

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),¹ Ig hypermutation and selection take place in germinal centers (GC) (1), specialized histologic structures of secondary lymphoid tissues (2–4). Splenic GC support oligoclonal 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 *Igh*- and *I* 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_H 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-

¹ Abbreviations used in this paper: CG, chicken gamma globulin; FW, framework region; GC, germinal center; NP, (4-hydroxy-3-nitrophenyl)acetyl; PALS, periarteriolar lymphoid sheath; PNA, peanut agglutinin.

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 antibody phenotype that is selected during antigen-driven competition.

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 *Igh^b* mice to NP is highly restricted, most primary anti-NP antibodies bear the $\lambda 1$ L chain, and the predominant H chain is encoded by the $V_H V186.2$, DFL16.1, and $J_H 2$ 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 $V186.2 V_H$ gene segment do not appear initially to dominate the antigen-induced, λ^+ GC populations. Instead, a majority of closely related V_H 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_H 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 μ g NP (Cambridge Research Biochemicals, Cambridge, UK) conjugated to chicken gamma globulin (CG; Accurate Chemical & Scientific Corp., Westbury, NY) and precipitated in alum (4). Cohorts of 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- μ m-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°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- λ L chain antibody (Southern Biotechnology As-

sociates, 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-aminocarbonyl carbazole and naphthol AS-MX phosphate/Fast Blue BB (Sigma Chemical Co., St. Louis, MO), respectively (4). Cells (~ 100) were recovered from λ^+ , 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 λ^+ , 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 $V186.2 V_H$ gene segment and to a region in the $J_H 2$ - $J_H 3$ intron (1, 28). 2 μ l 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 $J_H 2$ 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 pBlue-script SK+ (pBSK+; Stratagene Cloning Systems, La Jolla, CA) as described (1, 6). Competent DH5 α bacteria were transformed by electroporation and recombinant colonies screened with a ^{32}P -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 S107 V_H gene family in association with $J_H 1$ (36). HPCG-14 differs from TEPC-15 in the V region at positions 31 and 68 and in the CDR3 region (36). Rearranged $V1$ - $J_H 1$ 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 8–15, 5'ACGGATCCGGTGACCGTGGTCCCT3' (Kel-16), of the V1 gene segment and to the $J_H 1$ element, 5'GGAGGAAGCTTGGTACAAGCCTGGG3' (Kel-8). Kel-16 and Kel-8, respectively, contain recognition sequences for the HindIII and BamHI restriction endonucleases to facilitate cloning. After two rounds of 40 cycles of PCR amplification, amplified VDJ DNA was digested with HindIII and BamHI and cloned into pBSK+ as described above. Recombinant bacterial colonies containing TEPC-15 or HPCG-14 VDJ DNA were identified by colony hybridization using ^{32}P -labeled oligonucleotides specific for the TEPC-15 (5'TACTACGGTAGTAGCTAC3') or HPCG-14 (5'GTC-TACTATGGTTACGAC3') CDR3 regions. The specificity of hybridization was validated by sequencing selected colonies.

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 λ^+ GC. An average of 11.1 VDJ sequences (range, 8–23) were obtained for each GC population. 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 Artifactual 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, J_H2), 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 $3.0\text{--}3.7 \times 10^{-5}$ /bp per cycle, a value in excellent agreement with other reports (39). On average then, 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 artifactual 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

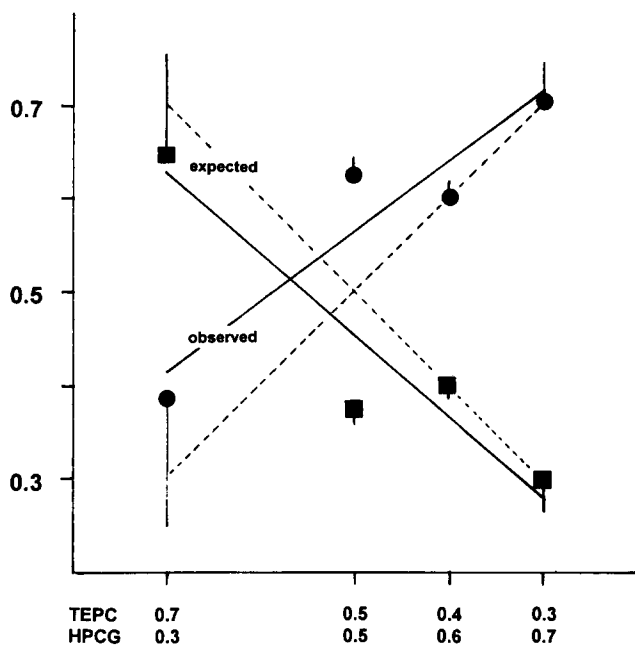


Figure 1. PCR of heterogeneous DNA population yields proportionate amplification. Two cloned hybridoma lines, TEPC-15 and HPCG-14, which use identical V_H and J_H gene segments that differ only by point mutations in CDR1 and FW3 and distinct CDR3 regions, were mixed in ratios 7:3 to 3:7. The VDJ segments of these mixtures were PCR amplified, cloned into plasmid vectors, and used to transform competent bacteria. Transformant colonies were screened for relative proportions of TEPC-15 and HPCG-14 VDJ regions by hybridization with ³²P-labeled CDR3-specific oligonucleotides. Specificity of the hybridization was confirmed by sequence analysis of representative clones. The observed and expected frequencies of VDJ segments for the mixed population are shown.

