

Facultative Role of Germinal Centers and T Cells in the Somatic Diversification of IgV_H Genes

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Summary

The development of memory B cells takes place in germinal centers (GC) of lymphoid follicles where antigen-driven lymphocytes undergo somatic hypermutation and affinity selection, presumably under the influence of helper T cells. However, the mechanisms that drive this complex response are not well understood. We explored the relationship between GC formation and the onset of hypermutation in response to the hapten phosphorylcholine (PC) coupled to antigenic proteins in mice bearing different frequencies of CD4⁺ T cells. PC-reactive GC were identified by staining frozen splenic sections with peanut agglutinin (PNA) and with monoclonal Abs against AB1-2, a dominant idiotope of T15⁺ anti-PC antibody. The nucleotide sequences of rearranged T15 V_H1 genes were determined from polymerase chain reaction amplifications of genomic DNA from microdissected GC B cells. T15⁺ GC became fully developed by day 6–7 after primary immunization of euthymic mice with either PC-keyhole limpet hemocyanin (KLH) or PC-chicken gamma globulin (CGG). Yet the V_H1 gene segments recovered from the primary GC as late as day 10–14 had low numbers of mutations, in contrast to responses to the haptens nitrophenyl or oxazolone that sustain high levels of hypermutation after GC formation. PC-reactive B cells proliferate in histologically typical GC for considerable periods with no or little somatic hypermutation; the signals for GC formation are independent of those for the activation of hypermutation. We then examined GC 7 d after secondary immunization with PC-KLH in euthymic mice, in *nu/nu* mice reconstituted with limited numbers of normal CD4⁺ cells before priming (CD4⁺-*nu/nu*) and in *nu/nu* mice. All of these animals develop T15⁺ GC after antigen priming, however, the patterns of V gene mutations in the secondary GC reflected the levels of CD4⁺ cells present during the primary response. VDJ sequences from secondary GC of euthymic mice were heavily mutated, but most of these mutations were shared among all related (identical VDJ joints) sequences suggesting the proliferation of mutated, memory B cells, with little *de novo* somatic hypermutation. In contrast, the patterns of V gene diversity in secondary GC from CD4⁺-*nu/nu* mice suggested that there was ongoing mutation and clonal diversification during the first week after rechallenge. The secondary GC from T cell-deficient, *nu/nu* mice showed little evidence for mutational and/or recombinational diversity of T15⁺ B cells. We conclude that the participation of CD4⁺ helper cells is required for full activation of the mutator in GC and takes place in a dose-dependent fashion.

Within secondary lymphoid tissues, two distinct cellular compartments, the Ab-forming cell (AFC)¹ foci and germinal centers (GC), participate in the primary Ab response; both are discrete, pauciclonal cell populations (reviewed in 1, 2). Foci are aggregates of plasmablasts and plasma cells at the periphery of the periarteriolar lymphatic sheaths and are the major source of early circulating Ab. GC

are well-defined structures in the lymphoid follicles that contain B cells undergoing antigen-driven proliferation and are the source of the affinity-selected mutant clones that dominate the memory response (3–5). B cells in GC are readily identified by avid binding of the lectin, PNA (6).

The somatic evolution of B cells has been studied intensively by analysis of hybridomas produced at different stages of the Ab response (reviewed in 7–9). Recently, it has become possible to recover antigen-specific B cells from individual GC and foci by microdissection, allowing direct analysis of their rearranged Ig V region genes (3, 10, 11). These studies, which examined primary Ab responses to the hapten, (4-hydroxy-3-nitrophenyl)acetyl (NP), showed that somatic hypermutation of V genes is confined to B cells within the

¹ Abbreviations used in this paper: AFC, antibody-forming cells; ALPH, alkaline phosphatase; Ars, *p*-azophenylarsonate; CGG, chicken gamma globulin; EPC, *p*-nitrophenyl-6-(*o*-phosphocholine)hydroxyhexanoate; FDC, follicular dendritic cell; GC, germinal center; Id, idiotope; NP, (4-hydroxy-3-nitrophenyl)acetyl; PC, phosphorylcholine; phOx, 2-phenyl-oxazolone; SA, streptavidin.

GC and that mutant VDJ sequences arose in all splenic GC analyzed by day 8 of the response (11). These results were elegantly confirmed by McHeyzer-Williams et al. (12) in studies on single, NP-specific/PNA-binding B cells. Similarly, using the hapten 2-phenyl-oxazolone (phOx), Berek et al. (5) readily found mutations in V genes among PNA-binding B cells derived from GC 10–12 d after immunization. Thus, it appears that the GC environment becomes competent to activate hypermutation in hapten-reactive B cells by the beginning of the second week of the primary response.

GC formation is a complex process that requires interactions of B cells with T cells (13–15), follicular dendritic cells (FDC) (16–18), antigen–Ab complexes (19), and complement (20–21). Further, it has been suggested that not all B cells are competent to enter the GC differentiation pathway (22). These considerations raise a number of questions regarding the relationship between the GC reaction and the initiation of somatic hypermutation. Two of these questions are addressed in the present study: (a) Is hypermutation constitutively active in all GC B cells? (b) How do T cells influence the formation of GC and IgV gene hypermutation? We have explored these issues using a well-characterized antigen, the phosphorylcholine hapten (PC) (23, 24). Abs specific for PC are encoded by the V_H1 segment of the V_HS107 gene family in combination with the DFL16.1 and J_H1 gene segments, and share the T15 idiotype (Id). A single immunization with PC-protein conjugates induces a strong GC response in the spleen (25) and primes the host for memory responses (23). PC-reactive GC are readily identified in frozen sections by staining with anti-Id (25), as in the case of the response to NP. Given the restricted V gene use and similar kinetics of Ab production and GC formation between the anti-PC and anti-NP responses, it seemed appropriate to investigate somatic hypermutation of the T15 genes of anti-PC Ab using the NP system as a reference point. Regarding the contribution of T cells, we have recently observed that T cell-deficient *nu/nu* mice immunized with PC-protein conjugates develop histologically normal GC in the spleen in the absence of a primary Ab response (25). This model has been used in the present study to delineate the relationship between GC formation and somatic hypermutation in the pathway to the B cell memory.

Materials and Methods

Mice. BALB/c Tac, C57BL/6 Tac, and BALB/c NTaC athymic (*nu/nu*) mice (all 2 mo of age) were purchased from Taconic Farms Inc. (Germantown, NY). All mice were maintained in a restricted animal room in sterile microisolator cages (Lab Products, Inc., Maywood, NJ) on a 12-h day/night cycle.

Antigens and Immunization. *p*-nitrophenyl-6(*o*-phosphocholine) hydroxyhexanoate (EPC) conjugated to KLH (Sigma Chemical Co., St. Louis, MO) according to Spande (26) (EPC-KLH) was kindly provided by Dr. James J. Kenny (National Cancer Institute, Frederick, MD). Preparation of PC conjugates of chicken gamma globulin (CGG) and BSA (both from Sigma Chemical Co.) was previously described (25). Conjugates of NP (Cambridge Research Biochemicals, Cambridge, UK) with KLH and BSA were also described previously (27). All antigens were administered as a single

intraperitoneal injection of 100 μ g of haptened protein in PBS on day 0 (primary response) and day 35 (secondary response). In one experiment, NP-KLH (100 μ g) was administered in alum (27).

