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

By Xuhao Yang, Jaroslav Stedra, and Jan Cerny

From the Department of Microbiology and Immunology, School of Medicine, University of Maryland at Baltimore, Baltimore, Maryland 21201

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 (V_H) 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_H1 (S107) and $V_\kappa22$ gene segments (17), whereas aged mice

[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.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/96/03/959/12 $2.00
Volume 183 March 1996 959–970

utilized V_H segments from different gene families including 7183, J558, and X-24 (14,15) and they expressed V_κ genes other than $V_\kappa 22$ (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. $CD4^+$ T helper cells (T_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_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 Igh^b mice to NP is highly restricted; most primary anti-NP antibodies bear the λ1 L chain and the H chain is encoded by the V_H 186.2, DFL16.1, and J_H2 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 Igh^b B10 *scid* mice with $CD4^+$ lymphocytes and B cells from syngeneic, unimmunized 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-λ probes (32), B cells were recovered from individual GC and their rearranged Ig V_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.

Antigens, 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 immunochemical 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 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 (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® 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 $CD8^+$ T cells. The resulting T cell fraction 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 of the B10 *scid* recipients, 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., 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–rat 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-nitophenyl)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

tetramethylbenzidine hydrogen peroxide substrate kit (Bio-Rad Laboratories, Richmond, CA).

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-chloroindoylphosphate (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 mm^2 area of splenic section (34). 20 mm^2 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 $<1/20$ mm^2 (data not shown).

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

Amplification and Sequencing of VDJ DNA Recovered from Individual GC. The isolated λ^+/PNA^+ GC cells were transferred into a 0.5-ml microcentrifuge tube with 5 μl PBS and 10 μl H_2O. 5 μl of 4 mg/ml proteinase K solution (Boehringer-Mannheim Biochemicals, Indianapolis, IN) was added and the tube was incubated in a 37°C water bath overnight. The proteinase K was then inactivated at 96°C, for 10 min. DNA amplification was carried out by two rounds of PCR using pairs of nested primers (18, 30). The initial round of amplification used primers (5′-CCTGACCCAGATGTCCCTTCTTCTCCAGCAGG-3′ and 5′-GGGTCTAGAGGTGTCCCTAGTCCTTCATGACC-3′, corresponding to V186.2 genomic DNA 5′ of the transcription start site sequence and to the intron J_H2 sequence, respectively. In 50 μl reaction volume, the crude cell lysate was mixed in 1× *Taq* DNA polymerase incubation buffer (Boehringer Mannheim), 4 mM $MgCl_2$, 200 mM dNTP, 50 pM of each primer, and 2.5 U of *Taq* DNA polymerase and amplified by 50 cycles of 94°C, 1.4 min/70°C, 3 min. Reaction mixture (5 μl) from the first round was reamplified for an additional 40 cycles (92.5°C, 0.5 min/66°C, 2 min/70°C, 1 min, and one cycle of 72°C, 10 min) using nested primers 5′-TCTAGAATTCAGGTCCAACTGCAGCAGCC-3′, complementary to the initial 20 nucleotides of the V186.2 gene (with an additional recognition sequence for restriction enzyme PstI) and 3′ primer 5′-ACGGATCCTGTGAGAGTGGTGCCT-3′, complementary to the J_H2 gene segment with BamHI recognition site. The second reaction mixture (3–5 μl) was loaded on a 1% agarose gel electrophoresis for amplified DNA identification (~400-bp fragment).

The PCR product was then purified by ethanol precipitation and digested with the restriction enzymes BamHI and PstI (Boehringer Mannheim), isolated via agarose gel electrophoresis using the Gel Extraction Kit (Qiagen, Inc., Chatsworth, CA) and ligated into pBluescript SK II plasmid. Competent DH5α bacteria were transformed by electroporation, and recombinant colonies were screened with a biotin-endlabeled oligonucleotide corresponding to the V186.2 gene codons 71-76, (5′-CTGGAGGGTTTGTCT-3′), using the PhotoGene™ Nucleic Acid Detection System (GIBCO BRL). DNA from positive clones was sequenced

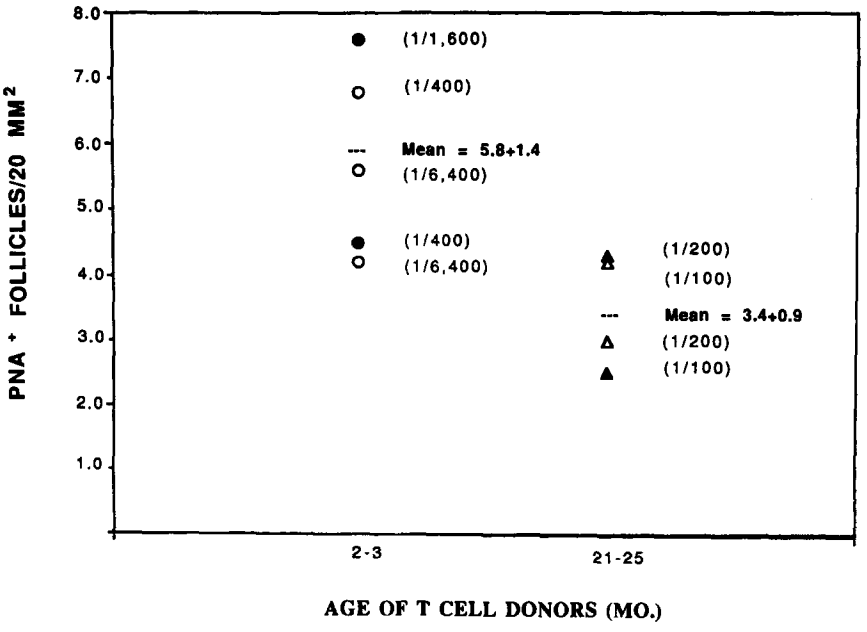

Figure 1. GC formation in B10 *scid* mice reconstituted with $CD4^+$ T cells from either young (*2–3 MO.*) or aged (*21–25 MO.*) donors in combinations with B lymphocytes from young (*open symbols*) or aged (*closed symbols*) donors. The symbols represents individual recipients. The GC were scored as PNA^+ follicles per 20 mm^2 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.