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 V_H and J_H 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 ($n = 172\text{--}258$) 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

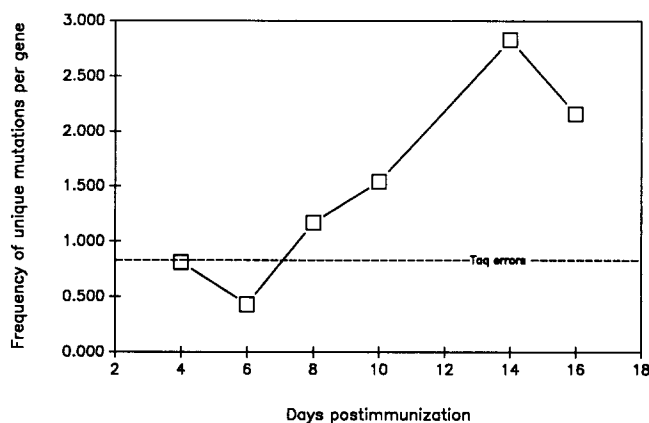


Figure 2. Kinetics of somatic mutations in GC of mice immunized with 50 μ g of NP-CG/alum. A total of 15 individual λ^+ GC were analyzed; 8–23 VDJ DNA clones were sequenced from each GC. Shared mutations within a single B cell clone, as judged by identical H chain CDR3 regions, were scored as one mutational event. The y-axis represents the frequency of unique somatic mutations per gene plotted against the time postimmunization. Dashed line represents the frequency of *Taq* polymerase error.

A

DAY 4 λ+ GERMINAL CENTER

H4	CDR1										CDR2												
	10	11	12	13	16	20	28	35	38	42	43	46	49	50	52	53	54	55	56	58	61	65	69
	GAG	CTT	GTG	AAG	GCT	CTG	ACC	CAC	AAG	CGA	CGA	GAG	GGG	AGG	GAT	AAT	AGT	GGT	GGT	AAG	GAG	ACC	CTG
1	--A	--G [†]	---	---	---	G-- [†]	---	---	---	--C [†]	-A- [†]	---	---	---	C-- [†]	TC- [†]	GA- [†]	A-- [†]	-A- [†]	--C [†]	C-A [†]	G-- [†]	T-- [†]
7	--A	--G [†]	---	---	--A	G-- [†]	---	---	---	--C [†]	-A- [†]	---	---	---	C-- [†]	TC- [†]	GA- [†]	A-- [†]	-A- [†]	--C [†]	C-A [†]	G-- [†]	T-- [†]
10	---	---	---	---	---	G-- [†]	---	---	---	--C [†]	-A- [†]	---	---	---	C-- [†]	TC- [†]	GA- [†]	A-- [†]	-A- [†]	--C [†]	C-A [†]	G-- [†]	T-- [†]
4	---	--G [°]	--A [°]	---	---	T-- [°]	--T [°]	---	---	---	-A- [°]	---	---	--T [°]	C-- [°]	---	---	---	A-- [°]	--C [°]	---	---	---
8	---	--G [°]	--A [°]	---	---	T-- [°]	--T [°]	---	---	---	-A- [°]	---	---	--T [°]	C-- [°]	---	---	---	A-- [°]	--C [°]	---	---	---
9	---	--G [°]	--A [°]	---	---	T-- [°]	--T [°]	---	---	---	-A- [°]	---	---	--T [°]	C-- [°]	---	---	---	A-- [°]	--C [°]	---	---	---
2	---	---	---	---	-G-	---	---	---	--A	---	-A- [°]	---	---	--T [°]	C-- [°]	---	---	---	A-- [°]	--C [°]	---	---	---
6	---	--G [°]	---	-G-	T-- [°]	---	---	--T [°]	---	AT- [°]	-A- [°]	--A [°]	--T [°]	-AC [°]	--C [°]	TC- [°]	GA- [°]	A-- [°]	-AA [°]	C-C [°]	C-A [°]	GA- [°]	T-- [°]

D-Region					D-Region								
74	76	81	91	94	TAC	TTT	GAG	TAC	TGG	CCC			
1	T-- [†]	--T	---	--C [†]	CC	CCT	ACT	ACG	GTA	CCC	CCG	TAC	CCC
7	T-- [†]	--T	---	--C [†]	CC	CCT	ACT	ACG	GTA	CCC	CCG	TAC	CCC
10	T-- [†]	---	---	--C [†]	CC	CCT	ACT	ACG	GTA	CCC	CCG	TAC	CCC
4	T-- [°]	---	--A [°]	--C [°]	---	CAT	CTC	TCC	TAC	---	---	---	---
8	T-- [°]	---	--A [°]	--C [°]	---	CAT	CTC	TCC	TAC	---	---	---	---
9	T-- [°]	---	--A [°]	--C [°]	---	CAT	CTC	TCC	TAC	---	---	---	---
2	T-- [°]	---	--A [°]	--C [°]	---	CAT	CTC	TCC	TAC	---	---	---	---
6	T-- [°]	---	---	--C [°]	---	GGG	CTC	---	---	---	---	---	---