Cohorts of mice were bled from the tail vein and killed by cervical dislocation at various intervals after primary or secondary immunization. Recovered spleens were processed either for AFC assay in suspension (see below) or for histology and DNA amplification (10, 25, 27). Serial, 6- μ m-thick frozen sections of spleen were cut in a cryostat microtome, thaw-mounted onto silanated glass slides (Digene Diagnostic, Inc., Beltsville, MD), air-dried for 20 min, fixed in acetone for 10 min, and stored at -70°C . In some experiments, about two thirds of each spleen was frozen and the remainder was used for AFC assay.

Lymphocyte Preparations and Cell Transfer. Splenocyte suspensions were prepared by teasing spleens in RPMI 1640 medium supplemented with 25 mM Hepes (GIBCO BRL, Gaithersburg, MD) and 0.5% BSA (Sigma Chemical Co.). T lymphocytes were depleted by two treatments with a cocktail of mAb H013-4 (anti-Thy 1.2), GK 1.5 (anti-CD4), and 3.155 (anti-CD8) from ascitic fluids (from the American Type Culture Collection, Rockville, MD) for 30 min at room temperature, followed by a pretested, normal rabbit serum as a source of complement for 40 min in a 37°C bath. The resulting B cell fraction contained <1% Thy 1.2-positive cells by FACS[®] analysis (Becton Dickinson & Co., Mountain View, CA).

T cell-enriched splenocyte populations were prepared by filtration through nylon wool columns (Wako BioProducts, Richmond, VA) using the manufacturer's protocol. Nonadherent cells were treated once with mAb 3.155 plus rabbit complement to eliminate CD8⁺ T cells. The resulting T cell fractions contained >80% of Thy 1.2⁺/CD4⁺ cells, <10% of sIg⁺ cells and <2% of CD8⁺ cells as determined by FACS[®] analysis.

Cells for adoptive transfer were resuspended in 0.5 ml of PBS containing 1% (vol/vol) of normal mouse serum and injected in the tail vein 16 h before immunization.

FACS[®] Analysis. Cells were incubated with biotinylated goat anti-mouse Ig (Fisher Biotech, Pittsburgh, PA) or with biotinylated anti-Thy 1.2 (Becton Dickinson & Co.) followed by staining with streptavidin-FITC (Fisher Biotech). The binding of mAb GK1.5 and 3.155 was visualized with goat anti-rat Ig-FITC conjugates (Fisher Biotech). Cells were analyzed by flow cytometry.

Serum Ab. Levels of PC-specific Ab were determined by standard ELISA techniques using PC-BSA as Ag in solid phase and goat anti-mouse (polyvalent) Ab labeled with β -galactosidase (Fisher Biotech) as probes. Serum Ab concentrations (μ g/ml) were determined by extrapolation from a standard curve.

Enumeration of AFCs. Splenocytes producing PC-specific Ab of different isotypes were enumerated by a modified ELISPOT assay (25). Spleen cells were suspended in RPMI 1640 medium with 5% FCS and distributed into 96-well, round bottom culture plates (Costar, Cambridge, MA) in triplicate aliquots of $1-3 \times 10^5$ cells in 100 μ l. Nitrocellulose filter sheets (0.45- μ m pore size; Schleicher & Schuell, Keene, NH) were coated with a solution of PC-BSA (100 μ g/ml in PBS) overnight at 4°C and then incubated in a blocking solution of 10% BSA in PBS for 2 h at 37°C . Filters coated with BSA were used as controls. Nitrocellulose filters were placed on the top of culture plates and held firmly in place with the lid using binder clips. Cells were transferred onto the nitrocellulose by turning the plate upside down and placing it in a 37°C incubator with 5% CO₂ for 4 h. Filters were then washed under tap water, treated with PBS-EDTA (10 mM) for 10 min and rinsed in PBS. Bound PC Ab "spots" were visualized by staining with biotin-labeled goat Abs to mouse IgM, IgG, and IgA followed by a streptavidin-alkaline phosphatase (SA-ALPH) conjugate (all from

Fisher Biotech) and a substrate (ProtoBlot[®], NBT/BCIP Color Development System, Promega Corp., Madison, WI). Spots were scored under a magnification of 15 and expressed as means from triplicates. Cells producing NP-specific Ab, which exhibit heteroclitic binding to the NP analogue, (4-hydroxy-5-iodo-3-nitrophenyl)acetyl (NIP), were enumerated on filters coated with a solution (100 µg/ml in PBS) of NIP-BSA conjugate (27); the procedure was otherwise the same as described for PC.

Immunohistochemistry. PC-specific B cells in situ were identified using the previously described (28) mAb AB1-2 with specificity for the T15 Id, which was conjugated with biotin-*N*-hydroxy succinamide (Vector Laboratories, Inc., Burlingame, CA), followed by SA-ALPH and nitroblue tetrazolium and 5-bromo-4-chloroindoylphosphate (Fisher Biotech) as substrate. Staining with AB1-2 overlaps with that of PC-BSA-biotin and splenic sections from mice immunized with antigens other than PC hapten do not stain with AB1-2, demonstrating the specificity of the AB1-2 probe for PC-binding B cells in situ (25, 29). GC B cells that bind PNA (PNA⁺) were visualized with PNA conjugated to horseradish peroxidase (HRP; E.Y. Laboratories, Inc., San Mateo, CA) and a substrate, 3-amino-9-ethyl-carbazole (Sigma Chemical Co.). FDC were detected with a rat mAb, FDC-M1 (a kind gift from Dr. Marie Kosco, Basel Institute for Immunology, Basel, Switzerland), followed by a goat anti-rat Ig Ab conjugated to the appropriate enzyme, ALPH or HRP (Fisher Biotech).

Frozen sections were rehydrated in PBS, blocked by incubation with PBS containing 10% BSA (1 h), incubated with the primary Ab (60 min), washed three times in PBS/BSA and incubated either with a secondary Ab-enzyme conjugate or with a SA-enzyme conjugate. The appropriate substrate was added according to the manufacturer's instructions.

Enumeration of GC and Isolation of B Cells. Splenic sections were double stained with AB1-2 and PNA as described above. GC were enumerated as PNA-stained areas within lymphoid follicles and the number was expressed as a percentage of all follicles in the section (mean from two sections). Cells (~100) were recovered from individual AB1-2⁺ and PNA⁺ GC using a sharpened micropipette controlled by an electrically powered micromanipulator (Narishige, Tokyo, Japan) as previously described (3, 10).

