Relative Contribution of T and B Cells to Hypermutation and Selection of the Antibody Repertoire in Germinal Centers of Aged Mice

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Summary

The immune system of aged individuals often produces antibodies that have lower affinity and are less protective than antibodies from young individuals. Recent studies in mice suggested that antibodies produced by old individuals may be encoded by distinct immunoglobulin (Ig) genes and that the somatic hypermutation process in these individuals is compromised. The present study employed Igh$^b$ scid mice reconstituted with normal lymphocytes from young (2–3-mo-old) and aged (20–25-mo-old) donors and immunized with a protein conjugate of the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP) to determine whether the molecular changes in antibody repertoire reflect senescence in the B cells or whether they are mediated by the aging helper T lymphocytes. The NP-reactive B cells from splenic germinal centers (GC) were recovered by microdissection of frozen tissue sections and their rearranged Ig heavy chain variable region (VH) genes of the V186.2/V3 families were sequenced. It was found that the $V_H$ gene repertoire of the GC B cells was strongly influenced by the source of the CD4$^+$ T cells. When T cells were donated by young mice, the anti-NP response in GC was dominated by the canonical V186.2 gene, even if the responder B cells came from aged donors. However, when the mice were reconstituted with T cells from aged donors, the expression of the V186.2 gene by young B cells was diminished and the response was dominated by the C1H4 gene, another member of the V186.2/V3 family. In contrast, the somatic hypermutation process in the GC B cells followed a different pattern. The mutation frequencies in the animals that were reconstituted with both B and T cells from young donors (1/50 to 1/150 bp) were comparable to the frequencies previously reported for NP-immunized intact young/adult mice. However, when either lymphocyte subset was donated by the aged mice, the mutation frequencies declined. Thus, mice reconstituted with T cells from the aged and B cells from the young had severely compromised mutational mechanism. Likewise, the recipients of aged B and young T cells had diminished mutations even though the repertoire of their anti-NP response was dominated by the canonical V186.2 gene. It appears that the change in germline-encoded repertoire and the decrease of somatic hypermutation represent distinct mechanisms of immunosenescence and that the aging of helper T cells plays a pivotal role in both of these processes.

The elderly population is vulnerable to infectious diseases, in part because of the functional impairment of antibody responses. Aged individuals often produce less antibody after immunization (1–3), but this is not always the case, and it may not be the sole reason for their susceptibility to infections. It has been shown that the aged immune system generates antibodies with lower avidity and/or affinity as compared to the young controls (4–8). Thus, even if the response to a specific antigen remains robust, as has been found in several studies on aged animals (9–12) and elderly individuals (13), the antibodies are functionally insufficient. Elucidation of the mechanisms of this phenomenon is of considerable theoretical and practical interest.

Recent studies on antibody responses to phosphorylcholine (PC)$^1$ (14, 15) and trinitrophenyl (16) haptens in mice suggested that the antibodies produced by an aged immune system may be encoded by genes distinct from those used in young adults. The change in the anti-PC response is particularly striking because the antibody molecules in young/adult mice are encoded almost exclusively by the $V_{\mu1}$ (S107) and $V_{\mu22}$ gene segments (17), whereas aged mice

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$^1$Abbreviations used in this paper: GC, germinal cell; HRP, horseradish peroxidase; NIP, (4-hydroxy-5-iodo-3-nitrophenyl)acetyl; NP, (4-hydroxy-3-nitrophenyl)acetyl; PC, phosphorylcholine; PNA, peanut agglutinin.
utilized V\textsubscript{H} segments from different gene families including 7183, J558, and X-24 (14,15) and they expressed V\textsubscript{H} genes other than V\textsubscript{H}22 (15). Moreover, the anti-PC antibodies produced by aged mice have lower affinities for the PC hapten and lower avidities for *Staphylococcus pneumoniae* expressing PC, and are less protective against the pneumococcal infection than antibodies produced by younger animals (12). Thus, decline of antibody affinity and protective function in the elderly may result from differential usage of germline IgV genes.

The common mechanism of generation of protective immune response is the process of antibody affinity maturation, which is the result of antigen-driven hypermutation and selection of B cells with high affinity receptors and which takes place within the germinal centers (GC) of secondary lymphoid tissues (18, 19). Thus, a defect in affinity maturation would likely render the antibody responses in the elderly less protective. Indeed, Miller and Kelsoe (20) have recently observed that the somatic hypermutation of Ig genes in response to the hapten, (4-hydroxy-3-nitrophenyl)acetyl (NP), is severely diminished in aged mice.