B

DAY 6 λ+ GERMINAL CENTER

J5	CDR1										CDR2																		
	6	9	10	11	12	13	16	20	35	42	43	49	50	52	53	54	55	56	58	61	65	69	74	81	82	91	93	94	
	GAG	GCT	GAG	CTT	GTG	AAG	GCT	CTG	CAC	GGG	CGA	GGG	AGG	GAT	AAT	AGT	GGT	GGT	AAG	GAG	AGC	CTG	CCC	CAG	CTC	TAT	GGA	AGA	
1	---	A-- [†]	--A [†]	--G [†]	---	---	---	---	---	---	-A- [†]	---	--AT [†]	A- [†]	-GC [†]	-A- [†]	---	---	--C [†]	---	---	T-- [†]	---	---	---	---	--T	G-T	
2	A--	---	--A [†]	--G [†]	---	--G-	---	---	---	---	-A- [†]	---	--AT [†]	A- [†]	-GC [†]	-A- [†]	---	---	--C [†]	---	---	T-- [†]	---	---	---	---	---	---	
4	A--	A-- [†]	--A [†]	--G [†]	---	---	---	---	---	---	-A- [†]	---	--AT [†]	A- [†]	-GC [†]	-A- [†]	---	---	--C [†]	---	---	T-- [†]	---	---	---	---	---	---	
5	A--	-G-	---	--G [°]	--A [°]	---	---	T-- [°]	---	---	-A- [°]	---	--T [°]	C-- [°]	---	-GC [°]	-A- [°]	---	---	--C [°]	---	---	T-- [°]	--A [°]	---	--C	---	--G [°]	
7	---	---	---	--G [°]	---	-G- [°]	T-- [°]	---	--T [°]	AT- [°]	-A- [°]	--T [°]	-AC [°]	--C [°]	TC- [°]	GA- [°]	A-- [°]	-AA [°]	C-C [°]	C-A [°]	GA- [°]	T-- [°]	---	---	---	---	---	---	
3	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
8	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
9	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
10	---	---	---	---	---	---	A--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---

D-Region					D-Region										
1	2	4	5	7	TAC	TTT	GAG	TAC	TGG	CCC					
1	GGT	AAC	TTT	TTT	CC-	---	---	---	---	---					
2	GGT	TGT	AGC	---	CC-	---	---	---	---	---					
4	GGT	AGT	AGC	---	C--	---	---	---	---	---					
5	ACG	GGC	TAT	GAT	G--	---	---	---	---	---					
7	TOG	CCG	GCT	CAG	GCT	AGG	GAG	TAC	---	---	---	---	---	---	
3	GGT	GGA	GGG	---	---	---	---	---	---	---					
8	TOG	CCT	ATT	ACT	ACG	GTA	GTA	GCT	TAC	---	---	---	---	---	---
9	GAT	GGA	GGG	GGC	---	---	---	---	---	---					
10	TCA	GGG	GTA	GTA	GGC	---	---	---	---	---	---	---	---	---	---

C

DAY 8 λ+ GERMINAL CENTER

M16	CDR1							CDR2										
	9	10	13	20	23	25	33	40	59	60	62	68	73	81	82	83	85	86
	GCT	GAG	AAG	CTG	AAG	TCT	TGG	AGG	TAC	AAT	AAG	ACA	AAA	CAG	CTC	ACA	GAG	GAC
1	---	---	---	---	---	---	---	---	---	---	---	-T-	---	---	---	---	--A	---
2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	T--	T--	---	--A
3	-T-	---	---	---	---	---	---	---	C--	---	---	---	---	---	---	---	--A	---
4	---	A--	G--	T--	---	---	---	---	--C	---	---	G--	---	---	---	---	--A	---
5	---	---	---	---	---	---	--A	---	---	---	---	---	---	---	---	---	--A	---
6	---	---	---	---	---	C--	--A	---	---	---	---	---	---	---	---	---	--A	---
7	---	---	---	---	---	---	T--	---	---	---	-G-	---	---	---	---	---	--A	--G
8	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	--A	---
9	---	---	---	---	--A	---	---	---	---	---	---	---	---	---	---	---	--A	---
10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	--A	---
11	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	--G	--A	---
12	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-C-	---	--A	---

D-Region					D-Region						
92	TGT	TAT	TAC	TAC	TAC	TTT	GAG	TAC	TGG	CCC	
1	---	TAT	TAC	TAC	GGT	AGT	AGC	---	---	---	
2	---	TAT	TAC	TAC	GGT	AGT	AGC	---	---	---	
3	---	TAT	TAC	TAC	GGT	AGT	AGC	---	---	---	
4	---	TAT	TAC	TAC	GGT	AGT	AGC	---	---	---	
5	---	TAT	TAC	TAC	GGT	AGT	AGC	---	---	---	
6	---	TAT	TGC	TAC	GGT	AGT	AGC	---	---	---	
7	---	TAT	TAC	TAC	GGT	AGT	AGC	---	---	---	
8	---	TAT	TAT	TAC	GGT	AGT	AGC	---	---	---	
9	---	TAT	TAT	TAC	GGT	AGT	AGC	---	---	---	
10	---	TAT	TAT	TAC	GGT	AGT	AGC	---	---	---	
11	---	TAT	TAT	TAC	GGT	AGT	AGC	---	---	---	
12	-A-	TAT	TAT	TAC	GGT	AGT	AGC	---	---	---	