Amplification and Sequencing of VDJ DNA Recovered from Individual GC. Cellular material microdissected from single AB1-2⁺ PNA⁺ GC was incubated with proteinase K for 16 h at 37°C. The protease was subsequently heat inactivated (96°C, 10 min) and the crude cellular extract subjected to two rounds of PCR amplification using nested primers, as previously described (3, 10). The initial round of 40 amplification cycles used primers identical to those described by Feeny and Theuraf (S107 and J_H 1+4) (30). Internal primers were made complementary to codons 8–15, 5'ACGGATCCGGTGACCGTGGTCTCT3' (Kel-16) of the V1 segment and to the J_H1 element, 5'GGAGGAAGCTTGGTACAAGCCTGGG3' (Kel-8). Kel-16 and Kel-8 contain recognition sequences for the HindIII and BamHI restriction endonucleases, respectively. After two rounds of 40 cycles of PCR amplification, DNA was extracted in phenol-chloroform, precipitated in ethanol, digested with BamHI and HindIII (Boehringer Mannheim Biochemicals, Indianapolis, IN) and ligated into pBluescript SK⁺ (pBSK⁺) (Stratagene Cloning Systems, La Jolla, CA) as described (3, 10). Competent DH5α bacteria were transformed by electroporation and recombinant colonies screened with a ³²P-labeled oligonucleotide corresponding to positions 60–67 of the V1 gene segment. DNA from positive clones was sequenced as previously described (3, 10).

Frequency and Distribution of Mutations Introduced by the PCR. A Taq polymerase error rate of 3.5 × 10⁻⁵ misincorporations/

bp/PCR cycle was determined by sequencing 71 VDJ clones recovered from six independent amplifications of 50–200, acetone-fixed hybridoma cells (11 and not shown). This rate and the kinds of mutations observed (not shown) are typical of this error-prone polymerase (31, 32). On average, we observed 0.72 artifactual mutations/V_H1 gene segment (258 bp), a frequency nearly identical to that observed for the V186.2 exon (11).

Within a collection of cloned VDJ fragments, artifactual mutations follow the Poisson distribution and the expected numbers of V_H segments containing 0, 1, 2, . . . *n* polymerase errors may be calculated (12, and see Fig. 4). Thus, in the absence of any mutational activity in vivo, 48.6% of amplified V_H1 segments will contain 0 mutations, 35.1% will contain 1 exchange, and 12.7, 3.0, 0.5, and 0.1% will contain 2, 3, 4, and ≥5 Taq errors, respectively. Significant departure from this baseline distribution indicates true Ig hypermutation and is a more sensitive measure than comparison of average mutation frequencies.

Although GCs have been demonstrated to contain pauciclonal B cell populations (10), little junctional diversity is found in VDJ joints of primary, anti-PC B cells (33). For this reason, we analyze VDJ fragments recovered in the primary response only in reference to the GC from which they originated. VDJ joints show greater diversity in secondary responses and are informative with regard to clonal relationships.

Results

Ab Responses to the PC Hapten in Normal and nu/nu Mice. Features of PC-specific Ab responses in normal, euthymic mice to immunization with EPC-KLH are shown in Table 1 (group A). The rise of primary PC-specific serum Ab coincided with the appearance of splenic AFC that were predominantly IgM producers. In addition, the majority of the splenic lymphoid follicles (>60%, Table 1) contained well-developed, PNA⁺ GC that included cells expressing AB1-2, a major T15 Id of PC-binding Ab (Fig. 1 a). This staining was not due to the deposition of immune complexes in the network of the FDC, as demonstrated by dual staining of adjacent splenic sections with anti-Id and the FDC-specific mAb, FDC-M1 (Fig. 1 b). Under high magnification, Id⁺ lymphocytes could be clearly separated from FDC (Fig. 1 c). Similar results were obtained by immunization with PC-CGG (25, and Miller, C., and J. Stedra).

Secondary challenge of euthymic mice with EPC-KLH at 4 wk after priming resulted in a sixfold increase of serum Ab and an isotype switch of the splenic AFC from IgM to IgG and IgA (Table 1). All lymphoid follicles in these spleens were occupied by secondary PNA⁺ GC (Table 1) that contained cells expressing the AB1-2 Id (not shown).

The role of T helper cells in the GC formation and development of humoral memory in response to EPC-KLH was studied by adoptive cell transfer into nu/nu mice. Nu/nu mice reconstituted with normal, syngeneic CD4⁺ lymphocytes (Table 1, group B) produced lower levels of PC-specific Ab than euthymic controls, but the patterns of GC development, isotype switching, and memory response were similar in both groups. It should be noted that nu/nu recipients of 2 × 10⁷ splenic CD4⁺ lymphocytes had approximately fivefold fewer splenic Thy 1.2⁺/CD4⁺ cells than did euthymic controls (Fig. 2). T cell engraftment remained at these low levels for

Table 1. Characteristics of Primary (1°) and Secondary (2°) Ab Responses to EPC-KLH

Mice*	1° response			2° response			
	PC-binding serum Ab	AFC ratio† (IgG + IgA) / IgM	GC‡	Group	PC-binding serum Ab	AFC ratio† (IgG + IgA) / IgM	GC‡
A Euthymic	782 ± 298	0.120 ± 0.03	62 ± 10	A	3,500 ± 452	0.9 ± 0.2	79 ± 2
B <i>m/m</i>	162 ± 35	0.07 ± 0.02	67 ± 25	B	970 ± 130	1.2 ± 0.12	78 ± 4
C <i>m/m</i>	<50	nt	38 ± 15	C-1	<50	nt	64 ± 12
				C-2	720 ± 270	0.05 ± 0.01	84 ± 4
				(plus CD4+ cells**)			

* Four to six mice/group.

† PC-specific AFC of IgM, IgG, and IgA isotypes were enumerated in the spleen by ELISPOT assay as described in Materials and Methods, and the ratio between the (IgG + IgA) AFC and IgM AFC was calculated.

‡ GC were enumerated as PNA-stained areas within lymphoid follicles and the number was expressed as a percentage (%) of all follicles in the section.

|| Mean of group ± SD.

†† Animals received 2×10^7 splenic CD4+ lymphocytes (i.v.) from unimmunized, syngeneic donors 1 d before 1° immunization.

** Antigen-primed *m/m* mice received 2×10^7 splenic CD4+ lymphocytes (i.v.) from unimmunized syngeneic donors 1 d before 2° immunization.

nt, not tested.

