Immunoglobulin Heavy Chain Variable Region Gene Replacement As a Mechanism for Receptor Revision in Rheumatoid Arthritis Synovial Tissue B Lymphocytes

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Abstract

Mature B cells can alter their antibody repertoires by several mechanisms, including immunoglobulin heavy chain variable region (V_H) replacement. This process changes the antigen combining site by replacing a portion of the original V_H/diversity/heavy chain joining region (V_H-DJ_H) rearrangement with a corresponding portion of a new V_H segment. This exchange can involve cryptic heptamer-like sequences embedded in the coding regions of V_H genes.

While studying the B lymphocytes that expand in the synovial tissues of patients with rheumatoid arthritis (RA), clones with V_HDJ_H variants that were apparently generated by V_H replacement were identified with surprising frequency (~8%). Examples of multiple independent V_H replacement events occurring in distinct progeny clones were also identified. These secondary V_H rearrangements were documented at both the cDNA and genomic DNA levels and involved several heptamer-like sequences at four distinct locations within V_H (three sites in framework region 3 and one in complementarity determining region 2). The identification of blunt-ended double-stranded DNA breaks at the embedded heptamers and the demonstration of recombinase activating gene (RAG) expression suggested that these rearrangements could occur in the synovial tissues, presumably in pseudo-germinal centers, and that they could be mediated by RAG in a recognition signal sequence–specific manner. The presence of V_H mutations in the clones that had undergone replacement indicated that these B cells were immunocompetent and could receive and respond to diversification signals. A relationship between these secondary V_H gene rearrangements and the autoimmunity characteristic of RA should be considered.

Key words: antibody diversity • recombinase activating genes • immune tolerance • autoimmunity • rheumatoid factor

Introduction

Antibody diversity is initially established in developing B lymphocytes in the bone marrow by the ordered rearrangement of V, (D), and J elements (1, 2). These rearrangements occur via site-specific recombination events that classically involve recognition signal sequences (RSS)[1] comprised of conserved heptamers and less conserved nonamers (3–5). As this process is largely random, receptors can be assembled that possess sufficient affinity for autoantigens to necessitate their elimination. However, such cells may be afforded another opportunity to express a tolerant receptor by undergoing secondary V region gene rearrangements that can involve either the H or L chain loci (6–13). These secondary rearrangements change a major portion of the antigen-combining site so the new receptor is significantly different from the original. The new receptor can consist of a completely new V_LJ_L rearrangement or a V_H-DJ_H rearrangement that is modified by the replacement of a

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[1]*Abbreviations used in this paper:* ds, double-stranded; FR, framework region; GC, germinal center; LM, ligation-mediated; RA, rheumatoid arthritis; RAG, recombinase activating gene; RF, rheumatoid factor; RSS, recognition signal sequence(s); RT, reverse transcription.

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portion of the originally rearranged V segment ("V_H replacement" [9]). If these secondary rearrangements provide the B cell with an acceptable antigenic specificity, it proceeds to the periphery as a functional B lymphocyte. This process of secondary rearrangement, presumably initiated by autoreactivity, has been termed receptor editing (6–8).

In the periphery, mature B cells can develop additional receptor/antibody diversity during the germinal center (GC) reaction. Although this process was long considered to be limited to the accumulation of point mutations (14), recent studies suggest that more extensive changes in the primary repertoire can occur by a variety of mechanisms. These mechanisms include the insertion and/or deletion of DNA segments of variable lengths (12, 15, 16), and the opportunity to again undergo V gene rearrangement. As these secondary rearrangements are thought to be initiated in those B cells undergoing the GC reaction that lack adequate affinity for the selecting antigen, this process appears to be different from receptor editing and therefore has been termed receptor revision (11, 12, 17, 18).

Furthermore, receptor revision could potentially lead to autoreactivity if the drive to clonal diversification created self-reactive receptors that were ineffectively selected against, because of either genetic or acquired defects in immunoregulation. Therefore, this process could play an important role in the development of certain autoimmune disorders.

Rheumatoid arthritis (RA) is a relatively common, chronic, destructive arthropathy that results from an inflammatory synovitis of peripheral joints (19). These inflammatory processes appear to be mediated by cells of the myeloid, macrophage, and lymphoid lineages (20). Although there has been considerable controversy regarding the relative importance of the various cell types and their products in the inflammatory reactions of RA, it seems likely that all cell lineages participate in disease pathogenesis. B lymphocytes may not be necessary to initiate the inflammatory reactions characteristic of RA (21), but B cells and their products can perpetuate and potentiate these responses (22–25). This perpetuation most likely involves at least two functions of B cells: their abilities to present antigen to T cells and to elaborate antibodies. Central to each of these functions is the (auto)antigenic specificity encoded in the V regions of the B cell's Ig molecules. These antigenic specificities include the classical anti-IgG/rheumatoid factor (RF) reactivity (26, 27) as well as other less well-defined reactivities that presumably are directed at tissue autoantigens (28–30) and possibly etiologic environmental antigens (31–33). Furthermore, a recent murine model that has some features similar to the human disease (34) suggests that B cells and their products could play an essential role in the disease process (35, 36).

Histologically, the synovial tissues of some RA patients contain collections of B and T lymphocytes and follicular dendritic cells that resemble GCs (37, 38). These pseudo-GCs possess at least some of the functions of typical GCs, i.e., they appear to be able to support clonal amplification and Ig V gene diversification as measured by the accumulation of new mutations. In the course of studying the clonally amplified B lymphocytes that develop V gene point mutations in the synovial tissues of RA patients, we identified a series of clones that exhibited V_HDJ_H variants that were apparently generated by V_H replacement. However, unlike previous studies of V_H replacement in murine B cell lines (39–42) and transgenic mice (6–8, 43) and in transformed (44–46) and normal human B lymphocytes (18, 45), these secondary V_H rearrangements occurred relatively frequently and involved several heptamer-like sequences at four distinct locations within V_H. Our data suggest that these rearrangements can occur in situ and could be mediated by products of the recombinase activating genes (RAGs) in an RSS-specific manner. A preliminary report of these findings was presented previously (47).

Materials and Methods

Patient Samples. Synovial tissue was obtained from three patients who fulfilled the American College of Rheumatology criteria for the diagnosis of RA (48). For patient 1 (a 29-yr-old Hispanic male), synovial tissue was collected from the right and left hips at the time of bilateral joint replacements; these samples are labeled ST1R or ST1L, respectively. For patient 2 (a 25-yr-old Black female) and 3 (a 56-yr-old White female), synovial tissue was collected from individual knee joints; these samples are labeled ST2 and ST3. Each sample was digested with collagenase type IV, DNase I (both from Worthington Biochemical Corporation), and hyaluronidase (Sigma-Aldrich) to obtain single cell suspensions. Mononuclear cells were isolated from these cell suspensions by density gradient centrifugation using Ficoll-Paque (Amersham Pharmacia Biotech) and then cryopreserved as viable cells in 20% fetal bovine serum with 10% dimethyl sulfoxide using a programmable cell freezing machine (CryoMed).

Isolation of DNA and RNA and Preparation of cDNA. Genomic DNA was isolated from mononuclear cells using the Puregene DNA isolation kit (Gentra Systems) and total RNA was isolated using Ultraspec RNA (Biotech Laboratories). 1 μg of RNA was reverse transcribed to cDNA using 200 U Moloney murine leukemia virus reverse transcriptase (GIBCO BRL/Life Technologies), 1 U RNase inhibitor (5 Prime 3 Prime), and 20 pmol oligo dT primer in a total volume of 20 μl. These reagents were incubated at 42°C for 1 h, heated to 65°C for 10 min to stop the reactions, and then diluted to a final volume of 100 μl.