D

DAY 10 λ+ GERMINAL CENTER

B8	CDR1										CDR2															
	9	10	11	13	16	22	23	35	42	43	46	49	50	52	53	54	55	56	58	61	64	65	68	69	74	91
	<u>GCT</u>	<u>GAG</u>	<u>CTT</u>	<u>AAG</u>	<u>GCT</u>	<u>CTG</u>	<u>AAG</u>	<u>CAC</u>	<u>GGA</u>	<u>CGA</u>	<u>GAG</u>	<u>GGA</u>	<u>AGG</u>	<u>GAT</u>	<u>AAT</u>	<u>AGT</u>	<u>GGT</u>	<u>GGT</u>	<u>AAG</u>	<u>GAG</u>	<u>AAG</u>	<u>AGC</u>	<u>ACA</u>	<u>CTG</u>	<u>CCC</u>	<u>TAT</u>
1	---	---	---	---	---	A--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
2	---	---	---	---	---	A--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	G--	---	---
4	---	---	---	---	---	A--	G--	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-G-	---	-TG	---
10	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-T-	---
3	A--†	--A†	--G†	---	---	---	---	---	---	-A-†	---	---	-AT†	A--†	-GC†	-A-†	---	---	--C†	---	---	---	---	---	T--†	---
5	A--†	--A†	--G†	---	---	---	---	---	---	-A-†	---	---	-AT†	A--†	-GC†	-A-†	---	---	--C†	---	---	---	---	---	T--†	---
9	A--†	--A†	--G†	---	---	---	---	---	---	-A-†	---	---	-AT†	A--†	-GC†	-A-†	---	---	--C†	---	---	---	---	---	T--†	---
8	A--†	--A†	--G†	---	---	---	---	---	---	-A-†	---	---	-AT†	A--†	-GC†	-A-†	---	---	--C†	---	---	---	---	---	T--†	---
7	A--	---	--G-	-G-°	T--°	---	-G-	--T°	AT-°	-A-°	-GA	--T°	-AC°	--C°	TC-°	GA-°	A--°	-AA°	C-C°	C-A°	---	GA-°	---	T--°	T--°	---

D-Region

	TAC	TTT	GAC	TAC	TGG	CCC
GAA	TTC	CTA	TTA	CTA	GGG	TAG
GAA	TTC	CTA	TTA	CTA	GGG	TAG
	TAC	GAC	TAC	GGT	AGT	
	TAC	GAC	TAC	GGT	AGT	
	TAT	GAT	GGT	TAC	TAC	
	TAT	GAT	GGT	TAC	TAC	
		GAG	AGG	AGT	AGT	
AGT	CCG	CCG	CTT	GAT	GGT	TGG

E

DAY 14 λ+ GERMINAL CENTER

D2	CDR1										CDR2									
	11	14	18	19	21	22	27	28	29	30	31	32	38	56	57	58	65	71	76	
	<u>CTT</u>	<u>CGT</u>	<u>GTG</u>	<u>AAG</u>	<u>TCC</u>	<u>TCC</u>	<u>TAC</u>	<u>ACC</u>	<u>TTC</u>	<u>ACC</u>	<u>AGC</u>	<u>TAC</u>	<u>AAG</u>	<u>GGT</u>	<u>ACT</u>	<u>AAG</u>	<u>AGC</u>	<u>GTA</u>	<u>AGC</u>	
1	T--	-T-	---	-G-	---	---	--T	-T-	--T	-T-	--T	---	---	---	-G-	-G-	---	---	---	
2	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
3	---	---	---	---	---	---	---	---	---	---	--T	--T	---	---	---	---	---	---	A--	---
4	---	---	---	---	---	---	---	---	---	---	-A-	---	---	---	---	---	---	---	---	---
5	---	---	--A	---	---	---	---	---	---	---	-C-	---	---	---	---	---	---	---	---	---
6	---	---	--A	---	---	---	C--	---	---	---	-AT	A--	---	TT-	---	---	GA-	---	-C-	
7	T--	---	---	---	---	A--	C--	CA-	C-T	CA-	-AT	A--	---	---	---	---	---	---	---	
10	---	---	---	---	---	C--	---	---	---	---	-AT	A--	---	---	---	---	---	---	---	
8	---	---	-C-	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-C-	-A-	
9	---	---	---	---	C--	---	---	---	---	---	-A-	---	--A	---	---	---	-C-	---	-A-	

D-Region

	TAC	TTT	GAC	TAC	TGG	CCC
1	---	---	---	---	---	---
2	---	---	---	---	---	---
3	---	---	---	---	---	---
4	-T-	---	---	---	---	---
5	A--	---	T--	-G-	---	---
6	---	---	---	---	--C	---
7	---	---	---	---	---	---
10	---	---	---	---	---	---
8	---	---	---	---	---	---
9	---	---	---	---	---	---

F

DAY 16 λ+ GERMINAL CENTER

61AA02	CDR1					CDR2					D-Region							
	25	33	50	52	53	54	55	56	65	81	82a	85						
a	<u>TCI</u>	<u>TGG</u>	<u>AGG</u>	<u>GAT</u>	<u>AAT</u>	<u>AGT</u>	<u>GGT</u>	<u>GGT</u>	<u>AGC</u>	<u>CAG</u>	<u>AGC</u>	<u>CAG</u>	TAT	GAT	GGT	TAC	TAC	CCC
c	---	-T-	---	---	---	---	-T-	-T-	---	---	---	--A	TAT	GAT	GGT	TAC	TAC	CCC
d	---	-T-	---	---	---	---	-C-	-T-	---	---	---	--T	TAT	GAT	GGT	TAC	TAC	CCC
e	---	-T-	-AT†	A--†	-GC†	-A-†	---	---	---	---	---	--T	TAT	GAT	GGT	TAC	TAC	CCC
k	---	-T-	---	---	---	---	-T-	-T-	--G	T--	---	---	TAT	GAT	GGT	TAC	TAC	CCC
l	---	-T-	---	---	---	---	-T-	-T-	---	---	---	---	TAT	GAT	GGT	TAC	TAC	CCC
m	---	-T-	---	---	---	---	-T-	-T-	---	---	---	--A	TAT	GAT	GGT	TAC	TAC	CCC
o	--G	-T-	---	---	---	---	-T-	-T-	---	---	---	---	TAT	GAT	GGT	TAC	TAC	CCC