The aim of the present study was to determine whether the molecular changes in the antibody repertoire of aged mice—the shift in germline gene usage and the decrease of somatic hypermutation—reflect senescence within the B cell compartment itself or whether they are mediated by the aging T lymphocytes. T lymphocytes play an important role in shaping the antibody repertoire. CD4\textsuperscript{+} T helper cells (T\textsubscript{H}) are required for GC formation (21-23) and the activation of somatic hypermutation in Ig genes (24, 25). Moreover, it has been suggested that T cells regulate the dominant expansion of B cells expressing a particular germline-encoded V\textsubscript{H} segment in response to specific antigen (25).

The NP hapten coupled to KLH was chosen as an immunogen in the present study based on the hypothesis that the anti-NP response in aged animals is likely to be subject to both mechanisms of repertoire changes discussed above. The antibody response of Ig\textsuperscript{h} mice to NP is highly restricted; most primary anti-NP antibodies bear the \(\kappa\) L chain and the H chain is encoded by the V\textsubscript{H} 186.2, DFL16.1, and J\textsubscript{H}2 segments (26-28). In this respect, the anti-NP response resembles the highly restricted anti-PC response in which the age-dependent shift in IgV gene usage was shown previously (14, 15). However, unlike the PC-reactive B cells, which are not subject to an intensive somatic hypermutation during the early primary responses (24, 29), NP-reactive B cells in splenic GC accumulate mutations from 8 d until day 14 after immunization (19, 30, 31). This mutational activity appears to be compromised in aged mice (20).

To assess the contribution of T and B cells to immunosenescence of anti-NP antibody repertoire, we reconstituted Ig\textsuperscript{h} B10 scid mice with CD4\textsuperscript{+} lymphocytes and B cells from syngeneic, immununized young/adult (2–3-mo-old) and aged (21–25-mo-old) donors, in reciprocal combinations and immunized the chimeras with NP-KLH. Antigen-reactive GC were identified by dual staining of frozen splenic sections with peanut agglutinin (PNA) and anti-\(\lambda\) probes (32), B cells were recovered from individual GC and their rearranged Ig V\textsubscript{H} genes were analyzed by PCR amplification of genomic DNA using the techniques of Jacob and Kelsoe (18, 33).

**Materials and Methods**

*Mice.* C57BL/6 mice aged (20–25-mo-old) and young (2–3-mo-old) were purchased from NIA/Charles River (Wilmington, MA). The B10 scid mice were obtained from the McLaughlin Research Institute (Great Falls, MT) and bred at our animal facility. 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.

**Antibodies, Immunization and Splenic Sections.** NP (Cambridge Research Biochemicals, Cambridge, UK) was conjugated to KLH (Sigma Chemical Co., St. Louis, MO) as described by others (32). Antigen was precipitated in alum and administered as a single intraperitoneal injection of 100 μg in PBS.

Mice were bled and killed at day 14 after immunization. The recovered spleens were embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN) by quick freezing in liquid nitrogen, and stored at −70°C. Serial, 6-μm-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, air-dried, and stored at −70°C until used for immunocytochemical staining and DNA amplification.

**Lymphocyte Preparations and Cell Transfer.** Splenocyte suspensions were prepared by teasing spleens from aged or young donor mice in RPMI 1640 medium supplemented with 25 mM Heps (GIBCO BRL, Gaithersburg, MD) and 0.5% BSA (Sigma Chemical Co.). T lymphocytes were depleted by two treatments with a cocktail of mAb H013 (anti-Thy 1.2), GK 1.5 (anti-CD4), and 3.155 (anti-CD8) from ascitic fluids (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\textsuperscript{\textregistered} analysis.

T cell-enriched splenocyte populations were prepared by filtration through nylon wool columns (Wako BioProduct, Richmond, VA) using the manufacturer’s protocol. Nonadherent cells were treated once with mAb 3.155 plus rabbit complement to eliminate CD8\textsuperscript{+} T cells. The resulting T cell fraction contained >80% of Thy 1.2\textsuperscript{+}/CD4\textsuperscript{+} cells, <10% of slg\textsuperscript{+} cells and <2% of CD8\textsuperscript{+} cells as determined by FACS\textsuperscript{\textregistered} 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 of the B10 scid recipients, 16 h before immunization.