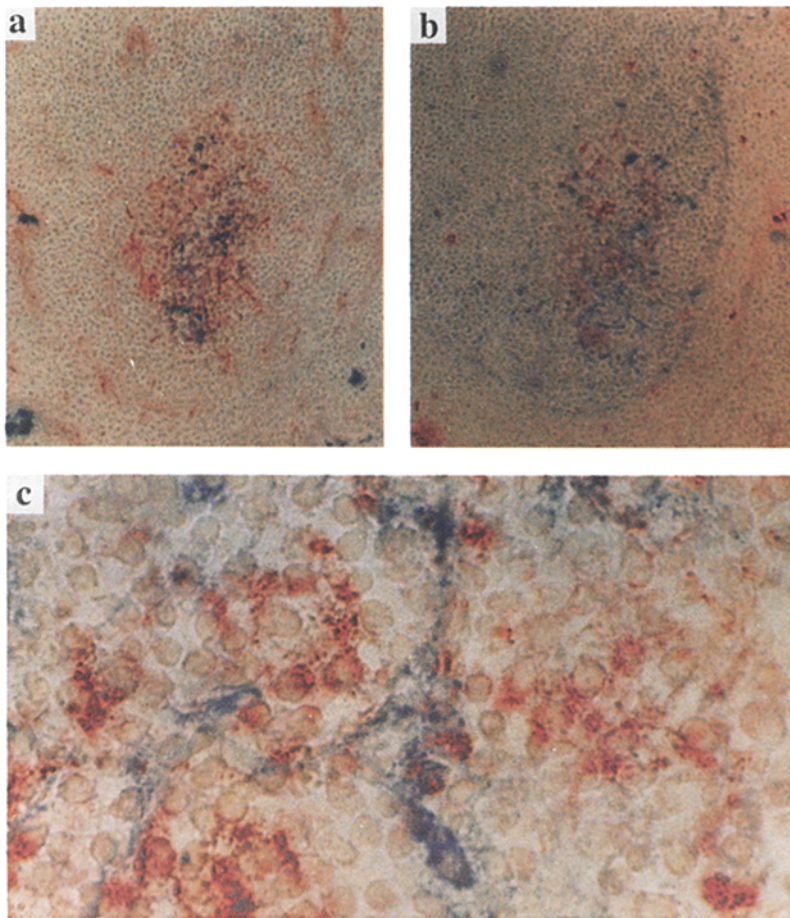


Figure 1. A splenic GC at day 7 after 1° immunization with EPC-KLH. Adjacent sections were stained (a) with PNA (red) and AB1-2 anti-Id (blue), and (b) with AB1-2 anti-Id (red) and the mAb FDC-M1 against FDC (blue). (c) Higher magnification ($\times 1,000$) of the GC from b. Note the distinct localization of Id⁺ lymphocytes (red) and FDC (blue).

the 35-d interval between antigen priming and challenge. This low T cell frequency may have produced the distinct pattern of somatic diversity of T15 V_H genes in these animals (see below).

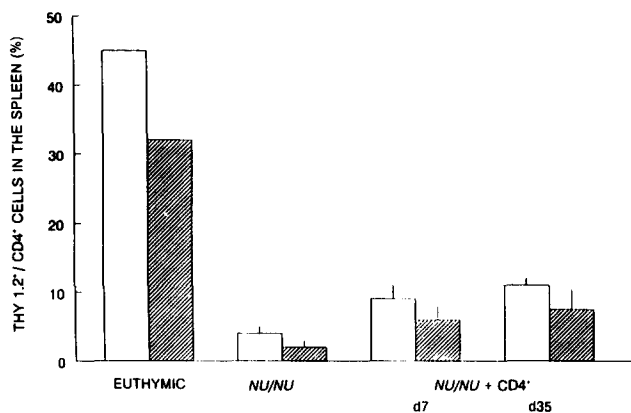


Figure 2. Relative numbers of T lymphocytes (percentage of nucleated cells) detectable with anti-Thy 1.2 (unshaded columns) or anti-CD4 (shaded columns) in the spleens of euthymic BALB/c mice (pool of three spleens), thymus-deficient (*nu/nu*) mice and *nu/nu* mice reconstituted with 2×10^7 normal syngeneic CD4⁺ T cells 7 or 35 d before the assay (mean from four animals \pm SD).

nu/nu mice that did not receive T cells failed to produce Ab to EPC-KLH, however, they did develop GC (Table 1, group C) containing antigen-specific, AB1-2 Id⁺ B cells, as reported in detail elsewhere (25). Moreover, these mice were primed for a vigorous anamnestic IgM response to EPC-KLH, which became apparent upon reconstitution of the animals with normal, unprimed CD4⁺ lymphocytes immediately before secondary challenge (Table 1, group C-2). Secondary Ab responses of the animals in group C-2 averaged sixfold higher than the primary responses of group B (CD4⁺-*nu/nu*) and were comparable to their secondary responses.

The humoral immunity elicited by immunogenic conjugates of PC is distinctive in that the IgM isotype dominates the primary anti-PC response in BALB/c (*Igh^a*) mice. In contrast, the response of C57BL/6 (*Igh^b*) mice to NP-CGG switches to IgG by day 7–8 after immunization (27). As shown in Table 2, the primary AFC responses of C57BL/6 mice to EPC-KLH and NP-KLH were mostly IgM and IgG, respectively, suggesting that the PC- and NP-reactive B cells maintain their characteristic properties for isotype switch regardless of the protein carrier and responder mouse strain.

T15 V_H Sequences from Primary GC of Euthymic Mice Contain Few or No Mutations. VDJ fragments were recovered from four AB1-2⁺/PNA⁺ GC after primary immunization of euthymic mice with either EPC-KLH (GC K.1 and K.2)

Table 2. *Ig Isotypes in Primary Ab Responses of C57BL/6 Mice to EPC-KLH and NP-KLH*

Antigen*	Hapten-specific AFC/10 ⁶ splenocytes [†]			Ratio IgG/IgM
	IgM	IgG	IgA	
EPC-KLH	344 ± 81 [§]	33 ± 5	25 ± 8	0.09
NP-KLH	37 ± 6	170 ± 24	<1	4.6
NP-KLH/Alum	501 ± 83	>1,500	14.2	>3.0

* C57BL/6 mice (three to four/group) were immunized with 100 µg i.p. of indicated antigen.

† AFC were enumerated by hapten-specific ELISPOT assay on day 7 after the primary immunization as described in Materials and Methods.

§ Mean/group ± SD.

|| The count was uniformly >150 spots/10⁵ splenocytes plated.

or PC-CGG (GC C5.1 and C8.1) (Figs. 3 and 4). The K.1 and K.2 GC were taken from different animals at day 10 and day 12, respectively, after immunization; the C5.1 and C8.1 GC were dissected from different sites in the same spleen at

day 12. From each GC, 10 sequences (258 bp) of rearranged V_H1J_H1 DNA segments were compared with the canonical TEPC15 VDJ sequence and nucleotide substitutions in V_H (to codon 95) were analyzed (Fig. 3).