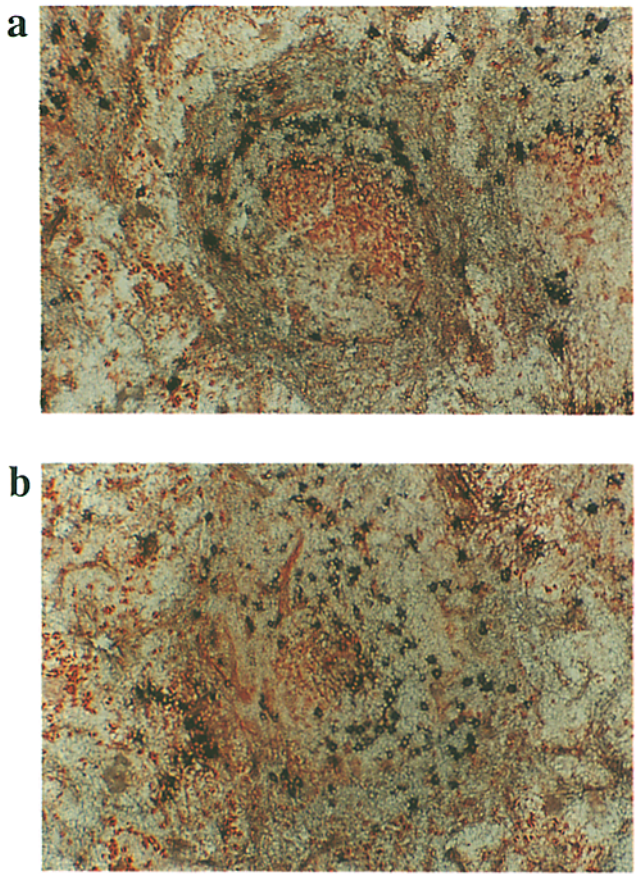

Figure 2. Histological appearances of splenic PNA$^+$ GC in (*a*) T^{young}/B^{young} and (*b*) T^{aged}/B^{young} groups. Note the dark staining with antibody to λ1 in the cytoplasm of cells around the follicles and on the membrane of cells within the GC.

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 2.5×10^{-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 J_H2). On average, we observed 0.6 artifactual 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 mm^2

Table 1. *Summary of Rearranged V_H Segments Recovered from λ$^+$ GC of the Lymphocyte-reconstituted NP-KLH-immunized scid Mice*

Age of donors (mo)					Percent V_H genes identified (%)							No. of distinct V_H genes/GC	
T cells	B Cells	No. of mice	No. of GC	No. of V_H segments	186.2	CH10	C1H4	23	165.1	3	102	24.8	Individual	Mean
Young (2–3)	Young (2–3)	2	6	29 (all)	12 (42%)	7 (24%)	5 (17%)	1 (3%)	–	3 (11%)	–	1 (3%)	1,3,2,2,2,2	2.0
				15 (clones)*	6 (40%)	4 (26%)	2 (13%)	1 (3%)	–	1 (7%)	–	1 (7%)		
Aged (21–25)	Young (2–3)	2	5	48 (all)	3 (6%)	5 (10%)	23 (48%)	8 (17%)	3 (6%)	3 (6%)	2 (5%)	1 (2%)	4,1,6,4,4,	3.8 ($p = .05$)‡
				24 (clones)*	2 (8%)	3 (12%)	10 (42%)	3 (13%)	2 (8%)	2 (8%)	1 (4%)	1 (4%)		
Young (2–3)	Aged (21–25)	2	4	21 (all)	7 (33%)	–	3 (14%)	3 (14%)	5 (24%)	1 (5%)	2 (10%)	–	4,3,3,4	3.5 ($p < .05$)‡
				13 (clones)*	5 (39%)	–	2 (15%)	2 (15%)	2 (15%)	1 (8%)	1 (8%)	–		

*Sequences with shared CDR3 were scored as one.
‡Statistical significance between the experimental group and control (T^{young}/B^{young}) as determined by the rank sum test.

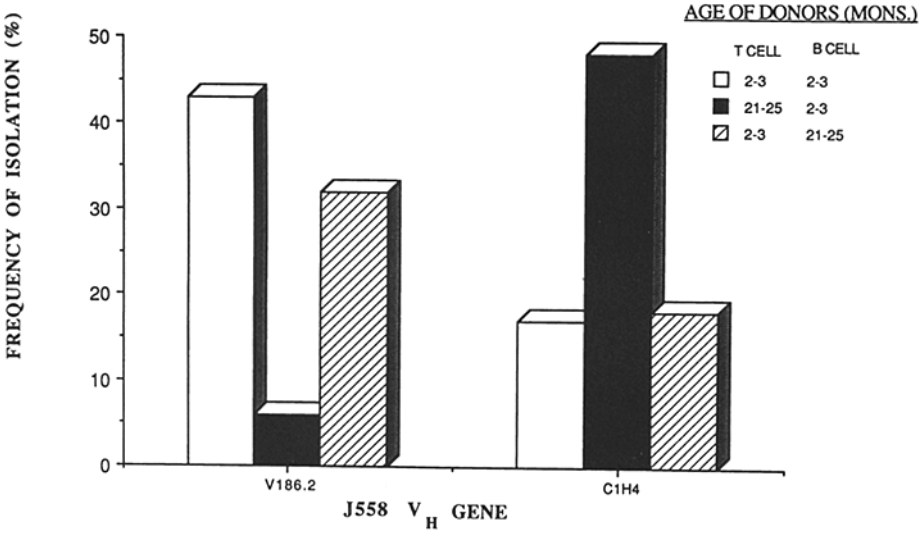

Figure 3. Recovery of V_H segments 186.2 and C1H4 from $\lambda1^+$GC of *scid* recipients T^{young}/B^{young} (*empty columns*), T^{aged}/B^{young} (*black columns*), and T^{young}/B^{aged} (*hatched columns*). Data are presented as percent of all V_H clones shown in Table 1.

