFL16.1 was most common (Table 2). Representation of DFL16.1 increased during the response, from 25-33% on days 4 and 6 postimmunization to 67% by days 14 and 16. These changes are correlated with increased recovery of V<sub>n</sub> V186.2 gene segments (see above) and most likely reflect antigen-driven selection. However, it is clear that multiple V186.2 + D<sub>n</sub> associations generate NP-specific paratopes.

Alternate V<sub>n</sub> Use in the Anti-NP Response. V<sub>n</sub> gene segments other than V186.2 were isolated in this study of λ<sup>+</sup> GC. Of the 178 VDJ sequences analyzed, 154 (87%) were identified as V186.2, while the remainder were closely related analogues of the V186.2 gene segment: CIH4, V23, CH10, V102, 24.8, and V165.1 (43) (Table 3). The CIH4, V23, and CH10 gene segments comprised 80% of the recovered analogue genes and were repeatedly isolated from different GC and mice. Although these V186.2 analogues could derive from cells not participating in the anti-NP response, at least three lines of evidence suggest that this is not the case. First, similar PCR amplifications of genomic DNA from unselected μ<sup>−</sup>δ<sup>−</sup> (B220<sup>−</sup>) cells (43, 44) generated different patterns of analogue V<sub>n</sub> genes. For example, while CIH4 constituted 32% of analogue V<sub>n</sub> genes recovered from GC (Table 3), CIH4 represented only 6% of the VDJ rearrangements recovered from pre-B cells (44) and was absent in the sample generated from μ<sup>−</sup>δ<sup>−</sup> B lymphocytes (43). The observed bias for CIH4 in λ<sup>+</sup> GC could represent recruitment by antigen. Second, although the probability of imprecise microdissection should remain constant, the frequency of non-V186.2 V<sub>n</sub> gene segments in GC decreases with time (Fig. 7). Early in the response to NP (days 4–6), rearranged VDJ fragments containing non-V186.2 gene segments are common, sometimes (day 4) the only V<sub>n</sub> genes recovered from GC. However, the frequency of analogue V<sub>n</sub> gene segments then sharply declines, such that non-V186.2 segments become minor components of the VDJ sequences recovered from later (days 8–16) GC populations (Fig. 7). Loss of alternative V<sub>n</sub> genes in the λ<sup>+</sup> GC cells is a major component in the decreasing clonal diversity (Fig. 6) of these populations as the immune response progresses. Third, we have established by transfection that at least some recovered analogue V<sub>n</sub> gene segments complement the λ L chain to generate NP-specific antibody (our unpublished results).

Absence of Somatic Hypermutation in Analogue V<sub>n</sub> Gene Segments. Even though evidence suggests that recovered analogue V<sub>n</sub> genes represent cellular participants in the anti-NP response (see above), mutations in analogue V<sub>n</sub> segments rarely exceeded that expected for the η<sup>q</sup> polymerase (Fig. 3). Low frequencies of mutations were observed even when substantially mutated VDJ fragments containing the V186.2 gene segment were recovered from the same GC. Nonetheless, at least some cells expressing the analogue V<sub>n</sub> genes appear competent to undergo Ig hypermutation; mutated analogue VDJ sequences were recovered from the day 10 GC,

![Figure 7. V<sub>n</sub> gene usage in the primary anti-NP response of C57BL/6 mice. The frequency of V<sub>n</sub> gene segments isolated from GC is plotted against time postimmunization. (Filled bars) V186.2 V<sub>n</sub> gene segment; (open, hatched, and crosshatched bars) frequencies of V186.2 analogues, closely related members of the J558 V<sub>n</sub> gene family. The identity of the V186.2 analogues are indicated.](image-url)
GC B12. Six VDJ fragments containing V\textsubscript{H} V186.2 and three containing C1H4 were recovered from GC B12, with each group representing clonally related mutants. The frequency of unique mutations in the related C1H4 V\textsubscript{H} gene segments (1/118) was typical of the day 10 response (1/190) (Fig. 2) and comparable to the six V186.2 segments (1/209) also recovered from GC B12 (6).