	TAC	TTT	GAC	TAC	TGG	CCC
a	---	---	---	---	---	---
c	---	---	---	---	---	---
d	---	---	---	---	---	---
e	---	---	---	---	---	---
k	---	---	---	---	---	---
l	---	---	---	---	---	---
m	---	---	---	---	---	---
o	---	---	---	---	---	---

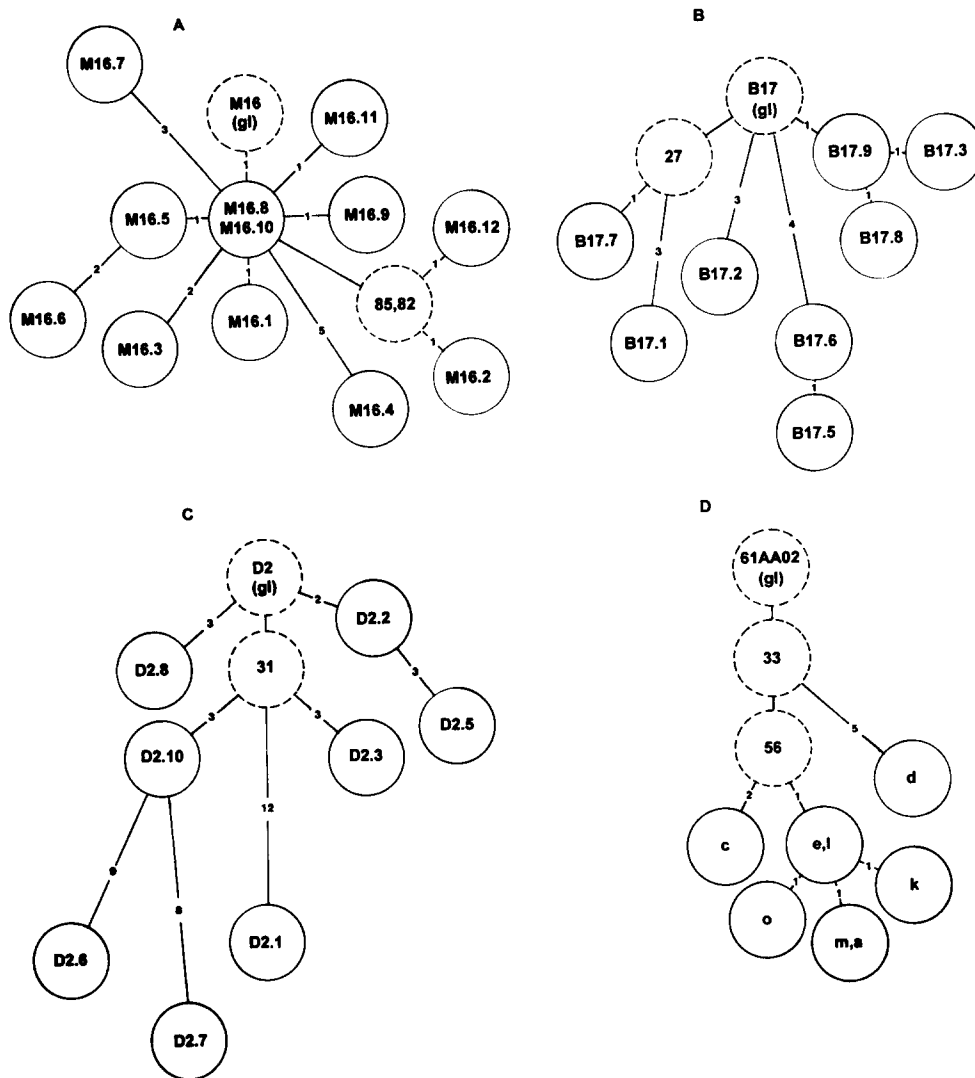


Figure 4. Genealogical relationship of VDJ DNA sequences derived from GC. Representative genealogies are shown, each typical of that time point (A, day 8; B, day 10; C, day 14; D, day 16) in the response. (gl) Germline; and (broken lines) hypothetical intermediates. Mutations are indicated by the codon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

(4), it is consistent with a mutation rate of $\sim 10^{-3}$ /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 J_H2 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_H gene segments. To distinguish these from mutants, codons identical to analogue V_H segments are indicated by: * C1H4, † V23, ° CH10, and § V102. (A) VDJ sequences recovered from the day 4 λ^+ GC, H4. Clones 1, 7, and 10 are V_H V102; 2, 4, 8, and 9 are V_H C1H4; and 6 is V_H CH10. (B) VDJ sequences from the day 6 λ^+ GC, J5. Clones 3, 8, 9, and 10 are V_H V186.2; 1, 2, 4, and 6 are V_H V23; 5 is V_H C1H4; and 7 is V_H CH10. (C) VDJ DNA sequences from the day 8 λ^+ GC, M16. All sequences use the V186.2 V_H gene segment. (D) Sequences from day 10 λ^+ GC, B8. Clones 1, 2, 4, and 10 are V_H V186.2; 3, 5, 8, and 9 are V_H V23; and 7 is V_H CH10. (E) VDJ sequences from the day 14 λ^+ 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. The 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/Gen Bank/DDBJ under accession numbers DS13953 and X67341-X67391.