PCR Amplification of Ig V_H Gene DNA and cDNA. The sequences of the primers used in these reactions were published previously (49). Genomic DNA (100 ng) was amplified using a sense V_H1 family–specific framework region (FR)1 primer in conjunction with an antisense J_H consensus primer. These reactions were carried out in 50 μl using 5 pmol of each primer and were cycled with a 9600 GeneAmp System (PerkinElmer) as follows: denaturation at 94°C for 40 s, annealing at 65°C for 45 s, and extension at 72°C for 40 s. After 35 cycles, extension was continued at 72°C for an additional 10 min. cDNA was amplified using the same sense primer paired with either a Cμ, Cγ, or Cα antisense primer (50). However, the reactions were cycled as follows: denaturation at 94°C for 45 s, annealing at 65°C for 45 s, and extension at 72°C for 45 s. After 35 cycles, extension was continued at 72°C for an additional 10 min.

Creation of DNA and cDNA Libraries and Sequencing of Selected Clones. V_H1-specific DNA and cDNA libraries were created from the PCR products described above. 10 ng of each PCR

product was ligated into pCR2.1 vector and transformed into INVαF′ competent cells using the TA cloning kit (Invitrogen). Transformed INVαF′ cell colonies were identified and isolated by color selection after overnight growth on Luria-Burtani (LB) agar plates containing X-Gal and ampicillin. Plasmids were isolated from each selected colony using Wizard minipreps (Promega) after overnight growth in LB broth containing kanamycin. The DNA sequences of individual molecular clones were determined using Big Dye DNA sequencing kit (PerkinElmer) with M13 forward and reverse primers and an automated genetic analyzer ABI PRISM 377 (Applied Biosystems). The error rate calculated for these sequence analyses was 8.36×10^{-4}, based on the detection of 13 mutations in 15,554 nucleotides of C_H genes of these and other human cDNA V_HDJ_H-C_H transcripts from RA synovial B cells. All V_H gene sequences discussed in this study have been entered in EMBL/GenBank/DDBJ under accession nos. AF308542–AF308567.

PCR Conditions for Ig V_H Gene Fingerprinting Assay. The original Ig V_H gene fingerprinting assay (51) was modified (52) into two stages, starting with either genomic DNA or cDNA as templates. The sequences of the primers used in these reactions and the details of the two PCR stages have been published previously (52).

Analyses of Ig DNA Breaks by Ligation-mediated PCR. To avoid adventitious breaks, genomic DNA was prepared by the agarose plug method (53) from $2–3 \times 10^5$ $CD19^+$ cells isolated using magnetic beads as described (54). To identify blunt-ended double-stranded (ds) DNA breaks, ligation-mediated (LM)-PCR was employed (55). In brief, linker ligation was performed in 25–50-μl reactions using T4 DNA ligase and the linker-ligated DNA was diluted with ligation buffer to adjust the amounts of DNA used in the subsequent seminested PCR (54). 2 μl of linker-ligated DNA was amplified using a sense V_H1 family–specific leader primer in conjunction with an antisense linker primer (GCGGTGACCCGGGAGATCTGAATTCAC). These reactions were performed in 25 μl using 5 pmol of each primer and were cycled as follows: denaturation at 94°C for 30 s, annealing at 63°C for 45 s, and extension at 72°C for 45 s. After 35 cycles, extension was continued at 72°C for an additional 10 min. Next, 2 μl of each PCR product was reamplified with 5 pmol of V_H1 family–specific FR1 primer and the same antisense linker primer. The reactions were carried out in 25 μl and cycled as described above. After 30 cycles, extension was continued at 72°C for an additional 10 min. The V_H LM-PCR products were visualized with ethidium bromide staining.

Analyses of RAG-1 Expression by Nested Reverse Transcription PCR. RAG-1 was amplified from cDNA by nested PCR using specific primer pairs that flank a 5.2-kb intron in the germline gene: sense, TGCAGACATCTCAACACTTTGGCCAG; antisense 1, TTTCAAAGGATCTCACCCGGAACAGC; antisense 2, AGCTTAAATTTCCATTCTGAATT. This PCR strategy has been used successfully to analyze RAG-1 expression in tonsilar GC B cells (56).

Results

Clonal Amplification among Synovial Tissue B Lymphocytes

HCDR3 length is a useful estimator of clonal diversity in polyclonal B cells (51). Comparisons of HCDR3 lengths generated from both the genomic DNA and cDNA of the same sample indicate the relative frequencies and activation states of individual B cell clones in polyclonal populations (52). Using this combined approach, we analyzed the B lymphocytes infiltrating the synovial tissues from four different joints of three RA patients. Widespread oligoclonality was found among the IgM-, IgG-, and IgA-expressing B cells in the four synovial tissue samples (52; see Figs. 3 and 4, and data not shown). Based on comparisons of the genomic DNA and cDNA fingerprinting assays, these clonal expansions involved both activated and memory B cells. Such clonal expansions are a common and characteristic feature of the B cell repertoires of RA patients (38, 52, 57–59), and they suggest in situ activation and growth of B lymphocytes with restricted B cell receptors (BCRs) that may be reactive with local (auto)antigens.

cDNA Sequence Analyses of V_H1-expressing B Cell Clones Reveal Evidence Consistent with V_H Replacement at Three Distinct Sites in FR3

To determine if diversification had occurred among the progeny of these expanded B cell clones, we prepared V_H1-IgM– and V_H1-IgG–expressing libraries from cDNA of the ST1R and ST1L synovial samples and determined the cDNA sequences of 95 random clones. The V_H1 family was chosen for analysis based on the results of the V_H fingerprinting analyses. As expected, several expanded B cell clones were found in each sample as indicated by the presence of identical HCDR3 sequences. In many instances, these clones contained distinct point mutations in their V_H genes, indicating that intraclonal diversification had occurred (data not shown).

Five sets of cDNA clones (Table I) were especially interesting because they contained clonal progeny that exhibited a significant discordance in the sequence between the 3′ and the 5′ regions of their rearranged V_HDJ_H genes (see Figs. 2–5). Among the members of these clones, there was an average of >99.9% similarity (usually 100% identity) from the distal portions of FR3 through HCDR3 to the end of the C_H sequence. In contrast, there was an average of <81.6% sequence similarity between the clonal members 5′ of these portions of FR3. This discordance is consistent with V_H replacement (represented schematically in Fig. 1). However, unlike previous examples of this phenomenon that involved rearrangements to a single site in FR3 (6–8, 18, 39–46), the rearrangements detected in the synovial tissue cDNA clones occurred at three distinct sites spanning FR3 (see Figs. 2–5).

Rearrangements Involving the 3′ FR3 Heptamer. Fig. 2 lists an example of V_H replacement occurring at the 3′ end of FR3 of two IgG-expressing clones from ST1L (clones G27 and G29). In this and the following figures, the sequence of the original B cell clone is listed above the new "replaced" B cell clone, with the most similar germline gene counterparts listed above and below the original and new rearrangements, respectively. The replacement in Fig. 2 occurs at an embedded heptamer of reverse orientation (TACTGTG; displayed with black background in the figure) that has been identified at the end of murine and human V_H genes (39–41, 60). These clones display 100%

Table I. *Molecular Characterization of the RA Synovial Tissue B Cell Clones That Underwent V_H Replacement*