Recovery of Hybrid VDJ Fragments Is Correlated to the Onset of Ig Somatic Hypermutation and Selection. In our initial study of Ig mutation in GC and foci (1), we noted that some 30% of VDJ fragments recovered from day 16 GC could be classified as hybrid sequences; i.e. sequences formed during PCR amplification by the fusion of distinct, yet homologous genes (45). Damaged DNA has been shown to promote the formation of gene hybrids during PCR amplification (45); broken DNA of one gene can act as a primer by hybridizing to a homologous gene and be extended by the Taq polymerase to yield a hybrid product (Fig. 8, inset). Thus, hybrid DNA products can be identified only when markers in the 5' (e.g., distinct CDR1 sequences) and 3' (e.g., different CDR3s) regions of the gene(s) are present.

Sequence analysis of VDJ DNA amplified from: (a) mixtures of fixed TEPC-15 and HPCG-14 cells; and (b) four, day 10 PALS-associated foci (6) yielded equivalent (≈ 6%) frequencies of hybrid sequences (data not shown). This finding demonstrates that the conditions necessary for histology and PCR are not responsible for the production of frequent hybrids. When VDJ sequences recovered from GC were analyzed for gene hybrids (Fig. 8), the frequencies of hybrids were initially identical to that of the hybridoma and focus controls (≤6%). Then, coincident with the onset of somatic hypermutation and selection, hybrid frequencies increased linearly to ~30% by day 16 of the response (Fig. 8). This observation implies that processes intrinsic to Ig mutation and/or antigen-driven selection promote the conditions necessary for the in vitro generation of hybrid VDJ amplification products. For example, site-specific DNA nicking has been proposed as a potential component of Ig hypermutation (16); apoptotic cell death in GC also results in DNA fragmentation (2, 31). Either (or both) processes would increase the likelihood of hybrid artifacts (45).

Discussion

This study confirms our earlier reports of Ig mutation in GC cells (1) and also supports previous work by other groups on the onset, accumulation, and selection of V region mutations (2, 10–14, 33, 36, 46–49). However, mutant B cells were not detected in early GC (Fig. 2) even though they were PNA+ and structurally obvious (6). Thus, the estimated three to six (Fig. 6) B cell founders of each GC undergo substantial proliferation before the initiation of Ig hypermutation. This observation may be significant, as most mutations are believed to vitiate the antibody paratope (50). Proliferation before mutation would increase the likelihood that the initial round(s) of mutagenesis produces cells bearing neutral or improved antibody phenotypes. VDJ fragments bearing mutations in excess of that expected from polymerase error were first recovered from GC cell populations d after immunization (Fig. 2), indicating day 7 of the response as the onset of mutagenesis. Mutation frequency then grew linearly until day 14, implying stepwise incorporation of single point mutations at a rate compatible with that calculated by Clarke et al. (10). On day 16 postimmunization, the average frequency of observed mutations actually declined (Fig. 2), an observation consistent with either the end of somatic hypermutation or increased selection stringency.

Iterative addition of point mutations was confirmed by the
analysis of clonal genealogies derived from single GC (Fig. 4). Related VDJ fragments could be assembled into increasingly complex trees in which most branch points were defined by one or two shared mutations. Although the error-prone \( \text{Taq} \) polymerase will, on average, introduce approximately one misincorporation into each amplified VDJ fragment, these artificial mutations occur as a Poisson distribution and are virtually never shared (1, 6); the representative clonal genealogies depicted reflect in vivo processes of genetic diversification and selection. The consequence of clonal proliferation before the initiation of Ig hypermutation can be seen in the extensive aborization of early genealogies (Fig. 4A). Most of the initial radiations are later lost due to phenotypic selection, confirming the improbability of neutral and/or beneficial mutations, leaving only a few major lineages (Fig. 4D).