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 unmutated 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_H V186.2 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., strand 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_H 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_H A → G transitions, the predominant error of the *Taq* polymerase (39). Indeed, VDJ DNA amplified from the antigen-activated but nonmutating cell populations of λ^+ 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

Table 1. Analysis of Mutations in GC B Cells

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
<u>Transversions</u>			
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
	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 $(O - E)^2/E$.

|| $\chi^2 = 77.2$; d.f. = 11; $p > 0.05$.

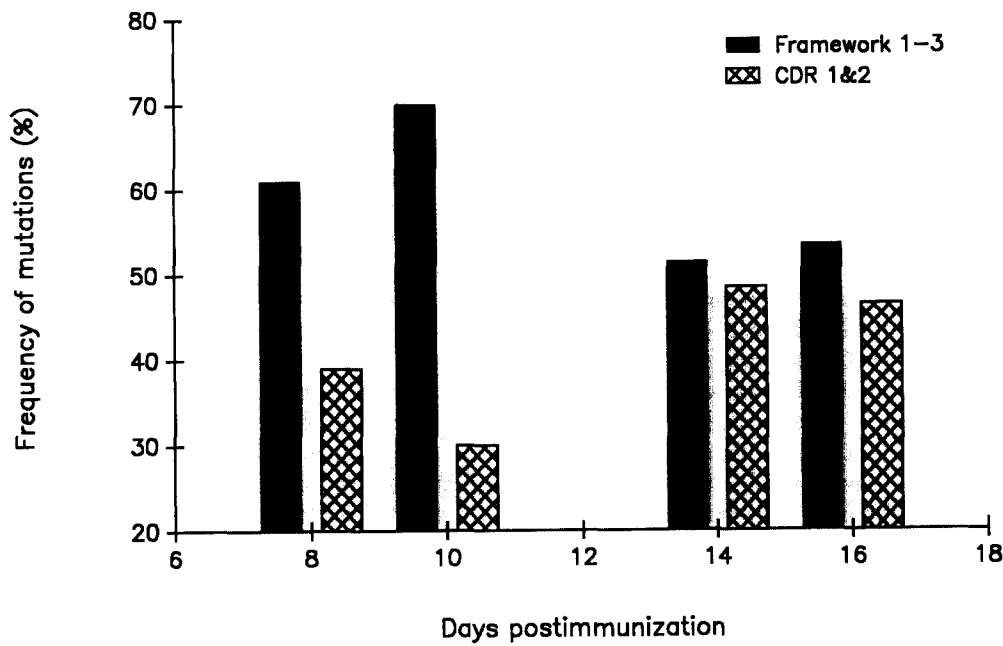


Figure 5. Distribution of somatic mutations in the GC Ig V regions of mice immunized 8–16 d earlier with 50 μ g NP-CG/alum. The frequencies of mutations that fall in FW 1–3 and CDR1 and -2 were determined. Data are presented as the mean frequency of somatic mutation in FW 1–3 (filled bars) and CDR1 and -2 (crosshatched bars) at days 8–16 postimmunization.