splenic area as compared to the recipients of "aged" T cells (T^{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^{aged} recipients were well above the background PNA staining observed in spleens of normal, unimmunized animals or spleens of immunized *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 titers against the NIP hapten (Fig. 1). Thus, aged B cells (B^{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 λ 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 λ^+ cells (not shown) which made it difficult to identify the NP-reactive GC; these animals were excluded from the subsequent molecular studies.

The V_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^+/λ^+ GC from two animals from each group of *scid* recipients reconstituted with various combinations of T and B cells from young and aged donors: T^{young}/B^{young}, T^{aged}/B^{young}, and T^{young}/B^{aged} (Table 1). The sequences of the V_H gene segments were found to be homologous to various germline V_H genes of the V186.2 and V3 subfamilies of the J558 V_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_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^{young}/B^{young}), the most frequently recovered V segment was the V_H186.2 gene (Table 1), which is known to encode the majority of H chains of the primary anti-NP antibodies in mice of Igh^b

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

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

*Sequences with shared CDR3 were scored as one clone.

a

CLONE	Vg GENE	Dg SEQUENCE	Dg GENE	Jg
7C13	CH10	GAC TAC GGT AGT AGC TAC GGG AGC	DFL16.1 (RF1)	2
7C28	V186.2	GAT TAC TAC GGT AGT AGC TAC AAC	DFL16.1 (RF1)	2
7C38	V186.2	GGG GGA TAC TAC GGT AGT AGC TAC GAC	DFL16.1 (RF1)	2
8C24	V186.2	TAT TAC TAC GGT AGC	DFL16.1 (RF1)	2
8C35	V186.2	TAT TAC TAC GGT AGC	DFL16.1 (RF1)	2
8C68	V186.2	TAT TAC TAC GGT AGC	DFL16.1 (RF1)	2
8C29	V186.2	TAT TAC TAC GGG AGC	DFL16.1 (RF1)	2
7C15	V186.2	GGG GAT AAC TGG GAC	DQ52 (RF3)	2
7C30	V186.2	GGG GAT AAC TGG GAC GTC	DQ52 (RF3)	2
7A07	C1H4	GGG ATC TAC TAT GAT TAC GAC CTC	DSP2.2 (RF1)	2
7A08	C1H4	GGG ATC TAC TAT GAT TAC GAC CTC	DSP2.2 (RF1)	2
7C53	CH10	?		
7C83	CH10	AAC	UNIDENTIFIED	2
7C80	CH10	AAC	UNIDENTIFIED	2
7C07	V186.2	AAC	UNIDENTIFIED	2
8B09	C1H4	CCC TCT GAC	UNIDENTIFIED	2
8B19	C1H4	CCC TCT GAC	UNIDENTIFIED	2
8B14	C1H4	CCC TCT GAC	UNIDENTIFIED	2
7E84	CH10	AAG AGG CTT	UNIDENTIFIED	2
8E82	V3	CTC GCT ACG GCT	UNIDENTIFIED	2
8E65	V3	GGG GGG TAC CCT	UNIDENTIFIED	2
7E51	V24.8	TAG GTA ACT TAG	UNIDENTIFIED	2
8C16	V186.2	TCG ACA GCT CAG AGA	UNIDENTIFIED	2
8C40	V186.2	TCG ACA GCT CCA GAG	UNIDENTIFIED	2
8C42	V186.2	TCG ACA GCT CCA GAG	UNIDENTIFIED	2
7E10	CH10	TCC GGT AAC GAC TAC TTT GAC CCT	UNIDENTIFIED	2
7E13	CH10	TCC GGT AAC GAC TAC TTT GAC CCT	UNIDENTIFIED	2
8E08	V23	CCC CTC TAC TAT GAT AAC TTA CGT GAC	UNIDENTIFIED	2
8E13	V3	AAT AAC CTT ATT ACT ACG GTA GTA AAC	UNIDENTIFIED	2