The kinds (Fig. 3) and distribution (Fig. 5) of mutations recovered from day 8 and 10 GC cells are consistent with random mutagenesis (41). Early in the response, crippling mutations (Fig. 3C; GCM16) are observed at frequencies that approximate expected values calculated on the presumption of random nucleotide substitution (not shown). Further, the distribution of mutations within CDR1 and -2 and F W 1-3 of mutated V186.2 \( V_\mu \) gene segments was initially comparable to the relative sizes of these regions (Fig. 5).

The 238 mutations found in this study (Table 1) provided evidence that the mechanism(s) responsible for these misincorporations operates distinctly from those processes responsible for spontaneous meiotic point mutations. In contrast to the unselected meiotic mutations collected by Li et al. (40), the frequencies of reciprocal transitions and transversions observed in mutated V186.2 template DNA were often biased (Table 1). In part, the biases are the result of PCR amplification by the \text{Taq} polymerase. A \( \rightarrow \) G transitions account for more than half of the misincorporations introduced by this error-prone polymerase (39). Nevertheless, frequent A \( \rightarrow \) G somatic mutations in/nearby V(D)J gene segments have also been observed by Golding et al. (16) in DNA sequences not subjected to the PCR. Also, the biased A \( \rightarrow \) T and A \( \rightarrow \) C transversions and the infrequent C \( \rightarrow \) A exchange are unassociated with \text{Taq} error. Asymmetrical transversions appear to be a leitmotif of the somatic hypermutation of the V186.2 gene segment.

As the immune response progressed, evidence for phenotypic selection of GC B cells became increasingly apparent; the proportion of VDJ sequences exhibiting crippling mutations fell, from a maximum of 33% on day 8 to <3% by day 14 (Fig. 5), and at the same time, mutations became focused within CDR1 and -2 (Fig. 5). Selection could also be inferred from decreasing CDR3 diversity of GC populations. Diversity in GC was maximal at days 4 and 6 of the response; early GC contained at least three to six clones of comparable size while later GC populations contained a single, dominant CDR3 sequence (Fig. 6). Initially, the usual D element of the anti-NP response, DFL16.1 (33), did not predominate in GC populations, but by day 16 postimmunization two-thirds of all recovered VDJ fragments contained this diversi-

ity segment (Table 2). And while newly formed \( \lambda^+ \) GC contained a majority of cells expressing \( V_\mu \) genes other than V186.2, later GC were almost exclusively populated with \( B \) lymphocytes expressing this canonical \( V_\mu \) segment (Fig. 7).

Surprisingly, the period of most intense selection on GC cell populations appeared between days 6 and 8 of the response. In this interval, the estimated average number of clones in individual GC fell from six to less than two (Fig. 6), DFL16.1 became favored (Table 2), and representation of \( V_\mu \) V186.2 in GC samples grew to nearly 100% (Fig. 7). This critical period is coincident with IgM \( \rightarrow \) IgG class switch in both foci and GC (6 and data not shown) and the initiation of somatic hypermutation (Fig. 2). The abruptness of this selection suggests that some factor(s) in addition to limiting antigen may act to reduce the diversity of the initial antigen-responsive GC B cell populations. A plausible candidate is the decreased density of mIg on \( PNA^+ \) GC cells. Shortly after entering the follicle, antigen-activated B cells lose mIgD expression, lowering the numbers of mIg available to interact with antigen almost 10-fold (51). Even after class switch, mIg levels of \( \text{PNA}^{hi} \) GC B cells remain \( \sim 1 \) log below that of \( \text{PNA}^{lo} \) B lymphocytes (52). George and Claffin (53) have demonstrated that an important effect of decreased mIg densities is a higher affinity threshold for cellular activation. A consequence of an increased affinity threshold would be the loss of initially responsive lymphocyte clones that expressed lower affinity antigen receptors (53). Such a physiologic restriction would neatly ensure that only those cells with the best starting affinities would later participate in mutation and selection within GC.