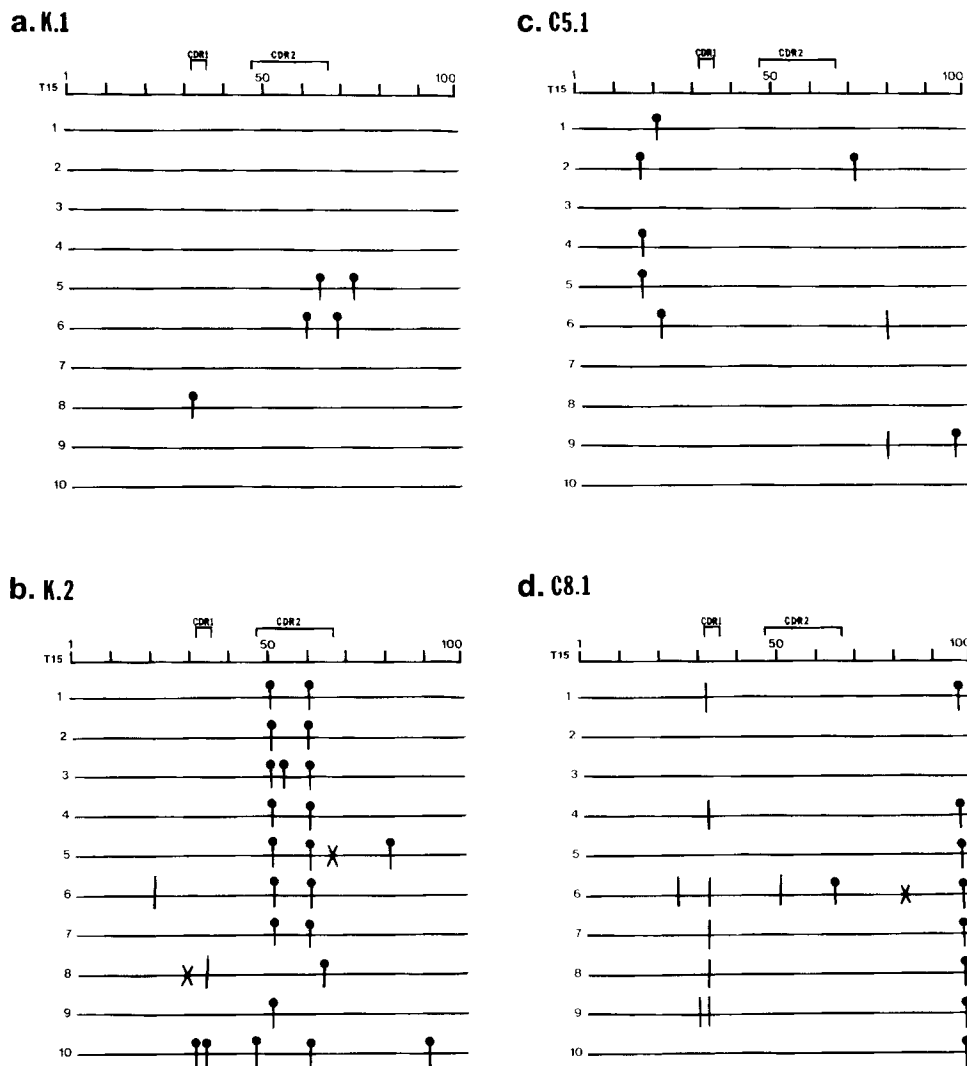


Figure 3. Comparison of rearranged V_H1 (T15) segments from splenic GC after 1° immunization of euthymic mice with EPC-KLH (a and b) and PC-CGG (c and d). Four individual GC were sampled at the following intervals after the immunization: (a) K.1, day 10; (b) K.2, day 12; (c) C5.1; and (d) C8.1, day 12. Vertical bars show the position of codons containing nucleotide differences between the clone and the germline V_H1 (T15) sequence. A black circle on the top of a bar represents a replacement mutation, no circle indicates a silent mutation, and crossed bars show stop codons.

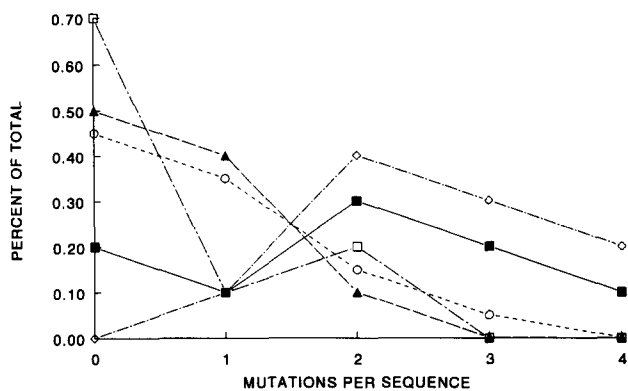


Figure 4. Distribution of mutations among the cloned V_H sequences from GC C5.1 (▲), C8.1 (■), K1 (□), and K2 (◇) as compared with the expected, normal distribution of artifactual mutations due to *Taq* polymerase errors (○).

On average, each 258-bp V_H1 exon rescued from a GC by PCR amplification is expected to contain about one mutation due to *Taq* polymerase error (1/330; see Materials and Methods). The mutation frequencies observed in three of the primary GC were within the range of polymerase error: K.1 = 1/660 bp, C5.1 = 1/375 bp, and C8.1 = 1/330 bp, suggesting that these B cells did not undergo hypermutation *in vivo*. In contrast, the K.2 sequences contained a significant excess of misincorporations (1/200) indicating Ig somatic hypermutation.

A more rigorous test for active hypermutation is analysis of the distribution of mutations observed among VDJ clones (Fig. 4) (see Materials and Methods). When this test was applied to the V_H1 segments recovered from primary GC, we found that the K.1 and C5.1 sequences contained distributions of mutations not significantly different from that expected from polymerase error. However, despite the low frequency of mutations in the C8.1 clones, these sequences contained excess multiple substitutions. Therefore, GC C8.1 appears to have supported a low level of active hypermutation.

However, both GC exhibiting signs of Ig hypermutation

K.2 and C8.1, contain frequent shared mutations, a pattern typical of secondary responses to PC (see below). Thus it is possible that the K.2 and C8.1 GC were founded by secondary B cell precursors stimulated and mutated by encounter with environmental antigens (34). We cannot rule out that some mutations occurred by day 12 of the primary response; if they did occur, however, their frequency (pooled average = 1/391) is considerably below that observed in other primary antihapten responses (7, 8, 10, 11, 35).

Hypermutation in T15 V_H Sequences from Secondary GC: Correlation with T Lymphocytes. Secondary responses to EPC-KLH were elicited by restimulation at 35 d after priming in one animal from experimental groups A (euthymic), B ($CD4^+$ cell-reconstituted *nu/nu*) and C-2 (*nu/nu*) (Table 2). Two GC were dissected from separate sites of each spleen on day 7 after secondary immunization. The names and origin of the recovered DNA clones and the main experimental findings are schematized in Table 3.

The VDJ gene sequences that were amplified from the secondary GC of an euthymic mouse, E403 and E409, represented heavily mutated V_H1 segments with mutation frequencies of 1/121 and 1/48, respectively (Fig. 5 a). Surprisingly, most of these mutations were shared among all related sequences from individual GC, suggesting the proliferation of previously mutated memory B cells rather than ongoing somatic hypermutation within the secondary GC. The clonal relationship of B cells in these GC was further apparent from the identity of the CDR3 sequences (Fig. 6 a). Despite their extensive somatic diversity, sequences from both E403 and E409 GC had the canonical V_H1 /DFL16.1 T15 joints between Asp in position 95 and Tyr in 96 which have been previously identified as key structural elements for the formation of PC-binding Abs (33, 36). However, the remaining D_H in E403 and E409 sequences could represent either somatically altered DFL16.1 genes or other, unidentified D_H segments.