Clone	Source	No. of identical sequences	V_H gene(s) used	Ig isotype	Type of diversification		No. of mutations	
					V_H mutation	V_H replacement	FR R/S	CDR R/S
ST1R M5	cDNA	1	V_H 1–58	IgM	−	−	0/0	0/0
ST1R M1	cDNA	1	V_H 1–58	IgM	+	−	0/1	0/0
ST1R M8	cDNA	1	V_H 1–58	IgM	+	−	0/1	0/0
ST1R M26	cDNA	1	V_H 1–58	IgM	+	−	1/0	0/0
ST1R M6	cDNA	1	V_H 1–58/V_H 1–18	IgM	+	+	5/2	1/0
ST1R M31	cDNA	1	V_H 1–58/V_H 1–24	IgM	+	+	1/2	0/0
ST1R M10	cDNA	1	V_H 1–58/V_H 1–69	IgM	+	+	10/5	4/1
ST1R M17	cDNA	1	V_H 1–08	IgM	+	−	4/4	4/1
ST1R M9	cDNA	1	V_H 1–08/V_H 1–69	IgM	+	+	0/1	0/0
ST1R G1	cDNA	1	V_H 3–09/V_H 1–02	IgG	+	+	17/3	18/1
ST1R G2	cDNA	1	V_H 3–09/V_H 1–69	IgG	+	+	11/6	3/2
ST1L G12	cDNA	1	V_H 1–69	IgG	+	−	8/3	2/1
ST1L G2	cDNA	8	V_H 1–69	IgG	+	−	7/4	2/1
ST1L G32	cDNA	1	V_H 1–69	IgG	+	−	7/4	2/2
ST1L G22	cDNA	1	V_H 1–69	IgG	+	−	9/4	2/1
ST1L G11	cDNA	1	V_H 1–69	IgG	+	−	9/5	5/1
ST1L G19	cDNA	1	V_H 1–69/V_H 1–08	IgG	+	+	10/6	4/1
ST1L G8	cDNA	1	V_H 1–08	IgG	+	−	10/5	4/1
ST1L G27	cDNA	1	V_H 1–08	IgG	+	−	13/4	4/1
ST1L G29	cDNA	1	V_H 1–08/V_H 1–69	IgG	+	+	7/2	3/1
ST2 14	DNA	5	V_H 1–46	Indeterminate	−	−	0/0	0/0
ST2 10	DNA	1	V_H 1–46/V_H 1–02	Indeterminate	+	+	5/3	6/2
ST2 6	DNA	2	V_H 1–58	Indeterminate	+	−	0/0	1/0
ST2 31	DNA	1	V_H 1–18/V_H 1–46	Indeterminate	+	+	3/1	0/0
ST2 2	DNA	2	V_H 1–46	Indeterminate	−	−	0/0	0/0
ST2 26	DNA	1	V_H 1–46/V_H 1–69	Indeterminate	+	+	6/2	0/0

The nucleotide sequences of 95 cDNA clones and 36 genomic DNA clones were determined. Only those clones that contained progeny exhibiting V_H replacement are listed. R, replacement; S, silent.

identity from the embedded heptamer to the end of the IgG C_H sequence determined. In contrast, the 2 clones differ at 48 positions 5′ of the embedded heptamer (80.2% similarity).

When a homology search was made for the most likely germline counterpart of the gene segments 5′ of the heptamer, G27 was 90.6% similar to the V_H 1–8 germline gene, whereas clone G29 was only 84.3% similar to the 1–8 germline gene. However, the 5′ segment of clone G29 was 94.6% similar to the V_H 1–69 germline gene. These comparisons suggest that the two clones diverged by interchanging major portions of the V_H 1–8 and 1–69 genes up to the position of the embedded heptamer.

Rearrangements Involving the 5′ FR3 Heptamer. The IgG-expressing clones listed in Fig. 3 demonstrate V_H replacement occurring at the 5′ end of FR3. These rearrangements utilize a heptamer (CACAGCC) embedded in an orientation like that found at the 3′ end of unrearranged

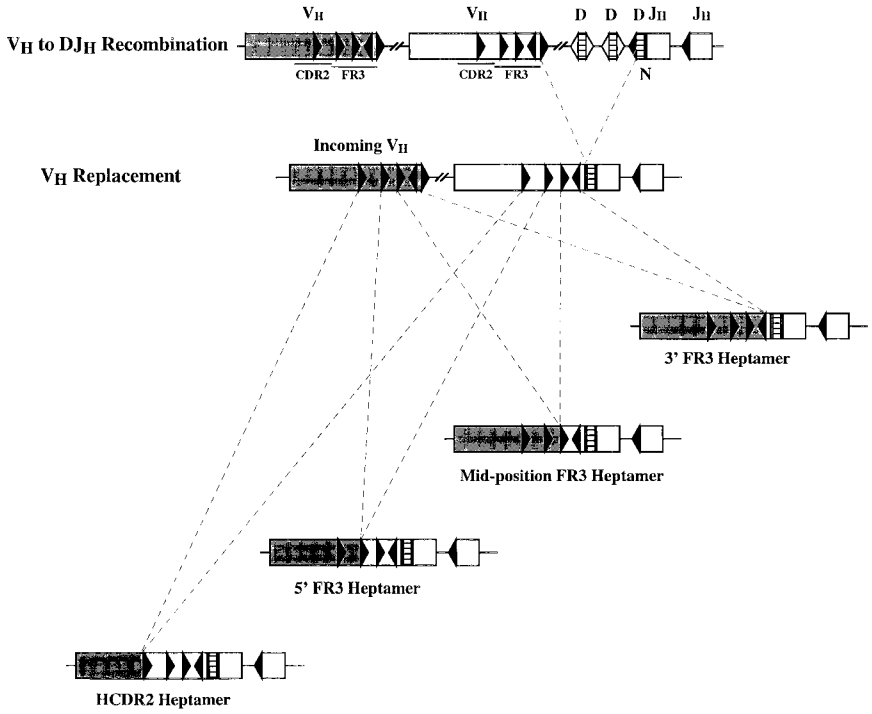

Figure 1. Schematic representation of V_H replacement occurring at the four heptameric sites defined in this study. The top panel illustrates a typical V_H to DJ_H rearrangement. The lower panels illustrate the four types of V_H replacement documented in this study. V_H segment replacement occurred at four discrete heptamer-like sequences, three located in FR3 (3′ FR3 heptamer, mid-position FR3 heptamer, and 5′ FR3 heptamer) and one located in CDR2 (CDR2 heptamer).

V_H genes. This heptamer lies 52 nucleotides upstream of the 3′ heptamer mentioned above.

As in the previous examples, there is 100% identity between the 2 clones from the embedded heptamer to the end of the C_H sequence, but only 82.6% similarity between these 2 clones upstream of the heptamer (33 differences). The upstream portion of clone G11 was most similar to the V_H 1–69 germline gene (93.7%), whereas the same portion of clone G19 was most similar to the V_H 1–08 germline gene (92.6%).

Rearrangements Involving a Heptamer Embedded between the 5′ and 3′ FR3 Sites. The clones listed in Fig. 4 illustrate V_H replacement occurring at a heptamer (CACGGCC) located between the two sites mentioned above. This "mid-position FR3 heptamer" is oriented like the 5′ FR3 heptamer. The examples listed in the figure (M17 and M9) are members of an IgM-expressing clone from a different anatomic site of patient no. 1. In this case, there is only a one-nucleotide difference between the two clones from the heptamer to the end of the IgM C_H sequence; this difference occurs outside of the HCDR3 in FR4. However, there are 43 nucleotide differences upstream of the heptamer (81.2% similarity). The 5′ portion of M9 derives from the V_H 1–69 germline gene (99.6% similar), whereas the same portion of clone M17 is from the V_H 1–8 germline gene (94.2% similar to 1–8 and only 81.9% similar to 1–69).