The relatively low frequency of the V816.2 \( V_\mu \) gene in early \( \lambda^+ \) GC populations was contrary to our expectation (24, 33). At days 4 and 6 of the response, some 65% of recovered VDJ fragments contained the closely related, analogue \( V_\mu \) genes C1H4, V23, CH10, V102, 24.8, and V165.2 (43). C1H4, V23, and CH10 were repeatedly isolated, accounting for 80% of the non-V186.2 genes sequenced. Although the frequency of recovered analogue \( V_\mu \) genes sharply declined by day 8 of the response, VDJ fragments containing \( V_\mu \) gene segments other than V186.2 were recovered from \( \lambda^+ \) GC as late as 16 d after immunization (Fig. 7). If these atypical \( V_\mu \) genes represent B cells actively participating in the early phases of the anti-NP response, selection plays a substantial role in the genetic restriction of \( N P \)-specific antibodies. Several lines of evidence suggest that analogue \( V_\mu \) genes do represent antigen-specific participants in the immune response; amplifications of unselected splenic B cells using the same 5' internal PCR primer used in this study do not generate equivalent patterns of recovered \( V_\mu \) genes (43); the most common analogues recovered, C1H4 and V23, can produce a (4-hydroxy-5-iodo-3-nitrophényl)acetyl (NIP)-binding phenotype in combination with the \( \lambda_4 \) L chain (54, 55); and a recent study of \( \lambda^+ \), \( N P \)-specific hybridomas recovered from C57BL/6 mice (56) also indicated that a significant number of \( V_\mu \) genes other than V186.2 are used in the primary response. Demonstration that at least some of the noncanonical \( V_\mu \) gene segments represent participants
in the anti-NP response comes from our expression of recovered VDJ fragments as complete H chains and subsequent transfection of the λL chain-producing cell line, J558L, with these constructs (our unpublished results). These studies have results confirmed that both CH4 and V23 may encode NIP-specific antibody, albeit of lower relative affinity than the canonical V186.2/DFL16.1 combination. None of the transfected antibodies studied have bound to the CG carrier protein (our unpublished results).

Another unexpected finding of this study was the absence of mutation in most VDJ fragments containing noncanonical, analogue Vα genes (Fig. 3). Of the 10 analogue VDJ clones recovered from λ+ GC at times after the onset of mutagenesis (Fig. 5), only 3 clonally related CH4 segments exhibited mutations in excess of expected polymerase error. In contrast, after day 6 of the response, no unmutated V186.2 gene was observed. Absence of mutation could simply reflect the restricted amplification of contaminant B cells. If, however, noncanonical VDJ fragments do represent GC cells participating in the anti-NP response (see above), the frequent absence of mutation suggests that dissociation of B cell proliferation in GC and Ig hypermutation is possible. Perhaps the threshold affinity required to induce B cell proliferation within GC lies below that needed to activate mutagenesis; the GC microenvironment may be necessary but not sufficient for somatic hypermutation.

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Note added in proof: Two recent reports support our observations and conclusions. Betz et al. (57) have also observed asymmetrical transversions in mutated κL chain transgenes and Lozano et al. (58) suggest that Ig density may be an important component of antigen-driven B cell selection.

References

1. Jacob, J., G. Kelsoe, K. Rajewsky, and U. Weiss. 1991. Intracellular generation of antibody mutants in germinal centres. *Nature (Lond)*. 354:389.

2. MacLennan, I.C.M., Y.J. Liu, J. Oldfield, J. Zhang, and P.J.L. Lane. 1990. The evolution of B-cell clones. *Curr. Top Microbiol. Immunol.* 159:37.