**FACS\textsuperscript{\textregistered} Analysis.** Cells were incubated with biotinylated goat anti-mouse Ig (Fisher Biotech, Pittsburgh, PA) or with biotinylated anti-Thy 1.2 (Becton Dickinson & Co., San Jose, CA) followed by staining with streptavidin-FITC (Fisher Biotech). The binding of mAb GK1.5 and 3.155 were visualized with goat anti-mouse Ig (Fisher Biotech, Pittsburgh, PA) or with biotinylated anti-mouse Ig-FITC conjugates (Fisher Biotech). Cells were analyzed with a flow cytometer (Becton Dickinson & Co.).

**Serum Antibody.** Levels of NP-specific antibody, which exhibit heteroclitic binding to the NP analogue, (4-hydroxy-5-iodo-3-nitrophenyl)acetyl (NIP), were determined by standard ELISA techniques using NIP-BSA conjugate as antigen (32) in solid phase and goat anti-mouse (polyvalent) antibody labeled with horseradish peroxidase (HRP; Fisher Biotech) as a probe, followed by a

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were identified as k+/PNA-binding by dual staining. Sections were incubated with goat anti-mouse k chain antibody conjugated to biotin (Fisher Biotech) for 60 min, washed three times in PBS-BSA, incubated with PNA-HRP (E.Y. Laboratories, Inc., San Mateo, CA) plus streptavidin-alkaline phosphatase (SA-ALPH; Sigma Chemical Co.) and nitroblue tetrazolium/5-bromo-4-chloroindophosphate (NBT/BCIP; Promega Corp., Madison, WI).

**Immunohistochemistry.** Frozen sections were warmed to room temperature, rehydrated in PBS, and blocked by incubation with PBS containing 10% BSA for 1 h. The NP-reactive, GC B cells were identified as λ+/PNA-binding by dual staining. Sections were incubated with goat anti-mouse λ chain antibody conjugated to biotin (Fisher Biotech) for 60 min, washed three times in PBS-BSA, incubated with PNA-HRP (E.Y. Laboratories, Inc., San Mateo, CA) plus streptavidin-alkaline phosphatase (SA-ALPH; Fisher Biotech) and then washed. The binding of the probes was visualized with substrates 3-amino-9-ethyl-carbazole (3-AEC; Sigma Chemical Co.) and nitroblue tetrazolium/5-bromo-4-chloroindophosphate (NBT/BCIP; Promega Corp., Madison, WI).

**Enumeration of GC and Recovery of B Cells.** Splenic sections were doubly stained with anti-λ and PNA as described above. GC were scored as PNA-stained areas within lymphoid follicles in at least 50 microscopic fields (10× objective) from two to three sections, and the results were expressed as number of PNA+ follicles/20 mm² area of splenic section (34). 20 mm² is the equivalent of 12 microscopic fields using a 10× objective. Cells (approximately 100) from individual λ+ and PNA+ GC were recovered using a sharpened micropipette controlled by an electrically powered micromanipulator (Narishige, Tokyo, Japan), as previously described (18, 33).

The background of PNA+ GC was assessed by staining spleen sections from normal, unimmunized mice or from scid mice reconstituted with purified B cells. Normal C57BL/6 mice obtained from the National Institute of Aging colony and maintained in sterile microisolators do not have any detectable PNA+ follicles in the spleen. B cell–reconstituted scid recipients immunized with NP-KLH have been included as a control in numerous adoptive transfer experiments. The number of PNA+ follicles in 12 such animals was <1/20 mm² (data not shown).

**Statistical Analysis.** Results were analyzed by the Wilcoxon-Mann-Whitney rank sum non-parametric test of means from different experimental groups. Differences with p < 0.05 were considered significant.

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### Table: GC score in B10 scid mice

| Tree Age | CD4+ T cell donors (MO.) | GC score | SD |
|----------|--------------------------|----------|----|
| 2-3      | 1/1,500                  | 7.0      |    |
|          | (1/400)                  |          |    |
|          | Mean = 5.8±1.4           |          |    |
| 21-25    | 1/1,400                  | 5.0      |    |
|          | (1/6,400)                |          |    |
|          | Mean = 3.4±0.9           |          |    |

*Figure 1.* GC formation in B10 scid mice reconstituted with CD4+ T cells from either young (2–3 MO.) or aged (21–25 MO.) donors in combinations with B lymphocytes from young (open symbol) or aged (closed symbol) donors. The symbols represent individual recipients. The GC were scored as PNA+ follicles per 20 mm² area of splenic section(s) (see Materials and Methods). (Horizontal broken lines) Mean GC score ± SD. The reciprocal titer of NIP-binding serum Ig in each animal at the time of killing (day 14) is indicated in parentheses.
Figure 2. Histological appearances of splenic PNA + GC in (a) T^young/B^young and (b) T^old/B^old groups. Note the dark staining with antibody to λ1 in the cytoplasm of cells around the follicles and on the membrane of cells within the GC.

by the Biopolymer Laboratory of the University of Maryland School of Medicine using an (model 373A; Applied Biosystems, Foster City, CA) automated DNA sequencing system.