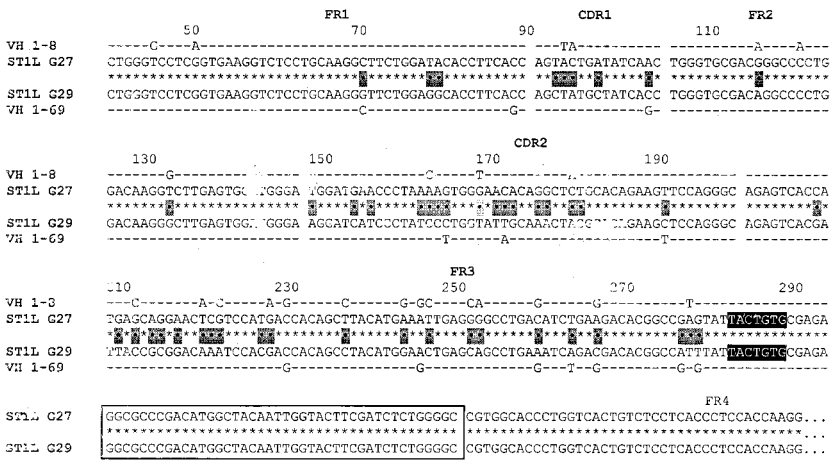

Figure 2. cDNA clones exhibiting V_H replacement at the 3′ FR3 embedded heptamer. Two V_H1-IgG$^+$ clones from ST1L (G27 and G29) are aligned centrally with their most similar germline gene counterparts listed above and below. The gaps in the sequences represent the boundaries between the various FR and CDR. The 3′ FR3 embedded heptamers are displayed with black backgrounds and the HCDR3 are boxed. Nucleotide differences between the two clones are indicated by dots and identities are indicated by asterisks. Nucleotide differences between the individual clones and their germline counterparts are indicated by letters and the identities are indicated by dashes. Note that the HCDR3 of the two clones are identical, whereas the V_H segments upstream of the heptamer are very different between the two clones and resemble different germline genes. Based on either genealogical trees or point mutations, the V_HDJ_H rearrangement of the original B cell clone is listed above the new V_HDJ_H rearrangement.

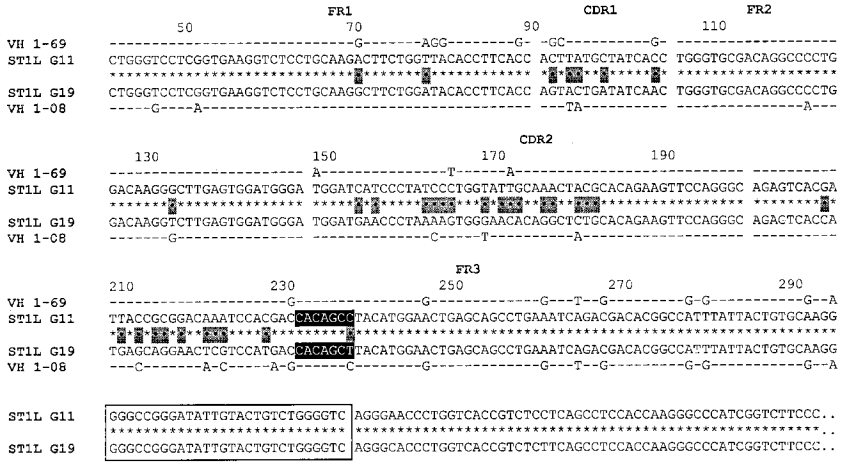

Figure 3. cDNA clones exhibiting V_H replacement at the 5′ FR3 embedded heptamer. Two V_H1-IgG$^+$ clones from ST1L (G11 and G19) are aligned as in Fig. 1. The original V_HDJ_H rearrangement is listed above the new V_HDJ_H rearrangement.

Multiple Distinct Rearrangements Using Different Heptamers within the Same Original B Cell Clones. We detected two examples of multiple independent V_H gene replacement events occurring in distinct progeny of single progenitor B cell clones. Fig. 5 A illustrates three replacements in the progeny of the ST1R M26 clone, each occurring at a different heptameric site. Thus, the rearranged M26 V_HDJ_H was replaced at the 3′ FR3 heptamer by a new V_H 1–69 germline gene to become clone M10. In addition, a replacement occurred in a different subclone involving a new V_H 1–18 germline gene at the mid-position FR3 heptamer, yielding clone M6. Finally, a replacement using the V_H 1–24 germline gene occurred at the 5′ FR3 heptamer, giving rise to clone M31.

The examples in Fig. 5 B represent two distinct V_H1 gene replacement events (V_H 1–2 and 1–69 segments) that occurred in a clone that originally used a V_H3 gene (V_H 3–09). Both of these rearrangements used the same mid-position FR3 heptamer. The identity of the original clone as a member of the V_H3 family is clear because the nucleotides downstream from the embedded heptamer are identical to V_H 3–09 and are not found in any V_H1 family genes.

DNA Sequence Analyses of V_H1-expressing B Cell Clones from a Different Patient Confirm and Extend V_H Replacement Events

Based on these findings, we searched for evidence of V_H replacement at the genomic DNA level to avoid the possibility that the cDNA results represented artifacts of reverse transcription (RT)-PCR. In addition, these studies were carried out using synovial tissue (ST2) from a different RA patient to assure that this phenomenon was not unique to a single patient.

Fig. 6 shows the three examples of V_H replacement found in the V_H1-expressing DNA library from sample ST2. Fig. 6, A and B, confirm the occurrence of V_H replacement occurring at the 5′ FR3 heptamer and the mid-position FR3 heptamer.

However, Fig. 6 C documents a V_H replacement event that we had not detected at the cDNA level. This replacement occurred at a heptamer (CACAGAA) embedded in HCDR2. As in the previous examples, the 2 clones are identical 3′ of the embedded heptamer, but only 82.3% similar 5′ of the heptamer (25 differences). Both clones are identical to their respective germline genes (V_H 1–46 and 1–69) 5′ of the heptamer.

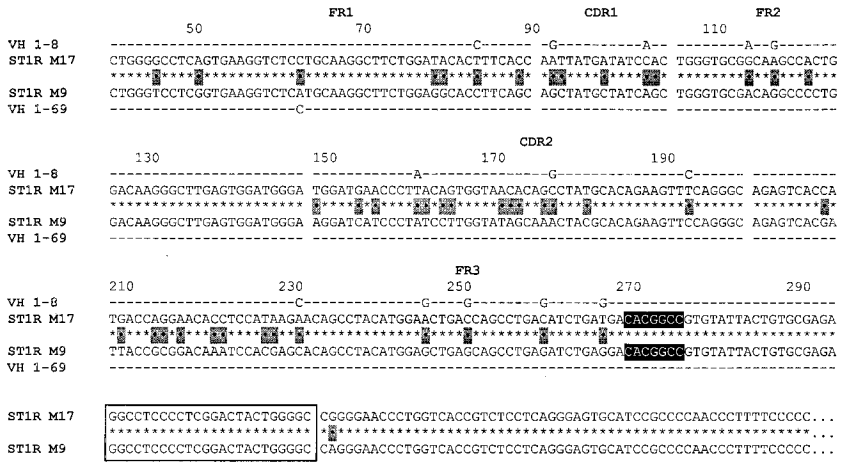

Figure 4. cDNA clones exhibiting V_H replacement at a heptamer embedded between the 5′ and 3′ FR3 heptamers (mid-position FR3 heptamer). See the legend to Fig. 1.