The VDJ fragments recovered from the NT01 and NT02 (Fig. 5 b) secondary GC of a *nu/nu* mouse reconstituted with $CD4^+$ lymphocytes ($CD4^+$ -*nu/nu*), were very different from those (E403 and E409) recovered from euthymic controls.

Table 3. Summary of Secondary GC

Experimental group*	GC designation	Mutation Frequency (per bp)	V/D junctions	D_H
A Euthymic	E403	1/121	T15 (D95/Y96)	Unknown
	E409	1/48 (shared mutations)	T15 (D95/Y96)	(mutated?)
B <i>nu/nu</i> + $CD4^+$ T Cells	NT01	1/238	T15 (D95/Y96)	DFL6.1
	NT02	1/188	Diverse	Unknown
C <i>nu/nu</i> †	N03	1/258	T15 (D95/Y96)	DFL6.1
	N04	1/372	Diverse	DFL6.1

* See Table 1.

† Antigen-stimulated *nu/nu* mice received unprimed $CD4^+$ cells 1 d before Ag challenge.

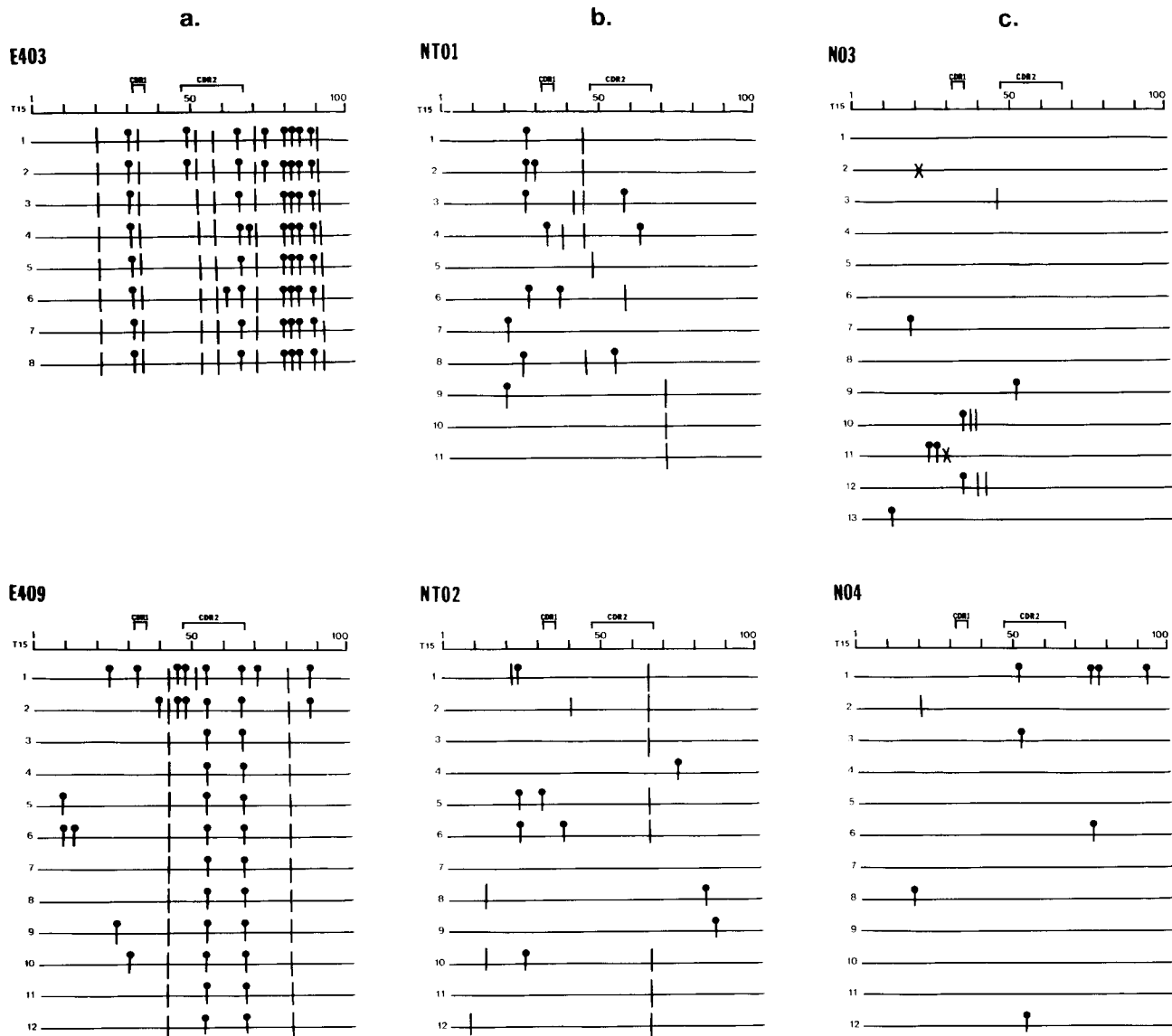


Figure 5. Comparison of rearranged V_H (T15) segments cloned from GC after 2° immunization with EPC-KLH. Mice were primed with EPC-KLH, challenged 35 d later, and sampled on day 7 after challenge. (a) GC E403 and E409 from a euthymic mouse; (b) GC NT01 and NT02 from a *nu/nu* mouse reconstituted with normal $CD4^+$ T cells before priming (Table 1, group B); (c) GC N03 and N04 from *nu/nu* mice that were primed without a T cell supplement (Table 1, group C). Nucleotide substitutions are marked as in Fig. 3.

Although the NT01 and NT02 sequences had significant numbers of excess mutations (1/188 and 1/238, respectively) nucleotide substitutions were not typically shared among clonally related genes. Indeed, several sequences from each GC could be readily arranged into genealogical trees (Fig. 7), indicating active mutational clonal diversification during the first week of the secondary response. It should be remembered that the $CD4^+$ -*nu/nu* mice had much lower numbers of T cells in the spleen compared to the euthymic mice (Fig. 2), which was reflected in less robust primary Ab responses (Table 1, groups B vs. A).

As shown in Fig. 6 *b*, the NT01 CDR3 sequences represent the canonical $V_H1/DFL16.1/J_H1$ gene rearrangement. In

contrast, GC NT02 contained three distinct CDR3 sequences: (a) canonical T15 joints (nos. 6, 9–11), (b) V_H1 joined to an unknown D_H segment (nos. 1–5, 8), and (c) a sequence (no. 7), showing an alternative non-T15 junction between V_H1 and DFL16.1 with Asp → Val in position 95, followed by Asp and Tyr. This junction has been found previously in hybridomas generated from secondary responses to PC coupled to a protein carrier (33).

