Somatic Hypermutation Shapes the Antibody Repertoire of Memory B Cells in Humans

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Abstract
High-affinity antibodies produced by memory B cells differ from antibodies produced in naive B cells in two respects. First, many of these antibodies show somatic hypermutation, and second, the repertoire of antibodies expressed in memory responses is highly selected. To determine whether somatic hypermutation is responsible for the shift in the antibody repertoire during affinity maturation, we analyzed the immunoglobulin lambda light chain (Igλ) repertoire expressed by naive and antigen-selected memory B cells in humans. We found that the Igλ repertoire differs between naive and memory B cells and that this shift in the repertoire does not occur in the absence of somatic hypermutation in patients lacking activation-induced cytidine deaminase (AID). Our work suggests that somatic hypermutation makes a significant contribution to shaping the antigen-selected antibody repertoire in humans.

Key words: immunoglobulin repertoire • activation-induced cytidine deaminase • somatic hypermutation • memory B cell • affinity maturation

Introduction
The affinity of antibodies for their cognate antigens increases during immune responses (1). In depth analysis of hybridoma antibodies specific for influenza hemagglutinin or for small chemical haptens such as 2-phenyl-5-oxazolone or 4-hydroxy-3-nitrophenyl acetyl (NP) revealed that somatic hypermutation is one of the mechanisms that produce this increased affinity (2–9). For example, the VH186–2 + Igλ antibodies dominate the initial antibody response to NP, and mutation from TrpH33 to LeuH33 brings about a 10-fold increase in affinity of these VH186–2 + Igλ antibodies (8, 10–13). Increased affinity is also accompanied by a shift in the antibody repertoire, and secondary high-affinity responses to NP are dominated by Igκ antibodies and not Igλ, suggesting that repertoire shifts contribute to affinity maturation (3, 10, 14, 15). Little is known about this shift in the repertoire and how it relates to somatic hypermutation.

Activation-induced cytidine deaminase (AID) is a germinal center B cell–restricted molecule that carries cytidine deaminase activity and is required for switch recombination and somatic hypermutation in mice and humans (16–18). In the absence of AID, B cells are unable to undergo somatic hypermutation or produce secondary antibodies despite germinal center formation (17, 18). Here we report on the Igλ antibody repertoire in humans deficient in AID. We find that AID is essential for the shift in repertoire between naive and antigen-selected memory B cells.

Materials and Methods
Patient Samples and Cell Preparation. AID-deficient patients and AID mutations have been described (18). Patients P1, P13, P14, P17, and P18 were 10, 11, 4, 14, and 2 yr old, respectively at the time of blood donation, and they did not suffer from chronic infections. They were treated with intravenous Ig supplementation. Control donors C1, C2, C3, C4, C5, C6, and C7 were healthy and 32, 11, 35, 2, 28, 41, and 33 yr old, respectively when blood samples were obtained. Blood mononuclear cells were isolated on Ficoll gradients. Control CD19+ B cells were fractionated into naive CD19+IgM+CD27− and memory CD19+IgM+CD27+ B cells by cell sorting on FACS Vantage™. Due to absence of secondary isotypes in AID-deficient patients, AID B cells are all IgM+ and were therefore sorted into naive CD19+CD27− and memory CD19+CD27+ B cells without IgM staining. Antibodies used for staining were FITC–anti-CD19,
Human Igλ Memory B Cell Repertoire Is Shaped by Somatic Hypermutation

**Results and Discussion**

To determine whether there is a shift in repertoire between naive and antigen-selected B cell compartments in humans, we compared the unmutated germline Igλ sequences to mutated Igλ sequences obtained from CD19+ peripheral B cells from four control donors. In humans, Igλ light chains are found in 30–40% of all antibodies, and among the 10 Vλ gene families three (Vλ1, Vλ2 and Vλ3) represent >80–90% of all Vλ genes (21, 22). We found that the distribution of Vλ1 and Vλ2, two of the most frequently used human Vλ families, differs between germline-encoded and mutated antibodies (Fig. 1 A; total of 239 individual sequences): Vλ1 is decreased and Vλ2 increased.

**Figure 2.** Igλ repertoire expressed in naive and memory B cells from control donors. (A) Vλ1 and Vλ2 gene usage in naive CD19+IgM+CD27− (open bars) and memory CD19+IgM+CD27+ (solid bars) B cells in three unrelated controls. 33, 33, and 52 Vλ1 sequences from naive B cells from donors C5, C6, and C7, respectively were compared with 39, 42, and 63 Vλ1 sequences from memory B cells from the same individuals. The percent Vλ utilization is indicated on the y axis. (B) Combined total Vλ1 and Vλ2 gene usage in germline and mutated Vλ1 sequences from control donors. 121 germline-encoded and 118 mutated sequences were obtained from the four control donors. Asterisk (*) indicates statistically significant difference (Vλ1, P = 0.022; Vλ2, P = 0.0004).