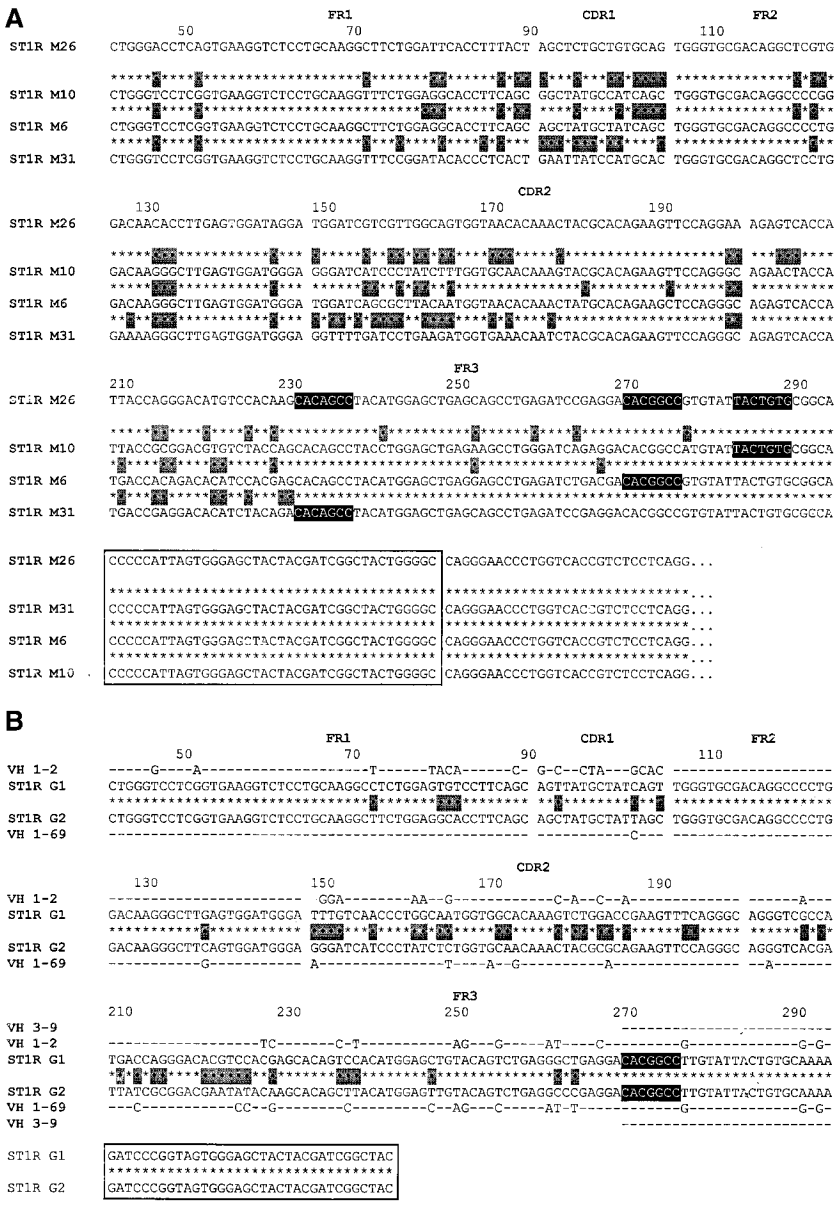

Figure 5. cDNA clones exhibiting multiple distinct secondary rearrangements using different heptamers. (A) Three distinct replacement events that occurred in the progeny of the parental ST1R M26 clone are listed below the original clone. Note that each clone used a different embedded heptamer to accomplish the replacement event. The most similar germline genes for M10 (1–69), M6 (1–18), and M31 (1–24) are not listed. (B) Two distinct replacement events that occurred in the progeny (ST1R G1 and G2) of a V_H3–09–expressing B cell clone. It is clear that the original clone expressed a V_H3 gene because the nucleotides downstream from the embedded heptamer are identical to V_H 3-09 and are not found in any V_H1 family genes.

Ig V_H Gene Diversification among the Clones Undergoing V_H Replacement

As indicated in Table I, the V_H replacement events detected in these synovial tissue samples usually occurred in B cells that had already had significant antigenic exposure and had accumulated V gene mutations. In addition, a comparison of the average numbers of mutations in the original clonal members (236 mutations/22 sequences; 10.73%) and the numbers of mutations in the "replaced" clones (144 mutations/11 sequences; 13.09%) indicates that the diversification process continued in those clones that had undergone V_H replacement. Therefore, these B cells remained immunocompetent after the secondary rearrangement event and received and responded to diversification signals.

Three genealogical trees that display examples of these clonal diversification events are presented in Fig. 7, along with the numbers, types, and locations of the V_H gene mutations that occurred in the various clonal members.

Use of LM-PCR to Detect DNA Breaks at the Sites of V_H Replacement

We used V_H family–specific LM-PCR to probe for blunt-ended dsDNA breaks at the embedded heptamers in the ST2 and ST3 synovial samples. Such products could represent intermediates of V_H replacement. Human bone marrow cells were used as positive controls and fibroblasts as negative controls in these studies. V_H-containing products were obtained from both the synovial tissue and bone marrow B cell samples, whereas no products were obtained from the fibroblast DNA. For the synovial tissue samples, two products of ~300 and ~250 bp were detected (Fig. 8). The DNA sequences of V_H1-expressing clones (n = 50) from the PCR products of ST3 revealed the linker ligated

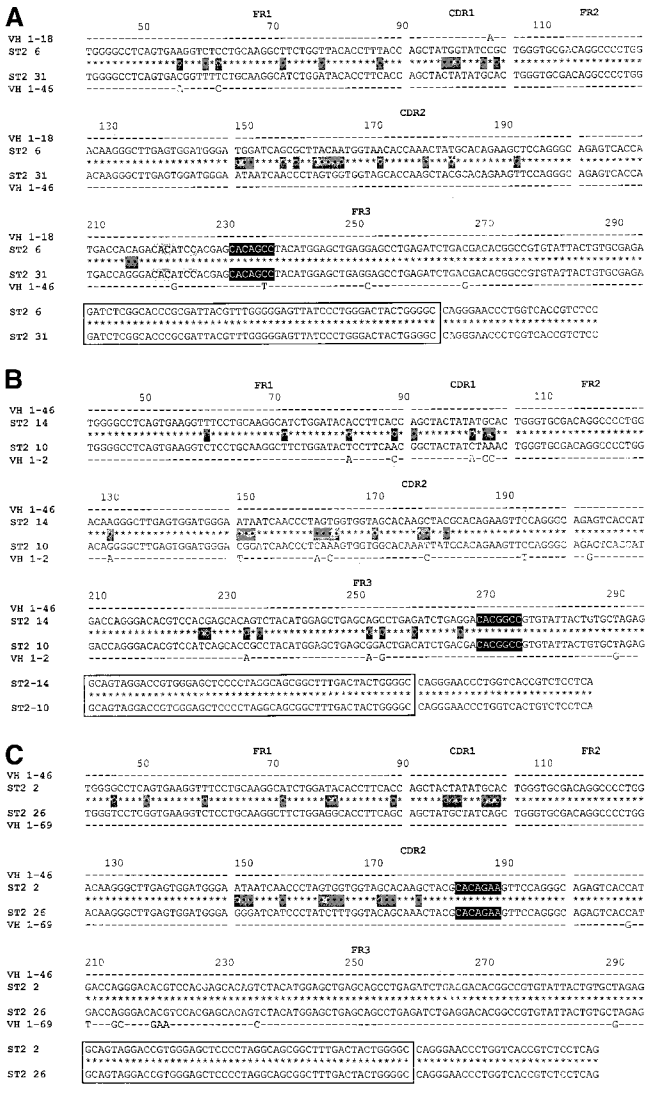

Figure 6. DNA clones exhibiting V_H replacements at the 5′ FR3 heptamer and the mid-position FR3 heptamer and at a heptamer embedded in HCDR2. (A) An example of a replacement that occurred at the 5′ FR3 heptamer. Note that there is another potential heptameric site located 12 bp upstream of the 5′ FR3 heptamer (highlighted in light gray). (B) An example of a replacement that occurred at the mid-position FR3 heptamer. (C) An example of a replacement that occurred at a heptamer located in HCDR2.

at or within the 5′ FR3 heptamer (300-bp product) and the 3′ FR3 heptamer (250-bp product), indicating that blunt dsDNA ends were available at these sites (data not shown).

RAG-1 Gene Expression in the Synovial Tissues of RA Patients

As V(D)J recombination requires RAG proteins, the identification of RAG gene expression would provide a possible mechanism for the identified DNA breaks and the secondary V_H gene segment rearrangements. Therefore, we used RT-PCR to search for RAG-1 gene expression in synovial tissue samples.

As shown in Fig. 9, RAG-1 mRNA was readily detected in several synovial tissue samples (lanes 2–5) and from human bone marrow (lane 1). The lack of RAG-1 gene expression in human fibroblasts (lane 6) and direct sequence analyses of these products (data not shown) confirmed the specificity of these reactions. As the primers used for these reactions flank a 5.2-kb intron in the RAG-1 gene, these 180-bp products could not have been generated from genomic DNA templates. Furthermore, preliminary single-cell PCR and cDNA sequencing analyses suggest expression of RAG-1 in a subset (~20–30%) of $CD19^+$ B cells from ST2 and ST3 (data not shown).