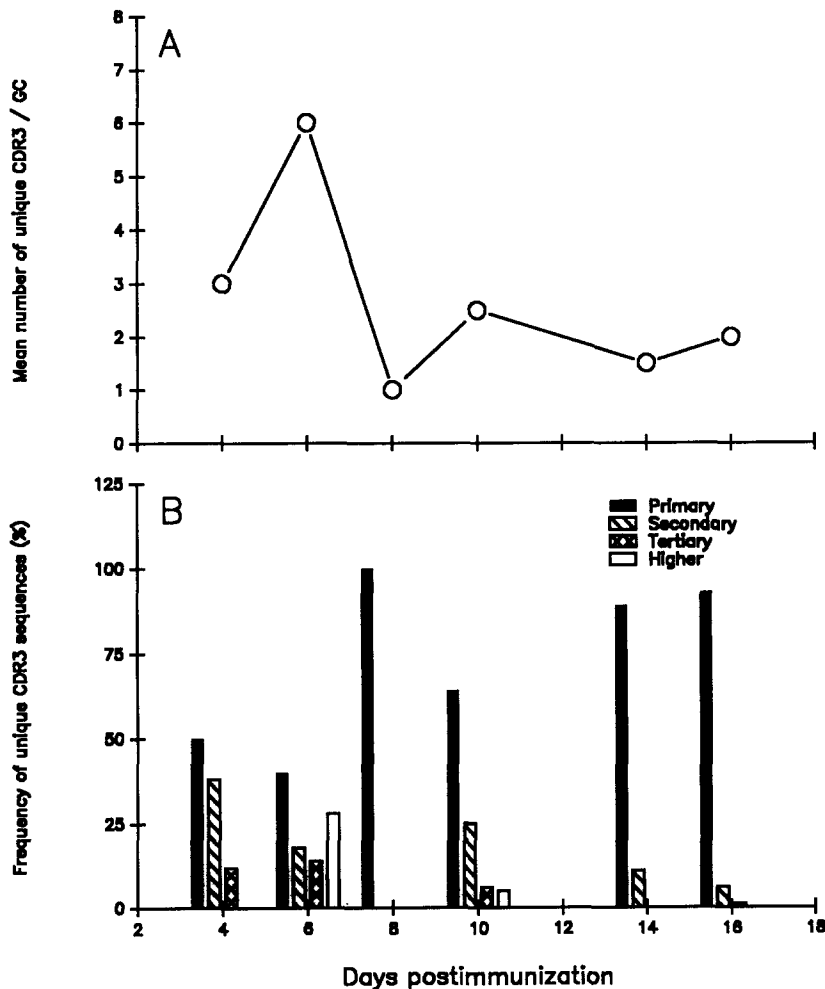


Figure 6. Estimated clonal diversity of individual GC decreases with time. (A) Clones within individual GC were identified as unique CDR3 sequences; the mean number of distinct CDR3 regions/GC is plotted against time postimmunization. (B) Dominance of single CDR3s (clones) within individual GC occurs 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 V_H 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-

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

Day	Frequency in recovered VDJ (percent of clones)				V186.2 DFL16.1
	All V _H				
	DFL16.1	DSP2	DQ52	Unidentified	
			%		
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	

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

V _H	Frequency in GC*	V _H frequency†	Percent total V _H	Percent analogue V _H
V186.2	15/16	154/178	87	-
C1H4	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	4

* Represents numbers of GC containing specific V_H/all GC studied.

† Represents numbers of VDJ fragments containing specific V_H/all VDJ fragments studied.

tinct 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_H 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 λ^+ 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_H V186.2 gene segments (see above) and most likely reflect antigen-driven selection. However, it is clear that multiple V186.2 + D_H associations generate NP-specific paratopes.

Alternate V_H Use in the Anti-NP Response. V_H gene segments other than V186.2 were isolated in this study of λ^+ 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 segment: C1H4, V23, CH10, V102, 24.8, and V165.1 (43) (Table 3). The C1H4, 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 $\mu^+\delta^+$ or

$\mu^-\delta^-$ (B220⁺) cells (43, 44) generated different patterns of analogue V_H genes. For example, while C1H4 constituted 32% of analogue V_H genes recovered from GC (Table 3), C1H4 represented only 6% of the VDJ rearrangements recovered from pre-B cells (44) and was absent in the sample generated from $\mu^+\delta^+$ B lymphocytes (43). The observed bias for C1H4 in λ^+ GC could represent recruitment by antigen. Second, although the probability of imprecise microdissection should remain constant, the frequency of non-V186.2 V_H 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_H genes recovered from GC. However, the frequency of analogue V_H 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_H genes in the λ^+ 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_H gene segments complement the $\lambda 1$ L chain to generate NP-specific antibody (our unpublished results).

Absence of Somatic Hypermutation in Analogue V_H Gene Segments. Even though evidence suggests that recovered analogue V_H genes represent cellular participants in the anti-NP response (see above), mutations in analogue V_H segments rarely exceeded that expected for the *Taq* 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_H genes appear competent to undergo Ig hypermutation; mutated analogue VDJ sequences were recovered from the day 10 GC,

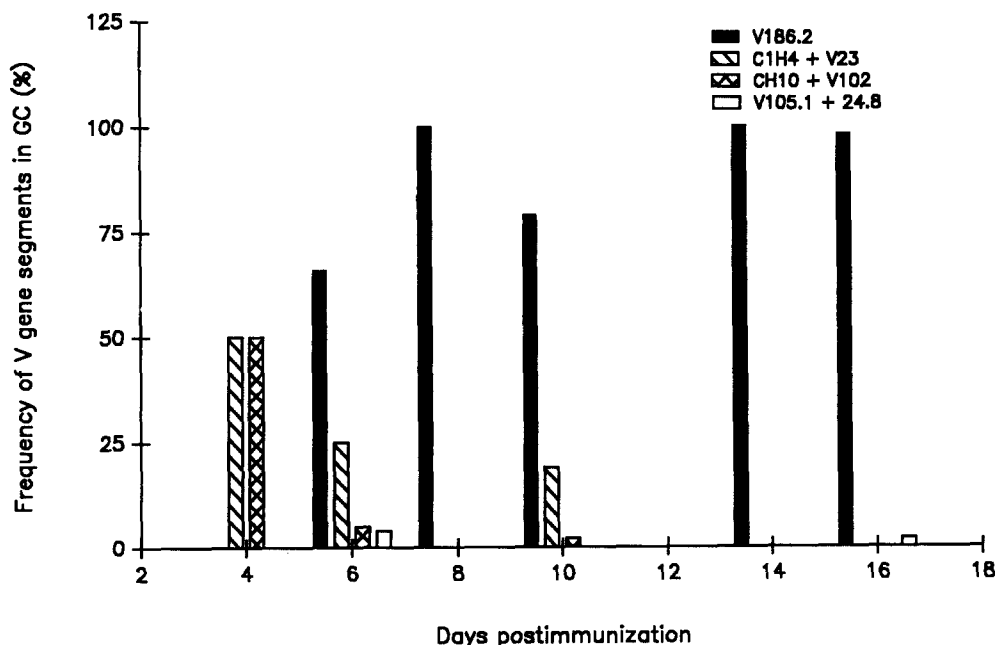


Figure 7. V_H gene use in the primary anti-NP response of C57BL/6 mice. The frequency of V gene segments isolated from GC is plotted against time postimmunization. (Filled bars) V186.2 V_H gene segment; (open, hatched, and crosshatched bars) frequencies of V186.2 analogues, closely related members of the J558 V_H gene family. The identity of the V186.2 analogues are indicated.