Frequency of Mutations Introduced by PCR. A Taq polymerase error rate of $2.5 \times 10^{-5}$ misincorporations/bp/PCR cycle was determined by sequencing 16 clones recovered from two independent amplifications of B1-8 hybridoma cells (V186.2, DFL16.1, and J3.2). On average, we observed 0.6 artificial mutations/V186.2 gene segment (273 bp), a frequency close to that observed by Jacob et al. (30). Thus, each VDJ fragment recovered from splenic tissue by 90 rounds of amplification is expected to contain approximately one mutation attributable to polymerase error. Mutations in excess of this value were assumed to have resulted from in vivo process. Shared mutations within a set of clonally related sequences were counted as one mutational event.

Results

The Age of CD4 Lymphocyte Donors Determines the GC Response. Examination of the spleens of scid mice reconstituted with T and B lymphocytes from young and aged donors revealed that the origin of CD4 T and not B cells influences the GC formation in response to NP-KLH. Recipients of T cells from young donors (T^young) developed on the average almost twofold more GC per 20 mm²

Table 1. Summary of Rearranged V\textsubscript{J} Segments Recovered from λ1 GC of the Lymphocyte-reconstituted NP-KLH-immunized aged mice

| Age of donors (mo) | T cells | B Cells | No. of mice | No. of V\textsubscript{J} segments | V\textsubscript{J} segments identified (%) | Percent V\textsubscript{J} genes identified (%) | No. of distinct V\textsubscript{J} genes/GC | No. of distinct V\textsubscript{J} genes/GC |
|-------------------|--------|---------|-------------|-------------------------------|---------------------------------|--------------------------|-------------------------------|-------------------------------|
| Young (2–3)       | 2      | 15      | 6           | 20 (all)                       | 12 (42%)                        | 5 (17%)                  | 2.0                          | 0 (0%)                        |
| Young (2–3)       | 2      | 48 (all)| 5           | 5 (100%)                       | 6 (40%)                         | 2 (13%)                  | 2.0                          | 0 (0%)                        |
| Aged (21–25)      | 2      | 24 (all)| 4           | 15 (all)                       | 3 (12%)                         | 2 (10%)                  | 2.0                          | 0 (0%)                        |
| Aged (21–25)      | 2      | 13 (all)| 4           | 21 (all)                       | 7 (33%)                         | 3 (14%)                  | 2.0                          | 0.5 (0.9)                     |
| Aged (21–25)      | 2      | 5       | 4           | 24 (all)                       | 1 (4%)                          | 2 (20%)                  | 2.0                          | 0.5 (0.9)                     |

Sequences with shared CDR4 were scored as unique.

*Statistical significance between the experimental group and control (T^young/B^young) as determined by the rank sum test.
splenic area as compared to the recipients of “aged” T cells \((T^{\text{-aged}})\), regardless of the age of B cell donors (Fig. 1), a difference that is statistically significant at \(p = 0.025\). However, the low numbers of PNA+ follicles in the T\(^{\text{aged}}\) recipients were well above the background PNA staining observed in spleens of normal, unimmunized animals or spleens of immunized \(\text{scid}\) mice reconstituted with B cells alone (see Materials and Methods). Consistent with the patterns of GC formation, the recipients of aged T cells had lower serum antibody titters against the NIP hapten (Fig. 1). Thus, aged B cells \((B^{\text{aged}})\) were competent in the formation of morphologically typical GC when given an appropriate source of help. Representative splenic GC in the recipients of young and aged T cells are shown in Fig. 2. There were no gross differences in staining of follicles with PNA, and B cells expressing the \(\lambda\) chain were readily detectable within these GC (Fig. 2). In contrast, the mice that received both T and B cells from aged donors had poorly developed GC with very few \(\lambda^+\) cells (not shown) which made it difficult to identify the NP-reactive GC; these animals were excluded from the subsequent molecular studies.