b

CLONE	Vg GENE	Dg SEQUENCE	Dg GENE	Jg
10B81	C1H4	TNT TAT TAC TAC GGT AGT AGC	DFL16.1 (RF1)	2
10B13	C1H4	GGC ATC TCA TAC GGT AGT AGC CCC	DFL16.1 (RF1)	2
10B55	C1H4	ATC TCA TAC GGT AGT AGC CCC	DFL16.1 (RF1)	2
11D08	C1H4	CGT TAC TAC GGT AGT AGC TAC GCC	DFL16.1 (RF1)	2
10E16	V24.8	TTT GGG AGT AAC TAC CAT	DFL16.1 (RF1)	2
10E39	V24.8	TTT GGG AGT AAC TAC CAT	DFL16.1 (RF1)	2
10E40	V24.8	TTT GGG AGT AAC TAC CAT	DFL16.1 (RF1)	2
10B27	CH10	AAA GGG TTA CTA CGT CAC	DFL16.1 (RF3)	2
10B30	CH10	AAA GGG TTA CTA CGT CAC	DFL16.1 (RF3)	2
10B25	CH10	AAA GGG TTA CTA CGT CAC	DFL16.1 (RF3)	2
10B68	CH10	AAA GGG TTA CTA CGT CAC	DFL16.1 (RF3)	2
10B19	C1H4	AAA GGG TTA CTA CGT CAC	DFL16.1 (RF3)	2
10B19a	C1H4	GGG GAT AAC TGG GAC GTC	DQ52 (RF3)	2
10D16	C1H4	GGG GAT AAC TGG GAC GTC	DQ52 (RF3)	2
10D43	C1H4	GGG GAT AAC TGG GAC GTC	DQ52 (RF3)	2
10D49	C1H4	GGG GAT AAC TGG GAC GTC	DQ52 (RF3)	2
10D50	C1H4	GGG GAT AAC TGG GAC GTC	DQ52 (RF3)	2
10D52	C1H4	GGG GAT AAC TGG GAC GTC	DQ52 (RF3)	2
10D56	C1H4	GGG GAT AAC TGG GAC GTC	DQ52 (RF3)	2
10D66	C1H4	GGG GAT AAC TGG GAC GTC	DQ52 (RF3)	2
10D84	C1H4	GGG GAT AAC TGG GAC GTC	DQ52 (RF3)	2
10D85	C1H4	GGG GAT AAC TGG GAC GTC	DQ52 (RF3)	2
10D86	C1H4	GGG GAT AAC TGG GAC GTC	DQ52 (RF3)	2
10E51a		GGG GAT AAC TGG GAC GTC	DQ52 (RF3)	2
10E79		GGG GAT AAC TGG GAC GTC	DQ52 (RF3)	2
10E75	V3	ACT GGG ACT	UNIDENTIFIED	2
11B68	V165.1	CAA GAG GAC ?		
10B49	V23	TCG GCT AGG CAG	UNIDENTIFIED	2
10B83	V23	TCG GCT AGG CAG	UNIDENTIFIED	2
10B75	V23	TCG GCT AGG CAG	UNIDENTIFIED	2
10E51	V186.2	AGG ATG GGG CTT	UNIDENTIFIED	2
11B09	C1H4	TCA ACC CGG TAC	UNIDENTIFIED	2
11B50	V186.2	TTT TGG GAT GGT TCC	?	
11B57	V186.2	TTT TGG GAT GGT TCC	?	
10B23	CH10	TCG GGG GAT TAC GAC	UNIDENTIFIED	2
10B43	C1H4	TCG GGG GAT TAC GAC	UNIDENTIFIED	2
10E50	V165.1	GAG AAT GGT TAC CTC	?	
10E59	V165.1	GAG AAT GGT TAC CTC	?	
11B19	V23	CAC TAT AGT AAC CGT	UNIDENTIFIED	2
11B29	V23	CAC TAT AGT AAC CGT	UNIDENTIFIED	2
11B52	V23	CAC TAT AGT AAC CGT	UNIDENTIFIED	2
11B49	C1H4	ACT ACG GCA GTA GAC	UNIDENTIFIED	2
11D85	C1H4	TAA ACC TGG GAC AGG	UNIDENTIFIED	2
10E29	V3	TCG AGT GAT TAC CTG TAC	UNIDENTIFIED	2
10E65	V3	TCG AGT GAT TAC CTG TAC	UNIDENTIFIED	2
11D39	C1H4	TTG GAT GGT AAC GAG GGG	UNIDENTIFIED	2
11D83	V23	GAT GAA TCT ATG ATG GCG	UNIDENTIFIED	2
10B63	V23	CTT CGA TTA CGA CGG GGG GGC	UNIDENTIFIED	2
10D34	C1H4	GGG GGG CTC TAT GAT GGT TAC TAC CCG	UNIDENTIFIED	2
10B66	V102	CCC CAT CTA CTA TGG TAC TAC TTG TAC	UNIDENTIFIED	2
10E38	C1H4	GAA GGG GGA CGA CTC AGG CTA CGA GGA GGT	UNIDENTIFIED	2

c

CLONE	V_H GENE	D_H SEQUENCE								D_H GENE	J_H
1A2-23	V165.1	TGG	AAT	TAC	TAC	GGT	AGT	AGC	TAC	GAC	DFL16.1 (RF1)	2
1A2-28	V165.1	TGG	AAT	TAC	TAC	GGT	AGT	AGC	TAC	GAC	DFL16.1 (RF1)	2
2A1-11	V23	GGG	GTC	TAC	TAC						DFL16.1 (RF1)	2
2A1-83	C1H4	GGG	GTC	TAC	TAC						DFL16.1 (RF1)	2
1A5-73	?	ATC	TAT	GAT	GGT	TAC	TTC	GGG			DSP2.6 (RF3)	2
1A5-61	?	ATC	TAT	GAT	GGT	TAC	TTC	GGG			DSP2.6 (RF3)	2
2A1-47	?	ATC	TAT	GAT	GGC	TTT	TTC	GGG			DSP2.6 (RF3)	2
2B1-48	V186.2	TCG	GGG	GTT	ACT	ACG	GTA	GTA	CCT		DFL16.1 (RF2)	2
2B1-78	V186.2	TCG	GGG	GTT	ACT	ACG	GTA	GTA	CCT		DFL16.1 (RF2)	2
2B1-26	V186.2	TCG	GGG	GTT	ACT	ACG	GTA	GTA	CCT		DFL16.1 (RF2)	2
1A2-08	V102	GGG	AAC	CTC							UNIDENTIFIED	2
1A2-25	V102	GGG	AAC	CTC							UNIDENTIFIED	2
1A5-52	V23	TTT	ATC	TCG							UNIDENTIFIED	2
1A5-83	V23	TTT	ATC	TCG							UNIDENTIFIED	2
2A1-67	V186.2	AGG	GGG	ATC							UNIDENTIFIED	2
2A1-63	V186.2	CGA	GAC	TAT	GAT						UNIDENTIFIED	2
2B1-46	?	AGG	GAA	CCT	CTT						UNIDENTIFIED	2
2B1-22	V165.1	AGG	GAA	CCT	CTT						UNIDENTIFIED	2
2B1-39	V165.1	AGG	GAA	CCT	CTT						UNIDENTIFIED	2
2B1-59	V165.1	AGG	GAA	CCT	CTT						UNIDENTIFIED	2
2A1-17	C1H4	CGG	GAC	GGT	AGT	TAC					UNIDENTIFIED	2
2A1-62	C1H4	CGG	GAC	GGT	AGT	TAC					UNIDENTIFIED	2
2B1-76	V186.2	TCA	ATA	GGT	AGC	GGA	CTA	CCG	CCC		UNIDENTIFIED	2
1A5-80	V186.2	AGG	AGC	ACT	GAT	TAC	GAC	CCT	CAT		UNIDENTIFIED	2
1A2-80	V3	CCC	TAT	AGT	AAC	TAC	GAG	ACC	TCG		UNIDENTIFIED	2