3. Nieuwenhuis, P., and D. Opstelten. 1984. Functional anatomy of germinal centers. *Am. J. Anat.* 170:421.

4. Jacob, J., R. Kassir, and G. Kelsoe. 1991. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)-acetyl. I. The architecture and dynamics of responding cell populations. *J. Exp. Med.* 173:1165.

5. Krose, M., A.S. Wubbena, H.G. Seijen, and P. Nieuwenhuis. 1988. The de novo generation of germinal centers is an oligoclonal process. *Adv. Exp. Med. Biol.* 237:245.

6. Jacob, J., and G. Kelsoe. 1992. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl)acetyl. II. A common clonal origin for PALS-associated foci and germinal centers. *J. Exp. Med.* 167:679.

7. Thorbecke, J.G., and F.J. Kreuning. 1956. Antibody and gamma globulin formation in vivo in hemopoietic organs. *J. Infect. Dis.* 98:157.

8. Sablitzky, F., D. Weisbaum, and K. Rajewsky. 1985. Sequence analysis of non-expressed immunoglobulin heavy chain loci in clonally related, somatically mutated hybridoma cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:3435.

9. Umar, A., P.A. Schweitzer, N.S. Levy, J.D. Gearhart, and P.J. Gearhart. 1991. Mutation in a reporter gene depends on proximity to and transcription of immunoglobulin variable transgenes. *Proc. Natl. Acad. Sci. USA.* 88:4902.

10. Clarke, S.H., K. Huppi, D. Ruefinksy, L. Staudt, W. Gerhard, and M. Weigert. 1985. Inter- and intra-clonal diversity in the antibody response to influenza hemaglutinin. *J. Exp. Med.* 161:687.

11. Berek, C., and C. Milstein. 1987. Mutation drift and repertoire shift in the maturation of the immune response. *Immunol. Rev.* 96:23.

12. Allen, D., A. Cumano, R. Dildrop, C. Kocks, K. Rajewsky, N. Rajewsky, J. Roes, F. Stablitzsky, and M. Siekevitz. 1987. Timing, genetic requirements and functional consequences of somatic hypermutation during B-cell development. *Immunol. Rev.* 96:5.

13. Manser, T., L. Wysocki, M.N. Margolies, and M.L. Gefter. 1987. Evolution of antibody variable region structure during the immune response. *Immunol. Rev.* 96:141.

14. Blier, P.R., and A.L.M. Bothwell. 1988. The immune response to the hapten NP in C57BL/6 mice: insights into the structure of the B-cell repertoire. *Immunol. Rev.* 105:27.

15. Lebecque, S.G., and P.J. Gearhart. 1990. Boundaries of somatic mutation in rearranged immunoglobulin genes: 5' boundary is near the promoter, and 3' boundary is approximately 1 kb from V(D)J gene. *J. Exp. Med.* 172:1717.
16. Golding, B.G., P.J. Gearhart, and B.W. Glickman. 1987. Patterns of somatic mutations in immunoglobulin variable genes. Genetics. 115:169.

17. Levy, N.S., U.V. Malipiero, S.G. Lebecque, and P.J. Gearhart. 1989. Early onset of somatic mutation in immunoglobulin V_{\text{\gamma}} genes during the primary immune response. J. Exp. Med. 169:2007.

18. Leanderson, T., E. Kallberg, and D. Gray. 1992. Expansion, selection and mutation of antigen-specific B cells in germinal centers. Immunol. Rev. 126:47.

19. Rogerson, B., J.J. Hackett, A. Peters, D. Haasch, and U. Storb. 1991. Mutation pattern of immunoglobulin transgenes is compatible with a model of somatic hypermutation in which targeting of the mutator is linked to the direction of DNA replication. EMBO (Eur. Mol. Biol. Organ.) J. 10:4331.

20. Both, G.W., L. Taylor, J.W. Pollard, and E.J. Steele. 1990. Distribution of mutations around rearranged heavy-chain antibody variable-regions genes. Mol. Cell. Biol. 10:5187.