**The \(V_{\text{H}}\) Gene Repertoire of GC B Cells is Influenced by the Age of CD4+ Lymphocyte Donors.** VDJ fragments were recovered from four to six different PNA+/\(\lambda^+\) GC from two animals from each group of \(\text{scid}\) recipients reconstituted with various combinations of T and B cells from young and aged donors: \(T^{\text{young}}/B^{\text{young}}\), \(T^{\text{aged}}/B^{\text{young}}\), and \(T^{\text{young}}/B^{\text{aged}}\) (Table 1). The sequences of the \(V_{\text{H}}\) gene segments were found to be homologous to various germline \(V_{\text{H}}\) genes of the V186.2 and V3 subfamilies of the J558 \(V_{\text{H}}\) gene family (35). Some of the sequences differed from their presumptive germline counterpart by several nucleotides. However, these differences typically fell into the positions that are shared between different germline members of the V186.2/V3 subfamily, suggesting that they represented either somatic mutations of the germline gene or PCR artifacts. The rare \(V_{\text{H}}\) sequences that could not be unambiguously assigned to a given germline gene (<10% of all sequences) were excluded from analysis. The homologous V genes recovered from each GC were scored in two ways (Table 1): (a) as a total number of \(V\) segments, and (b) as number of VDJ clones, in which case the sequences with shared CDR3 regions were scored as one.

In the control group of animals reconstituted with both T and B cells from young donors \((T^{\text{young}}/B^{\text{young}})\), the most frequently recovered \(V\) segment was the \(V_{186.2}\) gene (Table 1), which is known to encode the majority of H chains of the primary anti-NP antibodies in mice of IgHb.

### Table 1: D Segment Use in All VDJ Fragments Amplified from \(\lambda^+\) GC

| Age of donors (mo) | DFL16.1 | DQ52 | DSP2.2 | Unidentified |
|-------------------|---------|------|--------|-------------|
| T cells \(\lambda^+\) | All sequences | Clones* | All sequences | Clones* | All sequences | Clones* | All sequences | Clones* |
| Young (2–3) | 7 (25%) | 3 (19%) | 2 (7%) | 1 (6%) | 2 (7%) | 1 (6%) | 17 (61%) | 11 (69%) |
| Aged (21–25) | 12 (24%) | 6 (23%) | 13 (25%) | 2 (8%) | – | – | 26 (51%) | 18 (69%) |
| Young (2–3) | 17 (28%) | 3 (21%) | – | 3 (12%) | 2 (14%) | 15 (60%) | 9 (64%) |

*Sequences with shared CDR3 were scored as one clone.
was also seen when the mice received B cells from aged cells on the expression of V186.2 and C1H4 genes in the donors (groupations, namely CH10, C1H4, V23, V3, and 24.8 V, genes haplotype (25-27). Several other members of the V186.2/CLONE Y.L CENE ~.~ SEOUENCE pattern of D, usage is comparable to that observed previously although two other segments, DQ52 and DSP2.2, were the one most commonly found in all three experimental groups, ranging from 24 to 28% of all DVJ sequences, was the one most frequently recovered from the GC of the Ty~176 mice. However, the available data do not show any conspicuous change of D usage relative to the age of T and B cells is limited because of our failure to identify the D gene segment in about half (50-60%) of all VDJ sequences. However, the available data do not show any conspicuous change of D usage relative to the age of the lymphocytes. The DFL16.1 segment was the one most frequently recovered from the GC of young, intact C57BL/6 mice during the primary response to the NP hapten (30). The DFL16.1 segment was found in combination with the most frequently used VH genes in a given experimental group (Fig. 4, a-c). D segments were used most often in reading frame (RF) 1, in the nomenclature of Ichihara et al. (36) followed by R.F-3 and R.F-2 (Fig. 4, a-c), which is consistent with the pattern observed in mature B cells by Gu et al. (37).

### Use of D Families in GC B Cells

The analysis of D usage relative to the age of T and B cells is limited because of our failure to identify the D gene segment in about half (50-60%) of all VDJ sequences. However, the available data do not show any conspicuous change of D usage relative to the age of the lymphocytes. The DFL16.1 segment was the one most commonly found in all three experimental groups, ranging from 24 to 28% of all DVJ sequences, although two other segments, DQ52 and DSP2.2, were occasionally also identified (Table 2 and Fig. 4, a-c). This pattern of D usage is comparable to that observed previously in the GC of young, intact C57BL/6 mice during the primary response to the NP hapten (30). The DFL16.1 segment was found in combination with the most frequently used VH genes in a given experimental group (RF 1), consistent with the pattern observed in mature B cells by Gu et al. (37).