We were particularly interested in sampling the V gene repertoire of secondary GC from the C-2 experimental group of *nu/nu* mice (Table 1), because these T cell-deficient mice developed hapten-reactive GC after primary immunization with EPC-KLH without any detectable Ab response. They

		94	95	96	97	98	99	100	100A	B	C	D	101	102
		R	D	Y	Y	G	S	S	Y	W	Y	F	D	V
a.	E403	1	-	-	-	D	N	R	N	-	-	-	-	-
		2	-	-	-	D	N	R	N	-	-	-	-	-
		3	-	-	-	H	N	T	N	-	-	-	-	-
		4	-	-	-	H	N	T	N	-	-	-	-	-
		5	-	-	-	H	N	T	N	-	-	-	-	-
		6	-	-	-	H	N	T	N	-	-	-	-	-
		7	-	-	-	H	N	T	N	-	-	-	-	-
		8	-	-	-	H	N	T	N	-	-	-	-	-
b.	NT01	1	-	-	-	-	-	-	Y	W	-	-	-	-
		2	-	-	-	-	-	-	-	-	-	-	-	-
		3	-	-	-	-	-	-	-	-	-	-	-	-
		4	-	-	-	-	-	-	-	-	-	-	-	-
		5	-	-	-	-	-	-	-	-	-	-	-	-
		6	-	-	-	-	-	-	-	-	-	-	-	-
		7	-	-	-	-	-	-	-	-	-	-	-	-
		8	-	-	-	-	-	-	-	-	-	-	-	-
		9	-	-	-	-	-	-	-	-	-	-	-	-
		10	-	-	-	-	-	-	-	-	-	-	-	-
		11	-	-	-	-	-	-	-	-	-	-	-	-
		12	-	-	-	-	-	-	-	-	-	-	-	-
c.	NO3	1	-	-	-	-	-	-	-	-	-	-	-	-
		2	-	-	-	-	-	-	-	-	-	-	-	-
		3	-	-	-	-	-	-	-	-	-	-	-	-
		4	-	-	-	-	-	-	-	-	-	-	-	-
		5	-	-	-	-	-	-	-	-	-	-	-	-
		8	-	-	-	-	-	-	-	-	-	-	-	-
		10	-	-	-	-	-	-	-	-	-	-	-	-
		11	-	-	-	-	-	-	-	-	-	-	-	-
		12	-	-	-	-	-	-	-	-	-	-	-	-
		13	-	-	-	-	-	-	-	-	-	-	-	-
		c.	NO4	1	-	-	-	-	-	-	-	-	-	-
2	-			-	A	-	-	-	-	-	G	-	-	-
3	-			-	-	-	-	-	-	-	-	-	-	-
4	-			-	D	-	-	-	-	-	-	-	-	-
5	-			-	-	-	-	-	-	-	-	-	-	-
6	-			-	A	-	-	-	-	-	G	-	-	-
7	-			-	-	-	-	-	-	-	-	-	-	-
8	-			-	-	-	-	-	-	-	-	-	-	-
11	-			-	-	-	-	-	-	-	-	-	-	-
12	-			-	-	-	-	-	-	-	-	-	-	-
13	-			-	-	-	-	-	-	-	-	-	-	-

Figure 6. Amino acid sequences of CDR3 regions of VDJ segments recovered from 2° GC (Fig. 5) compared with the canonical T15 CDR3 region. Amino acids are identified by single-letter code: Ala = A; Asp = D; Val = V; Tyr = Y. These sequence data are available from EMBL/GenBank/DDBJ under accession number U20978.

received normal, unprimed CD4⁺ cells, 1 d before challenge and the secondary GC were sampled on day 7 (Fig. 5 c). Interestingly, the V_H1 sequences from one (N03) of the two GC analyzed demonstrated a frequency of mutations, 1/258, marginally greater than could be accounted for by Taq error; three sequences (nos. 10–12) contained three mutations each. Nonetheless, the frequency and distribution of multiple mutations in these animals are very similar to those observed in the K1 and C5.1 primary GC of euthymic mice (Fig. 4). All CDR3 segments from GC N03 resulted from canonical T15 (V_H/DFL16.1/J_H1) gene rearrangements (Fig. 6 c). The second GC, NO4, had an insignificant mutation frequency

of 1/372, however, three sequences from this GC showed V/D joint diversity with addition of Ala or Asp between positions 95 and 96 (Fig. 6 c). Such V/D diversity is a hallmark of secondary responses to PC-protein conjugates (33). Thus, these GC show signs of mutational and/or recombinational diversity that are associated with an anamnestic anti-PC response even though the antigen priming was initiated in T cell-deficient mice.

Discussion

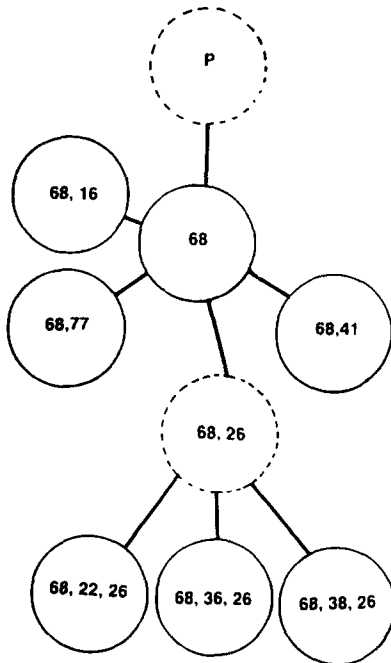
Paucity of Mutations in Primary PC-reactive GC. We found few mutations in the GC B cells by day 12 of primary responses to PC-protein conjugates. When mutations were observed, their pattern suggested that the GC may have contained B cells that had been sensitized by prior environmental encounters with PC, an antigenic determinant of common bacteria such as *Streptococcus pneumoniae* or *Proteus morganii* (37, 38).

This is in sharp contrast to other hapten-protein systems where the onset of IgV gene mutation in vivo has been reported to be as early as day 7 after primary immunization (11, 12, 39); intensive hypermutational activity has been observed 10 d after immunization with protein conjugates of oxazolone (7), Ars (8), and NP (10, 11, 35) haptens. Detailed studies by Jacob et al. (10, 11) found that all GC between days 10 and 12 after immunization with either NP-CGG or NP-KLH contained B cell clones with mutated IgV_H genes. Thus, unlike NP-reactive B cells, PC-specific B cells may proliferate in histologically typical GC for some time with little or no somatic hypermutation. This period may last for a week or more, considering that fully developed GC occupy the majority of splenic lymphatic follicles by day 6–7 after immunization (Table 1 and 25). Since the protein carriers used here were the same used in studies that found early Ig mutation, it seems probable that the impoverished mutation reflects intrinsic differences between the NP- and PC-reactive B cells rather than insufficient T cell help. Our findings are consistent with the report by Levy et al. (39) who observed extremely low mutation frequencies (1/2,500 bp) among 40 T15 V gene sequences recovered from mouse splenic cDNA libraries between day 5 and 13 after primary immunization with PC-KLH.

PC-reactive B cells employing the T15 V_H1 gene clearly are capable of initiating Ig hypermutation, but it appears that the requirements to activate this mechanism may not be present in all anti-PC responses. For example, Claffin et al. (40–42) studied the somatic diversification of the V_H1 gene paired with different V_K genes in response to the PC hapten expressed on different bacteria. They found only a modest degree of somatic mutation in ~50% of V_H1/V_K22 hybridomas generated after repeated immunizations with *S. pneumoniae* PC (40). In contrast, they found high levels of mutation in V_H1/V_K8 cells after a single immunization with *P. morganii* PC (41, 42), suggesting that an element of *P. morganii* plays an important role in activation of the mutation mechanism.