Discussion

In this study we identified four types of secondary V_H gene rearrangements that occurred among clonally related B cells that were expanded in the synovial tissues of different RA patients. Although chimeric V gene sequences can be generated artifactually by crossover events during PCR and cloning, we believe that it is highly unlikely that our findings represent artifacts because these rearrangements (a) were observed in three different synovial tissue samples from two RA patients, (b) were documented at both the cDNA and genomic DNA levels, (c) occurred only at sites in the V_H genes that exhibited heptamer-like sequences, and (d) were always in-frame and coded translatable proteins. (e) Finally, and perhaps most convincingly, is the finding that transcripts from progeny of two different clones displayed replacements of different genes either at the same heptamer (Fig. 5 A) or at distinct heptamers (Fig. 5 B). The possibility that replicates of the same DNA segments would have developed crossover artifacts involving different genes either at the same position or at different positions, each flanked by a cryptic RSS, is remote.

Because these V_H replacement events occurred in B cells that exhibited dsDNA breaks at heptamer-like sequences (Fig. 8) and expressed RAG-1 proteins (Fig. 9), we believe that these V gene replacements were RSS specific and RAG mediated. Such a process has been shown to occur in vitro (39–42, 61) and in vivo (6–9, 18, 43–46), and the identification of circular DNA containing the outgoing V_H gene joined to the RSS of the incoming V_H gene (42, 61) suggests that the process involves the RAG proteins and the V(D)J recombination process. However, these rearrangements require the formation of hybrid joints between the coding end of the incoming V_H gene and the signal end of the outgoing V_H gene segment (5, 9, 18).

Nevertheless, we cannot exclude the possibility that these VH replacement events were mediated by a non–site-specific, general recombination process such as gene conversion, a mechanism that is involved in the diversification of the λ5/14.1 component of the pre-BCR in humans (62) as well as the BCR of chickens (63, 64) and rabbits (65, 66). If gene conversion is responsible for these secondary recombination events, the site of cross-strand exchange could be located anywhere downstream of the first mismatch and upstream of the next mismatch between the incoming/donor germline V_H gene sequence and the outgoing/recipient rearranged gene segment.

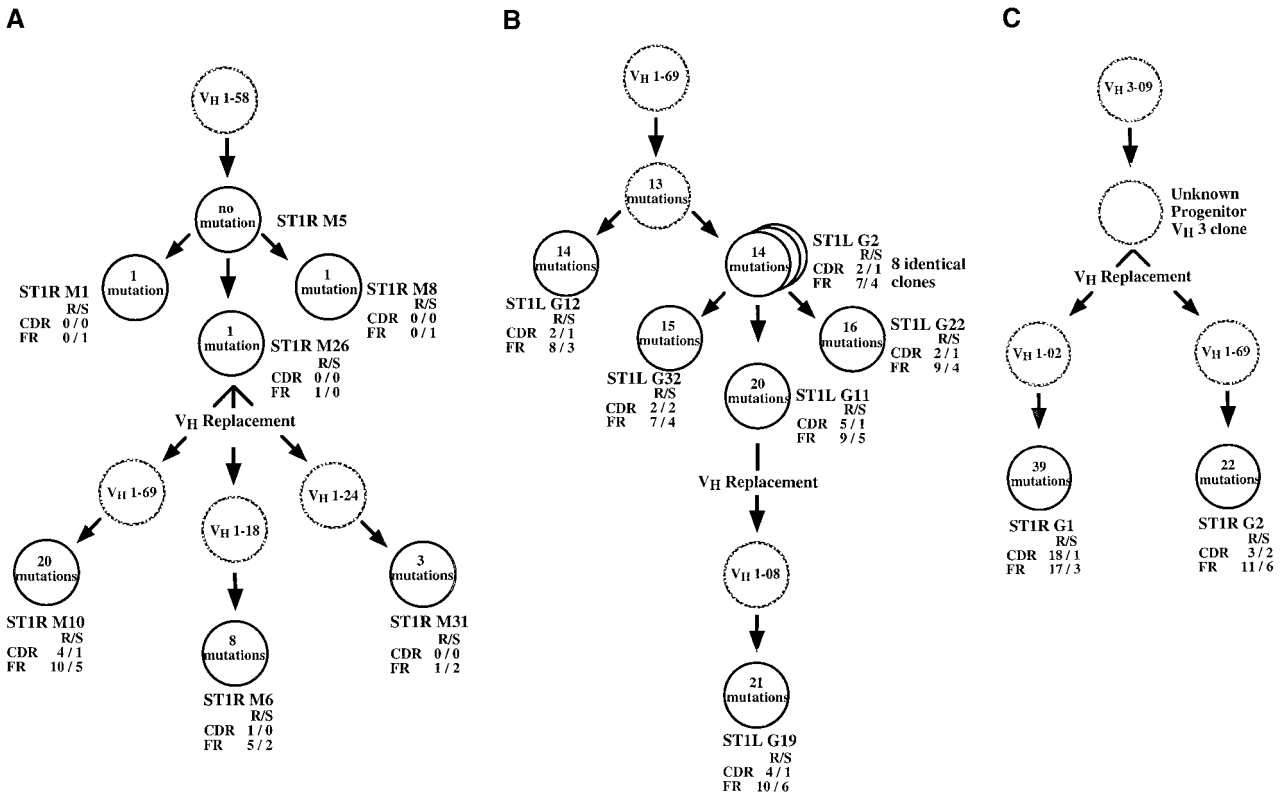

Figure 7. Representative examples of V_H diversification occurring in the B cell clones that exhibit V_H replacements. In each instance (A, B, and C), the identified cDNA clones are represented by filled circles and the presumed (unidentified) clones are represented by hashed circles. The numbers of mutations are contained within the circles and their locations (FR vs. CDR) and types (replacement [R] vs. silent [S]) are displayed adjacent to the circles.

The data that favor a RAG-mediated recombination event include: (a) the size of the fragments being replaced, as gene conversion usually involves smaller segments of DNA; (b) the apparent insertion of the new DNA fragment at specific sites (heptamer-like sequences), as the insertion of DNA via gene conversion is generally viewed as a non–site-specific process; (c) the presence of dsDNA breaks at these heptamer-like sequences, especially those clones with multiple distinct rearrangements (Fig. 5); and (d) the expression of RAG-1 in these B cell populations. The data that are atypical for classical V(D)J recombination include the absence of detectable nonameric sequences 12 or 23 bp from the cryptic heptamers (vide infra) and the lack of coding end processing at the junctions of the new rearrangements (Figs. 2–5). However, these latter findings are not completely incompatible with a RAG-mediated process, as nonamers are not essential for in vitro rearrangements (67) and in situations in which hybrid joint formation is facilitated (e.g., Ku knockout mice [68]), the hybrid junctions frequently do not display evidence of N-insertions and exonuclease activity (69). Thus, both site-specific and general recombination mechanisms may be responsible for these V_H replacement events, and the two mechanisms may not be mutually exclusive. Indeed, a mechanism involving RAG-mediated cleavage at cryptic RSS followed by DNA segment exchange mediated by homologous recombination may not be unreasonable. Clearly, further study will be required to determine the relative contributions of these two mechanisms. Nevertheless, at this point our data suggest a likely role for cryptic heptamers and RAG in these secondary V_H gene rearrangements.

In this study we identified four cryptic heptamers that appeared to be involved in these secondary rearrangements. Three of these heptamers were within FR3 (Figs. 2–5, and Fig. 6, A and B) and one in HCDR2 (Fig. 6 C). The hep-

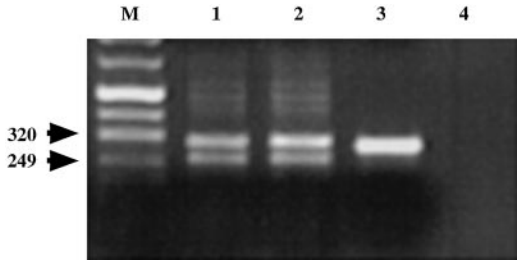

Figure 8. LM-PCR identifies dsDNA fragments generated by cleavage at the 5′ and 3′ heptamers. Genomic DNA was prepared from $CD19^+$ cells from two synovial tissue samples (lanes 1 and 2) and from human bone marrow (lane 3) by the agarose plug method (reference 53). LM-PCR was then used to identify blunt-ended dsDNA breaks (reference 55). The V_H-containing products of these reactions were visualized with ethidium bromide staining. The 320- and 249-bp bands contained DNA fragments with the linker attached at or in the 5′ (320 bp) and 3′ (249 bp) heptamers. Lane 4 is a template-deprived negative control. M, marker lane.