21. Manser, T. 1990. The efficiency of antibody affinity maturation: can the rate of B-cell division be limiting? Immunol. Today. 11:305.

22. Steele, E.J., and J.W. Pollard. 1987. Hypothesis: somatic hypermutation by gene conversion via the error prone DNA-RNA-DNA information loop. Mol. Immunol. 24:667.

23. Maizels, N. 1989. Might gene conversion be the mechanism of somatic hypermutation of mammalian immunoglobulin genes? Trends Genet. 5:4.

24. Cumano, A., and K. Rajewsky. 1986. Clonal recruitment and somatic mutation in the generation of immunologic memory to the hapten NP. EMBO (Eur. Mol. Biol. Organ.) J. 5:2459.

25. Zelenetz, A.D., T.T. Chen, and R. Levy. 1992. Clonal expansion in follicular lymphoma occurs subsequent to antigenic selection. J. Exp. Med. 176:1137.

26. Berek, C., A. Berger, and M. Apel. 1991. Maturation of the immune response in germinal centers. Cell. 67:1121.

27. Shlomchik, M., M. Maccelli, H. Shan, M.Z. Radic, D. Pisetsky, A. Marshall-Rothstein, and M. Weigert. 1990. Anti-DNA antibodies from autoimmune mice arise by clonal expansion and somatic mutation. J. Exp. Med. 171:265.

28. Weiss, U., and K. Rajewsky. 1990. The repertoire of somatic antibody mutants accumulating in the memory compartment after primary immunization is restricted through affinity maturation and mirrors that expressed in the secondary response. J. Exp. Med. 172:1681.

29. Allen, D., T. Simon, F. Sablitzky, K. Rajewsky, and A. Cunamo. 1988. Antibody engineering for the analysis of affinity maturation of an anti-hapten response. EMBO (Eur. Mol. Biol. Organ.) J. 7:1995.

30. Kocks, C., and K. Rajewsky. 1988. Stepwise intraclonal maturation of antibody affinity through somatic hypermutation. Proc. Natl. Acad. Sci. USA. 85:8206.

31. Liu, Y.J., D.E. Joshua, G.T. Williams, C.A. Smith, J. Gordon, and I.C.M. MacLennan. 1989. Mechanism of antigen-driven selection in germinal centres. Nature (Lond.) 342:929.

32. Foote, J., and C. Milstein. 1991. Kinetic maturation of an immune response. Nature (Lond.) 352:530.

33. Bothwell, A.L.M., M. Paskind, M. Reth, T. Imanishi-Kari, K. Rajewsky, and D. Baltimore. 1981. Heavy chain variable region contribution to the NP family of antibodies: somatic mutation evident in a γ 2a variable region. Cell. 24:625.

34. Bothwell, A.L.M., M. Paskind, M. Reth, T. Imanishi-Kari, K. Rajewsky, and D. Baltimore. 1982. Somatic variants of murine immunoglobulin lambda light chains. Nature (Lond.) 298:380.

35. Liu, Y.J., J. Zhang, P.J.L. Lane, E.Y.T. Chan, and I.C.M. MacLennan. 1991. Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens. Eur. J. Immunol. 21:2951.

36. Gearhart, P.J., N.D. Johnson, R. Douglas, and L. Hood. 1981. IgG antibodies to phosphorycholine exhibit more diversity than their IgM counterparts. Nature (Lond.) 291:29.

37. Feeney, A.M. 1990. Lack of N regions in fetal and neonatal mouse immunoglobulin V-D-J sequences. J. Exp. Med. 172:1377.

38. Saiki, R.K., D.H. Gelfand, S. Stoffel, S.S. Scharff, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science (Wash. DC) 239:487.

39. Tindall, K.R., and T.A. Kunkel. 1986. Fidelity of DNA synthesis by Thermus aquaticus DNA polymerase. Biochemistry. 27:6008.