Figure 4. CDR3 sequences (from position 95) amplified from the $\lambda 1^+$GC of (*a*) T^{young}/B^{young}, (*b*) T^{aged}/B^{young}, and (*c*) T^{young}/B^{aged} *scid* mice. 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 (?).

haplotype (25–27). Several other members of the V_H186.2/V3 gene family were also identified in decreasing proportions, namely CH10, C1H4, V23, V3, and 24.8 V_H genes (Table 1). The dominant use of the V_H186.2 gene segment was also seen when the mice received B cells from aged donors (group T^{young}/B^{aged}). If instead, the T cells came from the aged donors (group T^{aged}/B^{young}), the V186.2 segment was one of the least frequent among the eight V genes that we identified whereas the C1H4 segment was dominant (Table 1). The dramatic influence of the age of T cells on the expression of V186.2 and C1H4 genes in the GC B cells is apparent from a graphic presentation of the results in Fig. 3.

We noted that the age of lymphocytes also influenced the diversity of V_H gene repertoire within the individual GC of the recipient mice (Table 1). On average, only two (2.0) distinct V_H genes were recovered from single GC in the T^{young}/B^{young} group of mice. However, that number nearly doubled (3.8 and 3.5, respectively) if either the B or the T cells came from aged donors (Table 1); these increases in diversity are significant at $p = 0.05$ and $p < 0.05$, respectively.

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_H 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 V_H 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 RF-3 and RF-2 (Fig. 4, *a–c*), which is 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_H sequences amplified from six different rearranged VDJ segments from the GC of the T^{young}/B^{young} 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 T^{young}/B^{young} *scid* mice. However, similar mutation frequencies were observed in other members of the V_H186.2/V_H3 gene family that were recovered from this group of mice. As an example, the VDJ segments recovered from two λ^+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_H) and four segments contained a CH10-like gene with an average of 1 mutation/89 bp (3.1/V_H). GC 8C, from another mouse, yielded seven V186.2 segments with an average mutation frequency of 1/101 bp (2.7/V_H). One mutation, a TGG→TTG 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.

The GC from mice that received either T^{aged} or B^{aged}

Table 3. *Mutations in V_H Sequences in the Amplified VDJ Fragments*

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

cells contained VDJ segments with mutations in V_H genes ranging from 1/100 to 1/400 bp and 1/140 to 1/855 bp, respectively (Table 3). Many of these V segments were either unmutated or contained mutations that would be expected from the *Taq* polymerase error (1/420 bp or 0.6 mutations/sequence) in the present study (see Materials and Methods). Fig. 6 shows an example of V186.2 and C1H4 genes recovered from two distinct λ^+GC, 2AI and 2AB, from the spleen of a T^{young}/B^{aged} recipient. The V186.2 sequences contained an average of 1 mutation/182 bp (1.5/V_H) and 1/819 bp (0.3/V_H), respectively. The three C1H4 sequences from the GC 2AI averaged 1 mutation/273 bp (1/V_H).

The average number of mutations in the sequences of any given V 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 V_H gene mutations in B cells from either the T^{aged}/B^{young} or the T^{young}/B^{aged} group were lower ($p < 0.05$) than the control group (T^{young}/B^{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 ≥ 3 mutations/sequence in the control group, were recovered from the T^{aged}/B^{young} and T^{young}/B^{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^{young}/B^{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 T^{aged} cells, and the low number of mutations in mice with B^{aged} cells preclude any meaningful analysis of R/S ratios (Table 3).

Discussion

The anti-NP response in the GC of Igh^b *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 V_H gene, V186.2, was the one most frequently recovered from the λ^+ GC B cells from the T^{young}/B^{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 λ^+, NP-specific hybridomas recovered from C57BL/6 mice also indicated that a significant number of V_H 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 λ^+ 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 V_H 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 first new finding made in the present study was that