### Somatic Hypermutations in the V Segments Relative to the Age of T and B Cell Donors

A total of 28 V genes amplified from six different rearranged VDJ segments from the GC of the Ty~176/B(30) mice contained mutations ranging from 1/50 to 1/150 bp (Table 3), a frequency that is within the range of mutations found in the NP-reactive GC B cells from intact, young/adult mice (30). Most of these mutations were related to the V186.2 gene which was the one most frequently recovered from the GC of Ty~176/B(30) scid mice. However, similar mutation frequencies were observed in other members of the V186.2/ V3 gene family that were recovered from this group of mice. As an example, the VDJ segments recovered from two A4+GC dissected from different mice are shown in Fig. 5. In the GC 7C, five segments contained a V186.2 gene with an average mutation frequency of 1/65 bp (4.2 mutations/V) and four segments contained a CH10-like gene with an average of 1 mutation/89 bp (3.1/V). GC 8C, from another mouse, yielded seven V186.2 segments with an average mutation frequency of 1/101 bp (2.7/V). One mutation, a TGG→TG exchange in position 33 of the V186.2 segment, which increases the affinity of anti-NP antibody and is frequent in mature anti-NP responses (38), was not observed in our sample.

| Clone | Vn | Genotype | Dg | Sequence |
|-------|----|----------|----|----------|
| A2-23 | V165.1 | TGG | TAC | TAG GTG AGC TAC |
| A2-28 | V165.1 | TGG | TAC | TAG GTG AGC TAC |
| 2AI-11 | V23 | GGC | CTC | TAC |
| 2AI-83 | C1H4 | GGG | TAC | CAT |
| IA5-73 | ? | ATC | TAT | GTA GTG GTC |
| IA5-61 | ? | ATC | TAT | GTA GTG GTC |
| 2BI-48 | V186.2 | TGG | GTC | GTC AGC GTA TCA |
| 2BI-78 | V186.2 | TGG | GTC | GTC AGC GTA TCA |
| 2BI-26 | V186.2 | TGG | GTC | GTC AGC GTA TCA |
| IA2-08 | V102 | GGG | AAC | CTC |
| IA2-25 | V102 | GGG | AAC | CTC |
| IA5-52 | V23 | TTT | ATC | TCC |
| IA5-83 | V23 | TTT | ATC | TCC |
| 2AI-67 | V186.2 | AGG | TAC | CAT |
| 2AI-63 | V186.2 | AGG | TAC | CAT |
| 2BI-46 | V165.1 | AGG | GAA | CTT |
| 2BI-22 | V165.1 | AGG | GAA | CTT |
| 2BI-39 | V165.1 | AGG | GAA | CTT |
| 2AI-17 | C1H4 | CCG | GAC | GGT ACT |
| 2AI-62 | C1H4 | CCG | GAC | GGT ACT |
| 2BI-76 | V186.2 | TCA | ATG | AGC GGA CTA CCC CCC |
| 2BI-80 | V3 | CCG | GAC | AGT CAT |

The clones in the left column are identified by a number (animal) followed by a letter (GC). The assignment of V segments to the presumptive germline genes is shown including the ambiguous sequences (?) as observed in the GC 7C.
Table 3. Mutations in $\nu$ Sequences in the Amplified VDJ Fragments

| Age of donors | No. of sequences analyzed | Mutation frequency (range) | R/S mutation ratios |
|---------------|---------------------------|---------------------------|--------------------|
|               |                           |                           | Overall | Framework | CDR1+2 |
| T cells       | B cells                   |                           |         |           |       |
| 2–3 mo        | 2–3                       | 23                        | 1/50 (5.4/\nu)–1/150 bp (1.8/\nu) | 2:1 | 1.3:1 | 7.3:1 |
| 21–25         | 2–3                       | 41                        | 1/100 (2.7/\nu)–1/400 bp (0.7/\nu) | 1.2:1 | 1:1 | 2.6:1 |
| 2–3           | 21–25                     | 17                        | 1/140 (1.9/\nu)–1/855 bp (0.3/\nu) | 0.4:1 | ND | ND |