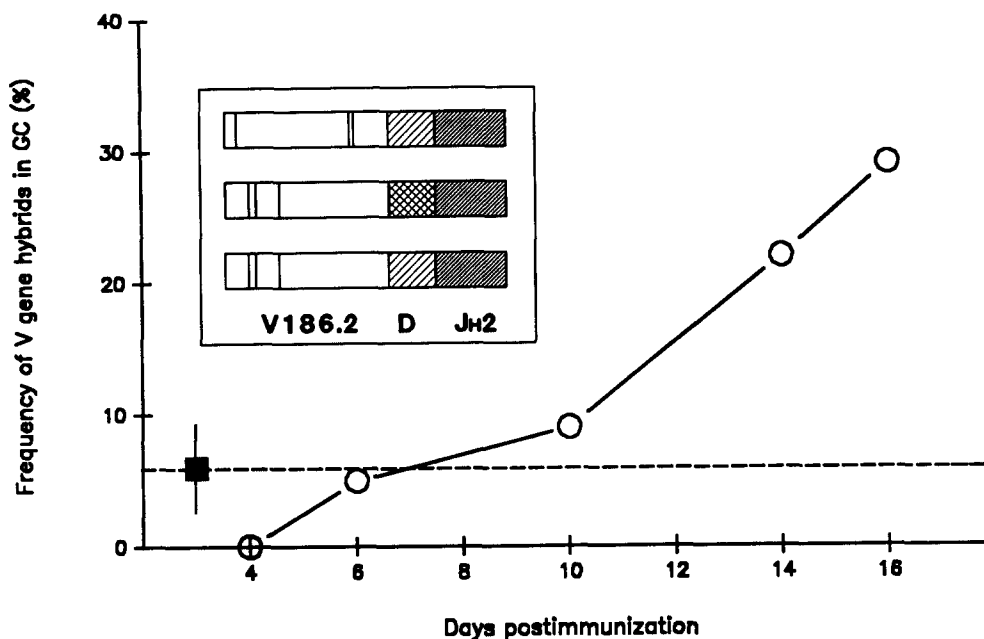


Figure 8. Hybrid gene formation in GC-derived VDJ DNA. Hybrid genes (*inset*) are formed by the fusion of different VDJ fragments during the PCR amplification of closely related DNA templates. Hybrid genes can be identified based upon mutations (*lines*) in the 5' end and 3' CDR3 sequences (*hatched* or *crosshatched*). Hybrid products can be distinguished when sequences contain the 5' region of one VDJ sequence and the 3' CDR3 region of another. The mean frequencies of hybrid genes produced were determined for GC derived at various times after immunization and plotted against time postimmunization. (*Dashed line*) Frequency of hybrid genes seen in focus-derived VDJ DNA and in the mixed hybridoma (Fig. 1) experiment.

GC B12. Six VDJ fragments containing V_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_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 ($\bar{x} \cong 6\%$) 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 ($\leq 6\%$). Then, coincident with the onset of somatic hypermutation and selection, hybrid frequencies increased linearly to $\sim 30\%$ 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 8 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 family trees in which most branch points were defined by one or two shared mutations. Although the error-prone (39) *Taq* polymerase will, on average, introduce approximately one misincorporation into each amplified VDJ fragment, these artifactual 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. 4 A). 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. 4 D).

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. 3 C; 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 FW 1-3 of mutated V186.2 V_H 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 amplifications by the *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 *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. 3), 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 diver-

sity segment (Table 2). And while newly formed λ^+ GC contained a majority of cells expressing V_H genes other than V186.2, later GC were almost exclusively populated with B lymphocytes expressing this canonical V_H 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_H 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 PNA^{hi} GC B cells remain \sim 1 log below that of 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_H gene in early λ^+ 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_H 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_H genes sharply declined by day 8 of the response, VDJ fragments containing V_H gene segments other than V186.2 were recovered from λ^+ GC as late as 16 d after immunization (Fig. 7). If these atypical V_H 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 NP-specific antibodies. Several lines of evidence suggest that analogue V_H 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_H genes (43); the most common analogues recovered, C1H4 and V23, can produce a (4-hydroxy-5-iodo-3-nitrophenyl)acetyl (NIP)-binding phenotype in combination with the λ_1 L chain (54, 55); and a recent study of λ^+ , NP-specific hybridomas recovered from C57BL/6 mice (56) also indicated that a significant number of V_H genes other than V186.2 are used in the primary response. Demonstration that at least some of the noncanonical V_H 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 λ_1 L chain-producing cell line, J558L, with these constructs (our unpublished results). These studies have results confirmed that both C1H4 and V23 may encode NIP-specific antibody, albeit of lower relative affinity than the canonical V186.2/DFL16.1 combination. None of the transfectant 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 V_H 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 C1H4 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 κ L chain transgenes and Lozano et al. (58) suggest that mIg density may be an important component of antigen-driven B cell selection.

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