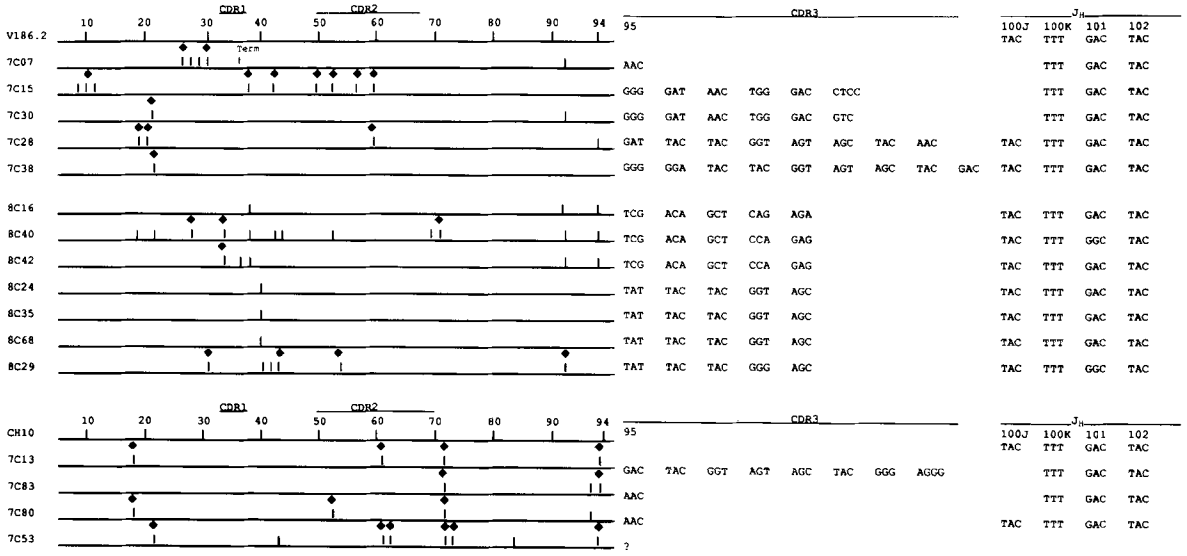

Figure 5. VDJ segments recovered from GC 7C and 8C from two different T^{young}/B^{young} *scid* mice. Amplification of the 7C DNA yielded five distinct clones with V segment homologous to the 186.2 gene and four clones containing C1H4 segments. The GC 8C yielded seven V186.2 sequences. The base differences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

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^{aged}), the V_H gene repertoire of λ^+ GC was more diverse than controls and was dominated by B cells that had rearranged the C1H4 V_H gene. To interpret this result, let us consider that both $V186.2^+$ and $C1H4^+$ 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 $V186.2^+$ 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_H cells would provide optimal help for the unmutated canonical V186.2 clones whereas the aged T_H 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^{young}/B^{aged}) was similar to that of the control group (T^{young}/B^{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_H genes other than V186.2 which were recovered from the λ^+ GC represent B cells engaged in anti-NP response, but indirect evidence supports the notion that at

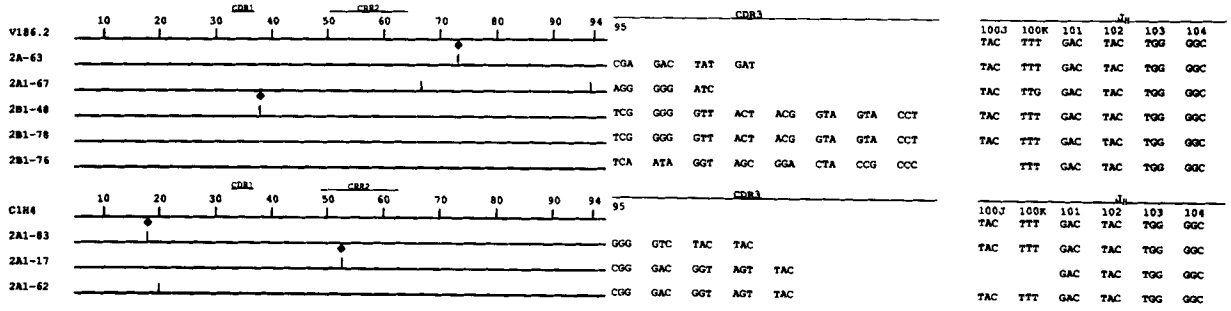

Figure 6. VDJ segments recovered from two GC, 2A1, and 2B1 from a mouse reconstituted with T^{young}/B^{aged} lymphocytes. The GC 2A1 yielded two V186.2 and three C1H4 sequences. Three V186.2 sequences from the GC 2B1 are also shown. Symbols are the same as in Fig. 5. (Sequences of V segments from Figs. 5 and 6 are available from GenBank under accession numbers U49667–U49690.)

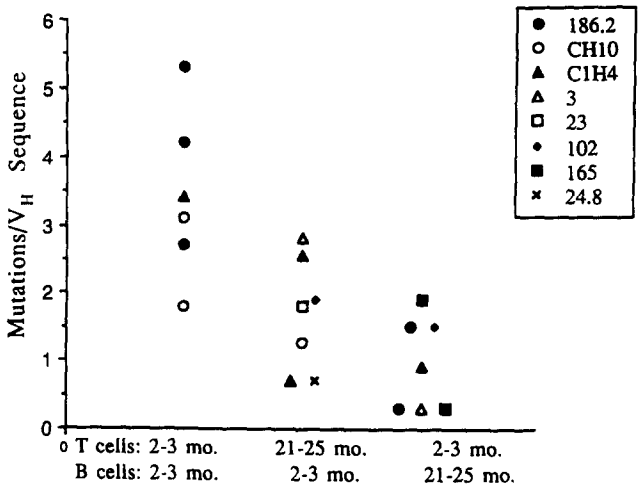

Figure 7. Mutations in the V segments recovered from $\lambda 1^+$ GC of *scid* mice reconstituted with T and B cells as indicated. Each point represents the mean from V segments that were recovered as multiple clones (n = 2–12) from a given GC: (●) 186.2; (○) CH10; (▲) C1H4; (△) 3; (□) 23; (◆) 102; (■) 165; (x) 24.8.

least some of them do. First, the amplification of unselected splenic B from unimmunized animals using similar PCR primers generated different patterns of V_H genes (35). Second, the V_H genes C1H4 and V23, which were among the most common recovered in our survey, can pair with the λ1 L chain to produce a NIP-binding antibody (43, 44). Third, Maizels and Bothwell (25) have shown that the NP-binding hybridomas generated from mice immunized a T-independent antigen, NP-Ficoll use a large repertoire of unmutated V_H genes including C1H4 and V23. This important result, which was the first to demonstrate that the dominance of $V186.2^+$ B cells depends on T cell help, provides a conceptual framework for our findings that the aging $CD4^+$ lymphocytes cannot sustain the preferential expression of the canonical NP-reactive cells.