The average number of mutations in the sequences of any given $\nu$ segment that was repeatedly cloned from individual GC from the three groups of mice are shown in Fig. 7. It is apparent that the frequency of $\nu$ gene mutations in B cells from either the $T_{\text{aged}}/B_{\text{young}}$ or the $T_{\text{young}}/B_{\text{aged}}$ group were lower ($p < 0.05$) than the control group ($T_{\text{young}}/B_{\text{young}}$), whereas the differences between experimental groups were not statistically significant ($p > 0.1$). Nonetheless, it is noteworthy that the low mutation frequency in the spleens of the experimental animals appears to be an attribute of individual GC. Whereas some GC support mutation rates that fall within the range of the control values, other GC contain only unmutated B cells. Also note that the $V$ segments C1H4 and 186.2, which were found to contain $\geq 3$ mutations/sequence in the control group, were recovered from the $T_{\text{aged}}/B_{\text{young}}$ and $T_{\text{young}}/B_{\text{aged}}$ groups, respectively, with $<1$ mutation/sequence (Fig. 7).

An increased ratio of $R/S$ mutations was observed in the CDRs (7:1) as compared to the framework regions (1.3:1; Table 3) in the $T_{\text{young}}/B_{\text{young}}$ mice, suggesting an incipient process of selection of antigen-binding mutants in this group. This trend was not found in the group that received Taged cells, and the low number of mutations in mice with Baged cells preclude any meaningful analysis of R/S ratios (Table 3).

Discussion

The anti-NP response in the GC of Ighb scid mice reconstituted with normal T and B lymphocytes from young donors appears to reproduce several characteristic features of the primary response of young adult C57BL/6 mice regarding the IgV genes use and the somatic hypermutation of these genes. The canonical $\nu$ gene, V186.2, was the one most frequently recovered from the $\lambda^+$ GC B cells from the $T_{\text{young}}/B_{\text{young}}$ scid mice. Other members of the V186.2/V3 gene family were also represented in our sample, however, this is not peculiar to the adoptive cell transfer system. Recent study of $\lambda^+$, NP-specific hybridomas recovered from C57BL/6 mice also indicated that a significant number of $\nu$ genes other than V186.2 are used in the primary response (39). Similarity also exists in the repertoires of rearranged D segments; in addition to the most frequently found DFL16.1, we also identified VDJ rearrangements containing the DQ52 and DSP2.2 segments which were also found in the $\lambda^+$ GC of young adult C57BL/6 mice (31). Finally, the frequencies of base substitutions observed in the V segments recovered from GC on day 14 after primary immunization (1/50–1/150 bp), in our study are comparable to those found in GC in situ (30) and in isolated GC B cells (31) from normal mice. Mutations occurred in the canonical $\nu$ segment, V186.2, as well as in C1H4 and CH10 segments (Figs. 5 and 6), suggesting that the antigen-driven GC response included B cells with receptors encoded by different members of the V186.2/V3 gene family. This finding is also consistent with that of Jacob et al. in intact mice (30).

On the other hand, we noted two differences in the anti-NP antibody repertoire of the lymphocyte-reconstituted scid mice as compared to the intact animals. First, the V segments CH10, C1H4, and 23 were identified in the GC of scid recipients on day 14 after the immunization (Table 1). In intact mice, however, the expression of these genes in GC is limited to the first week of the anti-NP response; later, the primary response is dominated by the V186.2 gene (30). Second, we failed to recover any V186.2/D/J segment containing the TGG--TTG mutation in position 33 which has been found in most GC-derived B cells from intact C57BL/6 mice on day 12 after immunization (31) and is a hallmark of high affinity antibody to NP (38). These results suggest that the dynamics of cell interactions and clonal selection in lymphocyte-reconstituted scid mice may be somewhat different from that in intact mice. It has been reported that scid mice develop follicular dendritic cells in the spleen only after the transfer of mature lymphocytes (40); an initial deficiency of these cells could delay the subsequent process of affinity maturation.
the dominance of B cells expressing rearranged V186.2 exons in response to the NP hapten is determined by the source of helper T cells. When help was provided by CD4+ lymphocytes from aged mice (T\textsuperscript{aged}), the V\textsubscript{\alpha} gene repertoire of \lambda\textsuperscript{+} GC was more diverse than controls and was dominated by B cells that had rearranged the C1H4 V\textsubscript{\alpha} gene. To interpret this result, let us consider that both V186.2\textsuperscript{+} and C1H4\textsuperscript{+} GC B cell clones become activated at an early stage of the anti-NP response in normal C57BL/6 mice; however, the V186.2 clonotype is later selected and expanded (30). The competitive advantage of the V186.2\textsuperscript{+} cells is presumably based on the primary affinity of their antigen receptor; recent studies have shown that the rearranged, unmutated (germline) C1H4 gene encodes NP-specific antibody of lower relative affinity than the unmutated canonical V186.2/DFL16.1 combination (G. Kelsoe, personal communication). It is reasonable to postulate that the strength of the receptor-mediated antigen signal must be balanced with the signals from helper cells in order to provide on optimal stimulus for B lymphocyte activation. In this scenario, the young T\textsubscript{\alpha} cells would provide optimal help for the unmutated canonical V186.2 clones whereas the aged T\textsubscript{\alpha} cells, which are known to be functionally different (41, 42), would help the clones expressing the lower affinity receptor(s) encoded by unmutated C1H4 and other rearranged germline genes. This interpretation is supported by our observation that the repertoire of GC from the mice reconstituted with aged B cells and young helper cells (T\textsuperscript{young}/B\textsuperscript{aged}) was similar to that of the control group (T\textsuperscript{young}/B\textsuperscript{young}, Fig. 3), i.e., the young T helper cells were able to sustain the dominance of the 186.2 gene expression in the population of aged B cells.