40. Li, W.-H., C.-I. Wu, and C.-C. Lo. 1984. Non randomness of point mutation as reflected in nucleotide substitutions in pseudogenes and its evolutionary implications. J. Mol. Evol. 21:58.

41. Weiss, U., R. Zoebelein, and K. Rajewsky. 1992. Accumulation of somatic mutants in the B cell compartment after primary immunization with a T cell dependent antigen. Eur. J. Immunol. 2:511.

42. Blier, P.R., and A.L.M. Bothwell. 1987. A limited number of Bcell lineages generate the heterogeneity of a secondary immune response. J. Immunol. 139:3996.

43. Gu, H., D. Tarlinton, W. Muller, K. Rajewsky, and I. Forster. 1991. Most peripheral B cells in mice are ligand selected. J. Exp. Med. 173:1557.

44. Schittek, B., and K. Rajewsky. 1992. Natural occurrence and origin of somatically mutated memory B cells in mice. J. Exp. Med. 176:427.

45. Schulz, A.R., A. Nirula, and J. Roth. 1989. Hybrid DNA artifact from PCR of closely related target sequences. Nucleic Acids. Res. 17:4409.

46. Weigert, M., I.M. Cesari, S.J. Yonkovich, and M. Cohn. 1970. Variability in the lambda light chains of mouse antibody. Nature (Lond.) 228:1045.

47. Rajewsky, K., I. Forster, and A. Cunamo. 1987. Evolutionary and somatic selection of the antibody repertoire in the mouse. Science (Wash. DC) 238:1088.

48. French, D.L., R. Laskov, and M.D. Scharff. 1989. The role of somatic hypermutation in the generation of antibody diversity. Science (Wash. DC) 244:1152.

49. MacLennan, I.C.M., and D. Gray. 1986. Antigen-driven selection of virgin and memory B cells. Immunol. Rev. 91:61.

50. Chen, C., V.A. Roberts, and M.B. Rittenberg. 1992. Generation and analysis of random point mutations in an antibody CDR2 sequence: many mutated antibodies lose their ability to bind antigen. J. Exp. Med. 176:855.

51. Butcher, E.C., R.V. Rouse, R.L. Coffman, C.N. Nottenberg, L.R. Hardy, and I.L. Weissman. 1982. Surface phenotype of peyer's patch germinal center calls: implications for the role of germinal centers in B cell differentiation. J. Immunol. 129:2698.

52. Havran, W.L., D.L. DiGiusto, and J.C. Cambier. 1984. mIgM: mIgD ratios on B cells: mean mIgD expression exceeds mIgM by 10-fold on most splenic B cells. J. Immunol. 132:1712.

53. George, J., and L. Claflin. 1992. Selection of B cell clones and
memory B cells. *Semin. Immunol.* 4:11.

54. Hawkins, R.E., and G. Winter. 1992. Cell selection strategies for making antibodies from variable gene libraries: trapping the memory pool. *Eur. J. Immunol.* 22:867.

55. McHeyzer-Williams, M.G., G.J. Nossal, and P.A. Lalor. 1991. Molecular characterization of single memory B cells. *Nature (Lond.)* 350:502.

56. Tao, W., F. Hardardottir, and A.L.M. Bothwell. 1993. Extensive somatic mutation in the Ig heavy chain V genes in a late primary anti-hapten immune response. *Mol. Immunol.* 30:593.

57. Betz, A.G., C. Rada, R. Pannel, C. Milstein, and M.S. Neuberger. 1993. Passenger transgenes reveal intrinsic specificity of the antibody hypermutation mechanism: clustering, polarity, and specific hotspots. *Proc. Natl. Acad. Sci. USA.* 90:2385.

58. Lozano, F., C. Rada, J.M. Jarvis, and C. Milstein. 1993. Affinity maturation leads to differential expression of multiple copies of a κ light-chain transgene. *Nature (Lond.)* 363:271.