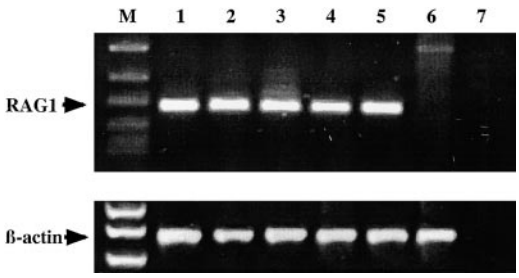

Figure 9. RAG-1 gene expression in the synovial tissues of RA patients. RAG-1 was readily detected by RT-PCR in the cDNA obtained from several synovial tissue samples (lanes 2–5) and from human bone marrow (lane 1). Lane 6 is a negative control (human fibroblast cDNA) and lane 7 is a template-deprived negative control. PCR products and DNA markers were visualized by ethidium bromide staining after agarose gel electrophoresis. M, marker lane.

tamer located at the end of FR3 was in an inverse orientation like that found at the 5′ end of D segments and corresponded exactly to that described previously in murine V_H gene replacement events (9, 39–41). The 5′ FR3 heptamer, the mid-position FR3 heptamer, and the heptamer in HCDR2 were oriented like those found at the 3′ ends of unrearranged V_H genes. All of these heptamers displayed the essential 5′ three nucleotides CAC (67), followed by variations on the expected AGTG sequence (e.g., AGCC, GGCC). In vitro studies (67) indicate that these sequences will permit recombination, albeit at reduced efficiencies. It is possible that any heptamer that has a CAC motif at its 5′ end can act as an embedded RSS for secondary rearrangements. In this regard, the genomic DNA clones listed in Fig. 6 A (ST2 6 and ST2 31) exhibit a heptameric sequence (CACATCC; highlighted in light gray in Fig. 6 A) 12 nucleotides upstream of the 5′ FR3 heptamer that could have functioned as an RSS in this instance. If this is the case, then this represents still another FR3 heptamer in addition to the three previously discussed.

Classical V(D)J recombination requires a heptamer, a spacer of either 12 or 23 bp, and a nonamer (ACAAAAACC-3′ [3, 4]). We could not define a standard nonamer sequence 12 or 23 bp from the heptamers described, although the clones listed in Fig. 5 B display a potential nonamer 12 bp downstream of the cryptic heptamer; this sequence spans the FR3–CDR3 junction. Nevertheless, the requirements for effective nonamers are far less stringent than for heptamers (67), and in vitro recombination studies suggest that nonamers may not be necessary (5). The inability to define a classical nonamer is consistent with previous studies of V_H replacement (9) and led to the suggestion that there might be a different nonamer sequence specific for V gene replacement (9).

Secondary V_H gene recombination events would be expected to involve incoming genes that are located upstream of the originally rearranged (and outgoing) V_H gene. In one combination it was impossible to determine which was the original gene (ST1R M17 and M9). However, in 10 instances this determination was clear, either because of critical nucleotide point differences (e.g., ST2 2→ST2 26; see the 9 nucleotides within the common downstream FR3 sequence in Fig. 5 C) or because of an obvious genealogical progression (e.g., ST1L G11→G19; ST1L G27→G29; ST1R M26→M10, M6, and M31; ST1R G1 and G2 [see Fig. 6]). In 5 of the 10 instances in which chronology was clear, the incoming gene was located 5′ of the original outgoing gene. However, in five events this was not the case. Therefore, in these instances the new rearrangement may have involved a V_H gene residing on the other chromosome or the original locus may have been inverted (e.g., by a D-J_H rearrangement that involved the heptamer at the 3′ end of a D segment [17]).

The V_H 1–69 gene was frequently involved in these V_H replacement events (6/11 instances), despite the fact that it is not overexpressed in the normal human B cell repertoire (70). This suggests that certain V_H genes may be favored in the replacement process. This is consistent with the findings that discrete V_H genes were nonstochastically involved in spontaneous (71) and induced (61) V_H replacement events in murine B cell lines.

We were surprised that these secondary V_H rearrangements occurred so frequently in these synovial tissue B cells. 8/95 V_H1-expressing cDNA clones and 3/36 V_H1-expressing genomic DNA clones from 3 anatomically distinct synovial tissues exhibited this phenomenon (Table I). This represents a frequency of 8.4%. As our DNA sequencing strategies restricted us to identifying V_H replacements that involved V_H1 family genes replacing V_H1 genes, the frequency of these events might have been higher had we searched for V_H replacements that involved V_H segments from other families being incorporated into V_H1.

It remains to be determined whether this high frequency of V_H replacement events represents a feature of normal GC reactions or of the ectopic GC reactions identified in RA (37, 38). In this regard, Wilson et al. (18) documented V_H replacement occurring at a 5′ FR3 heptamer found in V_H4 family genes in a subset of tonsilar B cells that express solely IgD and exhibit an especially high frequency of somatic point mutations (72). As our studies were performed with unfractionated B cells, we cannot determine the precise cellular subset in which these events occurred. However, as all of the cDNA transcripts analyzed in our study were of the IgM and IgG varieties (Figs. 2–5), V_H replacement is not limited to IgD^+IgM^- B cells. In addition, the appearance of point mutations in every case of V_H replacement (Table I) suggests that these B cells maintained a functional BCR that promoted viability (73) and permitted continued clonal expansion and somatic hypermutation in the "replaced" clone. Meffre et al. recently described a subset of human B lymphocytes marked by the coexpression of conventional and surrogate L chains that is enriched in the synovial tissues of certain RA patients (74). These cells express RAG mRNA and show evidence of receptor editing. It will be interesting to see if this unusual B cell subset is a component of the B cell expansions that are characteristic of RA and that can undergo V_H replacement.

These surprisingly frequent V_H replacement events may have significance for the autoreactivity seen in RA, as they

could either maintain or break self-tolerance. RA is characterized classically by the expansion of RF-producing B cells that often increase in affinity as the disease progresses (75) and that correlate with disease severity (76). Furthermore, B cells and plasma cells with this autospecificity are enriched to very high numbers within the synovial tissues of RA patients (77). As it is clear from transgenic mouse models of autoimmunity that secondary rearrangements of either V_H or V_L can lead to non–self-reactive receptors (6–9, 43), it seems reasonable that V_H replacement of autoreactive BCR may be occurring in RA. However, we should stress that we do not know the antigenic reactivities of the B cell clones involved in the V_H replacement events reported in this study.

However, it also seems reasonable that because receptor revision may be diversity (not tolerance) driven (56, 78), it could lead to autoimmunity. Indeed, several recent studies support this notion (11, 79). Most relevant is the study of Brard et al. documenting that anergic B cells from autoimmune prone mice could be activated to produce pathogenic autoantibodies after somatic mutation and receptor revision (11). The fact that the V_H 1–69 gene, which appears to be a preferred donor in the V_H replacement events detected in synovial tissue B cells, is frequently used to assemble RF (80) may support the notion that revision can inadvertently lead to autoreactivity.