We have no proof that all of the VDJ rearrangements containing V\textsubscript{\alpha} genes other than V186.2 which were recovered from the \lambda\textsuperscript{+} GC represent B cells engaged in anti-NP response, but indirect evidence supports the notion that at

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**Figure 5.** VDJ segments recovered from GC 7C and 8C from two different T\textsuperscript{young}/B\textsuperscript{young} scid mice. Amplification of the 7C DNA yielded five distinct clones with V segment homologous to the 186.2 gene and four clones containing C1H4 segments. The GC 8C yielded seven V186.2 sequences. The base differences from the germline sequence are shown as (  ) replacement, (I) silent, and (W=ml) termination codon. (?) Incomplete sequence.

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**Figure 6.** VDJ segments recovered from two GC, 2A1, and 2B1 from a mouse reconstituted with T\textsuperscript{young}/B\textsuperscript{aged} lymphocytes. The GC 2A1 yielded two V186.2 and three C1H4 sequences. Three V186.2 sequences from the GC 2B1 are also shown. Symbols are the same as in Fig. 5. (Sequences of V segments from Figs. 5 and 6 are available from GenBank under accession numbers U49667-U49690.)
expression of the canonical NP-reactive cells. This immunization with preformed NP/anti-NP complexes leads to activation of non-V186.2 clones in the GC and priming for a robust anamnestic response (Nie, X., S. Basu, and J. Cerny, manuscript in preparation).

The second finding of the present study confirms and extends the work of Miller and Kelsoe (20) which demonstrated the decline of somatic mutation activity in the GC B cells of aged mice. Reduced frequency of mutations was observed in scid mice reconstituted with either T or B cells from aged donors. Particularly illuminating is the pattern observed in the Ty~ group that appeared to have very low mutation frequency, whereas both the formation of morphologically typical GC (Fig. 2) and the dominant usage of the V186.2 gene (Fig. 3) in these mice were comparable to the control group (Ty~). This result suggests that the T cell signals for GC formation and B cell proliferation and differentiation may be different from those that are required for activation of the mutation mechanism.

Previous results from our laboratory (24) indicate that the rate of somatic mutations in GC B cells is proportional to the number of available T helper cells. This may help to explain the present observation of the variability of mutations within individual GC in the spleens of mice reconstituted with aged lymphocytes (Fig. 7). B cells in some of these GC appeared to mutate at a rate comparable with the young mice, whereas the B cells in other GC mutated less or below the level of detection. The lymphocytes within the B and T cell compartments of aged mice are known to be quite heterogeneous; some cells are functionally altered whereas other cells appear to function normally (2, 3). Because the individual GC are populated by very few founder lymphocytes (32, 33), chance may decide whether a given GC in the aging animal is founded by a competent or incompetent B or T cell.

Our results show that the molecular changes in the antibody repertoire of aged mice reflect senescence within both B and T cell compartments. Change in the germline-encoded repertoire and decreased hypermutation have now been defined as two distinct, but not mutually exclusive mechanisms that compromise the efficacy of the antibody response in aged animals. The aging of T helper cells appears to play a pivotal, albeit different role in both of these processes. The aging model proves to be a useful experimental tool to study T–B cell interactions that participate in the GC formation, Ig hypermutation, and B cell memory.

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