**Reverse Transcription PCR, Cloning, and Sequencing.** Total RNA was extracted from 10^6–10^7 purified cells using TRIzol Reagent (GIBCO BRL) and reverse transcribed in a 10-μl reaction with Superscript II (GIBCO BRL). For reverse transcription (RT)-PCR reactions, 1 μl of cDNA was amplified for 30–35 cycles of 30 s at 94°C, 30 s at 58°C (Vλ1-Cμ) or at 55°C (Vλ-Cμ) and 30 s at 72°C with a final 10-min extension at 72°C using HotStarTaq™ DNA polymerase (QIAGEN) and the following primers: Vλ1-Cμ sense, 5′-GGG(G/A)TC(T/G)TTCTCTGG(C/G)TCC-3′; Vλ9 sense, 5′-ATCCCTGATGCCTTCAGTCTTG-3′; Vλ10 sense, 5′-GATCTCAGAGAGATATTCTGCATCC-3′; and Cμ antisense, 5′-CAGTGTGGCCTTGTCCTTG-3′. Sense FR1 Vλ1 and antisense Cμ primers were as described previously (19, 20). RT-PCR products were run on 2% agarose gels, and PCR products were gel purified (Qiaquick™; QIAGEN) and cloned into TA vectors (Invitrogen). Double-stranded DNA sequences were obtained using anti-sense Cμ or Cλ primers and Dye Terminator Cycle Sequencing (PE Applied Biosystems). Sequences were analyzed using Ig BLAST®. When two or more identical sequences were found, they were counted as a single clone. Sequences were considered mutated when they displayed two or more nucleotide differences from their germline counterparts. Differences in gene distribution between naive and memory B cells were analyzed with chi-square tests (Cochran-Mantel-Haenszel test) adjusted by the Bonferroni method for multiple testing and they were considered significant when P values were equal to or less than 0.05.

**Figure 1.** Igλ repertoire expressed in peripheral B cells from control donors. (A) Vλ1 and Vλ2 gene usage in germline-encoded (open bars) and mutated (solid bars) sequences from CD19+ peripheral B cells in four unrelated controls. 25, 15, 46, and 34 germline Vλ1 sequences from donors C1, C2, C3, and C4 were compared with 33, 23, 33, and 29 mutated Vλ1 sequences from the same individuals. The percent Vλ utilization is indicated on the y axis. (B) Combined total of Vλ1 and Vλ2 gene usage in germline and mutated Vλ1 sequences from control donors. 121 germline-encoded and 118 mutated sequences were obtained from the four control donors. Asterisk (*) indicates statistically significant difference (Vλ1, P = 0.022; Vλ2, P = 0.0004).
among mutated IgAs, and this difference is independent of the age of the donors (Fig. 1, A and B).

To further analyze the shift in Ig\(\lambda\) repertoire between naive and memory B cells, we fractionated peripheral B cells using CD27 memory marker and isolated mutated Ig(H9) and memory (CD19\(^+\)IgM\(^+\)CD27\(^+\)) B cells from control donors (23, 24). The difference in V\(\lambda\) distribution was also found when comparing naive and memory B cell compartments (Fig. 2; total of 262 sequences). Antibodies cloned from memory B cells were predominantly mutated and showed decreased V\(\lambda\)1 and increased V\(\lambda\)2 gene usage (Fig. 2, A and B). We conclude that there is a shift in the Ig\(\lambda\) repertoire between the naive and antigen-selected memory B cell compartments in humans.

To determine whether the shift in Ig\(\lambda\) repertoire between the naive and memory compartments is related to somatic hypermutation, we analyzed the Ig\(\lambda\) genes expressed in naive and memory B cells from patients lacking activation-induced deaminase (AID) (17, 18). AID has been shown to be essential for both hypermutation and switch recombination but does not appear to be necessary for normal B cell development in mice and humans (17, 18). Patients with AID deficiency showed no secondary antibodies and no somatic mutation; nevertheless, these individuals displayed enlarged tonsils with germinal centers and showed normal numbers of CD19\(^+\)CD27\(^+\) B cells (18). The CD27\(^+\)IgM\(^+\) B cells found in AID-deficient patients resembled authentic CD27\(^+\)IgM\(^+\) memory B cells in that they showed normal selection against V\(\lambda\)1–69, a V\(\lambda\) gene that is frequently found in B lymphoid chronic lymphocytic leukemias producing autoreactive antibodies (Fig. 3) (19, 25, 26). However, the antibodies expressed in antigen-selected memory B cells in five AID-deficient patients differed from the three controls in that they showed no mutations, and there was no shift in the VA repertoire between naive B cells and antigen-selected memory B cells (compare Figs. 2 and 4; total of 330 sequences). In particular, there was no increase in VA2 gene expression and no relative decrease in VA1 (Fig. 4). In addition, V\(\lambda\)5–51 gene usage was favored in the memory CD27\(^+\) B cells from AID-deficient patients but not in normal controls (Fig. 3).

Somatic hypermutation is known to increase antibody affinity during immune responses. However, the contribution of mutation to shaping the antibody repertoire has not been determined. We have found a global shift in the Ig\(\lambda\)
antibody repertoire between naive and memory B cells from normal donors. This shift in repertoire is associated with somatic hypermutation and is AID dependent. We conclude that AID and hypermutation make a significant contribution to shaping the antigen-selected memory B cell repertoire in humans.

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