B cell clones expressing the germline low affinity receptors (encoded by the noncanonical V_H segments) have apparently undergone somatic mutation in the GC of lymphocyte-reconstituted *scid* mice (Figs. 5 and 7). Mutants which presumably acquired increased affinity for the antigen were likely to be further selected, allowing them to compete with the canonical clones and enter the pool of memory cells. We propose that the activation of B cells using noncanonical germline IgV genes by itself does not compromise the adaptability of the immune response as long as the mutation mechanism is operational. In support of this hypothesis, we have recently observed that 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 T^{young}/B^{aged} 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 (T^{young}/B^{young}). 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.

We thank Dr. George A. Carlson, McLaughlin Research Institute, for providing us with BIO *scid* mice, Dr. Garnett Kelsoe, University of Maryland School of Medicine, for review of the manuscript, and Ms. June Green for meticulous typing.

This work was supported in part by U.S. Public Health Service grant AG08193.

Address correspondence to Dr. Jan Cerny, Department of Microbiology and Immunology, University of Maryland School of Medicine, 655 West Baltimore Street, Bressler Research Building, Room 3-015, Balti-

more, MD 21201. J. Stedra's present address is Institute of Molecular Biology and Genetics, Czech Academy of Sciences, Prague, Czech Republic.

Received for publication 7 August 1995 and in revised form 29 September 1995.

References

1. Makinodan, T., and M.M.B. Kay. 1980. Age influence on the immune system. *Adv. Immunol.* 29:287–330.
2. Wade, A.W., and M.R. Szewczuk. 1984. Aging and the mucosal associated lymphoid system. *Adv. Immunol.* 36:143–188.
3. Miller, R.A. 1991. Aging and immune function. *Int. Rev. Cytol.* 124:187–215.
4. Kishimoto, S., T. Takahama, and H. Mizumachi. 1976. In vitro immune response to the 2,4,6-trintrophenyl determinant in aged C57BL/6 mice: changes in the humoral response to avidity for the TNP determinant and responsiveness to LPS effect with aging. *J. Immunol.* 116:294–301.
5. Goidl, E.A., J.B. Innes, and M.E. Weksler. 1976. Immunological studies of aging. II. Loss of IgG and high avidity plaque forming cells and increased suppressor cell activity in aging mice. *J. Exp. Med.* 144:1037–1048.
6. Zharhary, D., Y. Segev, and H. Gershon. 1977. The affinity and spectrum of crossreactivity of antibody production in senescent mice: the IgM response. *Mech. Ageing Dev.* 6:385–392.
7. Doria, G., G. D'Agostaro, and A. Poretti. 1978. Age dependent variations of antibody avidity. *Immunology.* 35:601–611.
8. Weksler, M.E., J.B. Innes, and G. Goldstein. 1978. Immunological studies of aging. IV. The contribution of thymic involution to the immune deficiencies of aging mice. *J. Exp. Med.* 148:966–1006.
9. Smith, A.H. 1976. The effects of age on the immune response to type III pneumococcal polysaccharide (SIII) and bacterial lipopoly saccharide (LPS) in BALB/c, SJL/J and C3H mice. *J. Immunol.* 116:469–474.
10. Zharhary, D., and N.R. Klinman. 1986. A selective increase in the generation of phosphorylcholine specific B cells associated with age. *J. Immunol.* 136:368–370.
11. Yin, J.-Z., S.R.S. Gottesman, M.K. Bell, and G.J Thorbecke. 1988. Resistance to low dose tolerance and enhanced antibody responses of aged as compared to young mice immunized with pneumococcal polysaccharides. *Aging Immunol. Infect. Dis.* 1:131–137.
12. Nicoletti, C., X. Yang, and J. Cerny. 1993. Repertoire diversity of antibody response to bacterial antigens in aged mice. III. Phosphorylcholine antibody from young and aged mice differ in structure and protective activity against infection with *Streptococcus pneumoniae*. *J. Immunol.* 150:543–549.
13. Ammann, A.J., G. Schiffman, and R. Austrian. 1980. The antibody responses to pneumococcal capsular polysaccharides in aged individuals. *Proc. Soc. Exp. Biol. Med.* 164:312–316.
14. Riley, S.C., B.G. Froscher, P.J. Linton, D. Zharhary, K. Marcu, and N.R. Klinman. 1989. Altered V_H gene segment utilization in response to phosphorylcholine by aged mice. *J. Immunol.* 143:3798–3805.
15. Nicoletti, C., and J. Cerny. 1991. The repertoire diversity and magnitude of antibody response to bacterial antigens in aged mice. I. Age-associated changes in antibody response differ according to the mouse strain. *Cell Immunol.* 133:72–83.
16. Goidl, E.A., X. Chen, and D.H. Schulze. 1990. B cell function in the immune response of the aged. *Aging Immunol. Infect. Dis.* 2:135–138.
17. Perlmutter, R.M., S.T. Crews, R.E. Douglas, G. Sorensen, N. Johnson, N. Nivera, P.J. Gerahart, and L. Hood. 1984. The generation of diversity in phosphorylcholine-binding antibodies. *Adv. Immunol.* 35:1–37.
18. Jacob, J., G. Kelsoe, K. Rajewsky, and U. Weiss. 1991. Intraclonal generation of antibody mutants in germinal centers. *Nature (Lond.).* 354:389–392.
19. Berek, C., A. Berger, and M. Apel. 1991. Maturation of the immune response in germinal centers. *Cell.* 67:1121–1129.
20. Miller, C., and G. Kelsoe. 1995. Somatic hypermutation is absent in the germinal centers of aged mice. *J. Immunol.* 155: 3377–3384.
21. Jacobson, E.B., L.H. Corporale, and G.J. Thorbecke. 1974. Effect of thymus cell injections on germinal center formation in lymphoid tissues of nude (thymus less) mice. *Cell. Immunol.* 13:416–430.
22. Gastkemper, N.A., A.S. Wubbena, F.J.H. Gimbrere, A. DeGraff, and P. Nieuwenhuis. 1981. Germinal centers and the B-cell system. V. Presence of germinal center precursor cells among lymphocytes of the thoracic duct in the rat. *Cell Tissue Res.* 219:281–289.
23. Vonderheide, R.H., and S.V. Hunt. 1990. Does the availability of either B cells or $CD4^+$ cells limit germinal center formation? *Immunology.* 69:487–489.
24. Miller, C., J. Stedra, G. Kelsoe, and J. Cerny. 1995. Facultative role of germinal centers and T cells in the somatic diversification of IgV_H genes. *J. Exp. Med.* 181:1319–1331.
25. Maizels, N., and A. Bothwell. 1985. The T-cell–independent immune response to the hapten NP uses a large repertoire of heavy chain genes. *Cell.* 43:715–720.
26. Cumano, A., and K. Rajewsky. 1986. Clonal recruitment and somatic mutation in the generation of immunologic memory to the hapten NP. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:2459–2468.
27. Bothwell, A.L.M., M. Paskind, M. Reth, T. Imanishi-Kari, K. Rajewsky, and D. Baltimore. 1981. Heavy chain variable region contribution to the NP^b family of antibodies: somatic mutation evident in a γ 2a variable region. *Cell.* 24:625–637.
28. Bothwell, A.L.M., M. Paskind, M. Reth, T. Imanishi-Kari, K. Rajewsky, and D. Baltimore. 1982. Somatic variants of murine immunoglobulin lambda light chains. *Nature (Lond.).* 298:380–382.
29. Levy, N.S., U.V. Malipiero, S.G. Lebecque, and P.J. Gearhart. 1989. Early onset of somatic mutation in immunoglobulin V_H genes during the primary immune response. *J. Exp. Med.* 169:2007–2019.
30. Jacob, J., J. Przylepa, C. Miller, and G. Kelsoe. 1993. 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 centers B cells. *J. Exp. Med.* 178: 1293–1307.
31. McHeyzer-Williams, M.G., M.J. McLean, P.A. Lalor, and G.J.V. Nossal. 1993. Antigen driven B cell differentiation in