The origin of PC-reactive, T15⁺ B cells is a controversial matter in the literature. Two groups of investigators, using

a. NT01



b. NT02

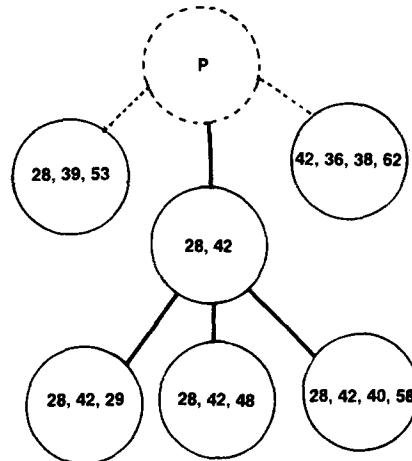


Figure 7. Genealogical relationships of V_H DNA sequences derived from 2° GC NT01 (a) NT02 (b) (from Fig. 5). Mutations are indicated by the codon number of the affected site within each circle. Broken lines denote hypothetical intermediates. *P* identifies the parental lineage. Independent parallel mutations at positions 28 or 42 appear to have taken place in the NT02 genealogy (b).

adoptive lymphocyte transfer methodologies, found that T15⁺ antibodies were produced by the B-1 subset of B cells rather than by the conventional, bone marrow-derived, B-2 lymphocytes (43, 44). However, these studies relied on the serological identification of the T15 Id. In contrast, Zarhary and Klinman (45) and Riley et al. (46) have reported a high frequency of precursor B cells in the bone marrow that give rise to T15 Id⁺ clones expressing the canonical $V_H1/V_{\kappa}22$ genes. The importance of this issue for the present study is underscored by the fact that the B-1 cell subset in the mouse is believed to lack the capability of somatic hypermutation, based on the sequences of rearranged V genes from B-1 cells and lymphomas (47–49) as well as from hybridomas produced by fusion of peritoneal B cells (49, 50). Moreover, Linton et al. (22) have shown that peritoneal B-1 lymphocytes do not efficiently form GC. It may be that the functional distinctions between the B-1 and B-2 cell lineages are not absolute (51). If the T15⁺ B cells which occupy the splenic GC of PC-immunized mice and which undergo somatic hypermutation when properly stimulated belong to the B-1 lineage, the notion that this cell lineage lacks the mutation mechanism and cannot form GC should be modified.

The only apparent phenotypic difference between NP-protein- and PC-protein-induced primary Ab responses is in Ig isotype (Table 2). Whereas the anti-NP Ab switch to IgG within a week after immunization, the anti-PC Ab remain predominantly IgM. Could the latter hold a clue to the delay of somatic hypermutation in T15 genes? It is generally accepted that hypermutation occurs in V genes rearranged to either C_{μ} or C_{γ} and that isotype switch and mutation are independent events (8, 41, 52–55). However, the two processes may take place concurrently during the immune

response, as suggested by Manser (8). There is a conspicuous concurrence of isotype switch and initiation of mutation 7 d after immunization with NP-CGG (10, 11).

Abundance of Mutations in Secondary PC-reactive GC. In marked contrast with the primary response, the T15 V_H1 genes from secondary GC of euthymic mice were heavily mutated. We cannot ascertain whether this reflects selective expansion of memory B cells that underwent mutation in the late stages of the primary response, or whether it is the result of a rapid burst of mutational activity and clonal selection within 7 d after antigenic challenge. However, the patterns of mutations argue in favor of the first alternative. Each secondary GC was apparently occupied by closely related cells that have identical V/D/J rearrangements and that shared most V_H mutations, suggesting that relatively few mutations were introduced during the first week after restimulation. This is consistent with observations that the anamnestic Ab response is sometimes borne by rapidly expanding memory B cells that no longer mutate (8, 41, 53, 56–58) and it raises the intriguing possibility that the mutator is more active in the late primary GC than in early secondary GC.

We note that mutations in the T15 V_H1 genes generally spared codons specifying positions 33, 52, 95, and 100b that represent the contact residues for binding of the PC hapten (59). A similar observation was made by Claffin et al. (40, 41) on PC-reactive hybridomas from mice immunized with *P. morganii*. They speculated that the maturation of the Ab repertoire against *P. morganii* PC involves the selection of molecules that bind PC in context with a larger carrier epitope. Perhaps a similar interpretation pertains to EPC-KLH.

T Cells Regulate the Pattern of Somatic Diversity. Insight into the process of somatic diversification of secondary GC

B cells was provided by the comparison of euthymic mice with the groups of CD4⁺ cell-reconstituted *nu/nu* mice (CD4⁺-*nu/nu*) and *nu/nu* mice (Table 3, Figs. 5–7). The memory B cells in CD4⁺-*nu/nu* contained highly mutated T15 V_H1 genes but, in contrast to euthymic animals, these B cells represented a heterogeneous population of clones that appeared to be genealogically related based on the shared V gene mutations (Fig. 7). In other words, it appears that at the time of Ag challenge, the development of the B cell repertoire in the GC of euthymic mice had already reached an advanced stage of selection and expansion of a few memory clones that did not actively mutate, whereas in CD4⁺-*nu/nu* animals, which had approximately fivefold fewer T cells in the spleen, the B cell repertoire was in an earlier evolutionary stage and the process of hypermutation was still active. Thus, the difference between euthymic and CD4⁺-*nu/nu* mice in somatic diversity of memory B cells correlates with the numbers of T helper cells available during the primary response. Although antigen-specific B cell proliferation occurs in the PNA⁺ GCs of *nu/nu* mice (25, and Table 1), this population expanded in a T-independent manner shows no evidence for accelerated maturation after restimulation in the presence of supplementary T cell help (Table 3, Group C).

The average frequency of V_H mutations in this group (1/315) was not significantly different from that found on days 10 and 12 of the primary response (1/391) in euthymic animals. These patterns of somatic diversity are best interpreted by the proposal that activation of hypermutation in GC via T cell help takes place in a dose-dependent fashion, reflecting perhaps, the probability of individual B cells encountering the critical helper signal(s) during their proliferation and differentiation in the GC microenvironment.

Collectively, our results show that the formation of GC and onset of IgV gene hypermutation are independent events and that both B and T cells contribute to their regulation. Some antigen-reactive B cells may proliferate in GC for lengthy periods without mutation, as exemplified by the differences observed between the responses to the NP and PC haptens. Indeed, there may be a mechanism that regulates the hypermutation process in *trans* between NP- and PC-reactive B cells (Miller, C., and G. Kelsoe, manuscript in preparation). Results from *nu/nu* mice indicate that the development of GC is much less dependent on T cell help than the activation of mutations, and that the availability of help from T cells influences the progression of somatic diversification of B cell clones.

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