vivo. *J. Exp. Med.* 178:295–307.
32. Jacob, J., R. Kassir, and G. Kelsoe. 1991. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl) acetyl. I. The architecture and dynamics of responding cell populations. *J. Exp. Med.* 173:1165–1175.
33. Jacob, J., and G. Kelsoe. 1992. In situ studies of the primary immune response to (4-hydroxy-3-nitrophenyl) acetyl. II. A common clonal origin for periateriolar lymphoid sheath–associated foci and germinal centers. *J. Exp. Med.* 176:679–687.
34. Stedra, J., and J. Cerny. 1994. Distinct pathways of B cell differentiation. I. Residual T cells in athymic mice support the development of splenic germinal centers and B cell memory without an induction of antibody. *J. Immunol.* 152:1718–1726.
35. Gu, H., D. Tarlinton, W. Muller, K. Rajewsky, and I. Forster. 1991. Most peripheral B cells in mice are ligand selected. *J. Exp. Med.* 173:1357–1371.
36. Ichihara, Y., H. Hayashida, S. Miyazaw, and Y. Kurosawa. 1989. Only DFC16, DSP2 and DQ52 gene families exist in mouse immunoglobulin heavy chain diversity gene loci, of which DFL16 and DSP originate from the same primordial D_H gene. *Eur. J. Immunol.* 19:1847–1854.
37. Gu, H., D. Kitamura, and K. Rajewsky. 1991. B cell development regulated by gene rearrangement arrest of maturation by membrane-found Dμ protein and selection of D_H element reading frames. *Cell.* 65:47–54.
38. Kocks, C., and K. Rajewsky. 1989. Stable expression and somatic hypermutation of antibody V regions in B-cell developmental pathways. *Annu. Rev. Immunol.* 7:537–559.
39. Tao, W., F. Hardardottin, and A.L.M. Bothwell. 1993. Extensive somatic mutation in the Ig heavy chain V genes in a late primary anti-hapten immune response. *Mol. Immunol.* 30: 593–602.
40. Tew, J.G., R.P. Phipps, and T.E. Mandel. 1980. The maintenance and regulation of the humoral immune response: persisting antigen and the role of follicular antigen-binding dendritic cells as accessory cells. *Immunol. Rev.* 53:175–201.
41. Lerner, A., T. Yamada, and R.A. Miller. 1989. Pgp-1^{hi} T lymphocytes accumulate with age in mice and respond poorly to concanavalin A. *Eur. J. Immunol.* 19:977–982.
42. Ernst, D.N., M.V. Hobbs, B.E. Torbett, A.L. Glasebrook, M.A. Rehse, K. Bottomly, K. Hayakava, R.R. Hardy, and W.O. Weigle. 1990. Differences in the expression profiles of CD45RB, Pgp-1 and 3G11 membrane antigens and in the patterns of T lymphokine secretion of splenic $CD4^+$ cells from young and aged mice. *J. Immunol.* 145:1295–1302.
43. Hawklins, R.E., and G. Winter. 1992. Cell selection strategies for making antibodies from variable gene libraries: trapping the memory pool. *Eur. J. Immunol.* 22:867–870.
44. McHeyzer-Willaim, G.C., G.J. Nossal, and P.A. Lalor. 1991. Molecular characterization of single memory B cells. *Nature (Lond.).* 350:502–505.