The ectopic GC reactions that take place in the synovial tissues of RA patients may compound this tendency. As these GC reactions occur within a microenvironment that does not normally support B cell diversification, they may not provide the same types and/or relative quantities of diversification signals as natural GCs. This could lead to differences in the degrees that various diversification mechanisms are employed (e.g., somatic mutation versus receptor revision) or in their regulation (e.g., lack of occurrence or selection of replacement mutations at appropriate rates and locations that could lead to dysfunctional BCRs and/or to the initiation of autoreactivity). In the latter regard, the V_H gene sequences derived from the synovial tissue B cells of our RA cases in many instances failed to exhibit the types of replacement to silent mutation ratios typically seen in antigen-selected immune reactions (Fig. 7 [81, 82]). Furthermore, they did not localize to RGYW motifs (83) with the expected frequencies (data not shown), a feature also seen in B lymphocytes from some patients with another autoimmune disorder, systemic lupus erythematosus (84). Thus, the nonphysiologic nature of the rheumatoid synovial GC–like environment or a primary defect in the mutation apparatus may support dysregulated B cell diversification events. These dysregulated events, combined with a defect in selection against autoreactivity, either genetic or acquired, and the provision of T cell help, might play an important role in the immunopathogenesis of autoimmune disorders such as RA.

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References

1. Tonegawa, S. 1983. Somatic generation of antibody diversity. *Nature*. 302:575–581.
2. Alt, F.W., E.M. Oltz, F. Young, J. Gorman, G. Taccioli, and J. Chen. 1992. VDJ recombination. *Immunol. Today*. 13:306–314.
3. Early, P., H. Huang, M. Davis, K. Calame, and L. Hood. 1980. An immunoglobulin heavy chain variable region gene is generated from three segments of DNA: VH, D and JH. *Cell*. 19:981–992.
4. Sakano, H., R. Maki, Y. Kurosawa, W. Roeder, and S. Tonegawa. 1980. Two types of somatic recombination are necessary for the generation of complete immunoglobulin heavy-chain genes. *Nature*. 286:676–683.
5. Gellert, M. 1997. Recent advances in understanding V(D)J recombination. *Adv. Immunol*. 64:39–64.
6. Gay, D., T. Saunders, S. Camper, and M. Weigert. 1993. Receptor editing: an approach by autoreactive B cells to escape tolerance. *J. Exp. Med*. 177:999–1008.
7. Radic, M.Z., J. Erikson, S. Litwin, and M. Weigert. 1993. B lymphocytes may escape tolerance by revising their antigen receptors. *J. Exp. Med*. 177:1165–1173.
8. Tiegs, S.L., D.M. Russell, and D. Nemazee. 1993. Receptor editing in self-reactive bone marrow B cells. *J. Exp. Med*. 177:1009–1020.
9. Chen, C., Z. Nagy, E.L. Prak, and M. Weigert. 1995. Immunoglobulin heavy chain gene replacement: a mechanism of receptor editing. *Immunity*. 3:747–755.
10. Bertrand, F.E., R. Golub, and G.E. Wu. 1998. V(H) gene replacement occurs in the spleen and bone marrow of non-autoimmune quasi-monoclonal mice. *Eur. J. Immunol*. 28: 3362–3370.
11. Brard, F., M. Shannon, E.L. Prak, S. Litwin, and M. Weigert. 1999. Somatic mutation and light chain rearrangement generate autoimmunity in anti–single-stranded DNA transgenic MRL/lpr mice. *J. Exp. Med*. 190:691–704.
12. de Wildt, R.M., R.M.A. Hoet, W.J. van Venrooij, I.M. Tomlinson, and G. Winter. 1999. Analysis of heavy and light chain pairings indicates that receptor editing shapes the human antibody repertoire. *J. Mol. Biol*. 285:895–901.
13. Lopez-Macias, C., U. Kalinke, M. Cascalho, M. Wabl, H. Hengartner, R.M. Zinkernagel, and A. Lamarre. 1999. Secondary rearrangements and hypermutation generate sufficient B cell diversity to mount protective antiviral immunoglobulin responses. *J. Exp. Med*. 189:1791–1798.
14. Kelsoe, G. 1996. Life and death in germinal centers (redux). *Immunity*. 4:107–111.
15. Wilson, P., Y.J. Liu, J. Banchereau, J.D. Capra, and V. Pascual. 1998. Amino acid insertions and deletions contribute to diversify the human Ig repertoire. *Immunol. Rev*. 162:143–151.

16. Goossens, T., U. Klein, and R. Kuppers. 1998. Frequent occurrence of deletions and duplications during somatic hypermutation: implications for oncogene translocations and heavy chain disease. *Proc. Natl. Acad. Sci. USA*. 95:2463–2468.
17. Nemazee, D., and M. Weigert. 2000. Revising B cell receptors. *J. Exp. Med*. 191:1813–1817.
18. Wilson, P.C., K. Wilson, Y.-J. Liu, J. Banchereau, V. Pascual, and J.D. Capra. 2000. Receptor revision of immunoglobulin heavy chain variable region genes in normal human B lymphocytes. *J. Exp. Med*. 191:1881–1894.
19. Harris, E.D., Jr. 1990. Rheumatoid arthritis. Pathophysiology and implications for therapy. *N. Engl. J. Med*. 322:1277–1289.
20. Feldmann, M., F.M. Brennan, and R.N. Maini. 1996. Rheumatoid arthritis. *Cell*. 85:307–310.
21. Good, R., and W. Mazzitello. 1957. The simultaneous occurrence of rheumatoid arthritis and agammaglobulinemia. *J. Lab. Clin. Med*. 49:343–357.
22. Rawson, A.J., J.L. Hollander, F.P. Quismorio, and and N.M. Abelson. 1969. Experimental arthritis in man and rabbit dependent upon serum anti-immunoglobulin factors. *Ann. NY Acad. Sci*. 168:188–194.
23. Holmdahl, R., C. Vingsbo, J.A. Mo, E. Michaelsson, V. Malmstrom, L. Jansson, and U. Brunsberg. 1995. Chronicity of tissue-specific experimental autoimmune disease: a role for B cells? *Immunol. Rev*. 144:109–135.
24. Zhao, Y.X., A. Abdelnour, R. Holmdahl, and A. Tarkowski. 1995. Mice with the xid B cell defect are less susceptible to developing *Staphylococcus aureus*-induced arthritis. *J. Immunol*. 155:2067–2076.
25. Taylor, P.C., C. Plater-Zyberk, and R.N. Maini. 1995. The role of the B cells in the adoptive transfer of collagen-induced arthritis from DBA/1 (H-2q) to SCID (H-2d) mice. *Eur. J. Immunol*. 25:763–769.
26. Waaler, E. 1940. On the occurrence of a factor in human serum activating the specific agglutination of sheep blood corpuscles. *Acta Pathol. Microbiol. Scand*. 17:172–188.
27. Rose, H.M., C. Ragan, E. Pearce, and M.O. Lipman. 1948. Differential agglutination of normal and sensitized sheep erythrocytes by sera of patients with rheumatoid arthritis. *Proc. Soc. Exp. Biol. Med*. 68:1–6.
28. Clague, R.B., K. Morgan, I. Reynolds, and H.J. Williams. 1994. The prevalence of serum IgG antibodies to type II collagen in American patients with rheumatoid arthritis. *Br. J. Rheumatol*. 33:336–338.
29. Snowden, N., I. Reynolds, K. Morgan, and L. Holt. 1997. T cell responses to human type II collagen in patients with rheumatoid arthritis and healthy controls. *Arthritis Rheum*. 40: 1210–1218.
30. Schellekens, G.A., B.A. de Jong, F.H. van den Hoogen, L.B. van de Putte, and W.J. van Venrooij. 1998. Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. *J. Clin. Invest*. 101:273–281.
31. Phillips, P.E. 1988. Evidence implicating infectious agents in rheumatoid arthritis and juvenile rheumatoid arthritis. *Clin. Exp. Rheumatol*. 6:87–94.
32. Kouri, T., J. Petersen, G. Rhodes, K. Aho, T. Palosuo, M. Heliovaara, H. Isomaki, R. von Essen, and J.H. Vaughan. 1990. Antibodies to synthetic peptides from Epstein-Barr nuclear antigen-1 in sera of patients with early rheumatoid arthritis and in preillness sera. *J. Rheumatol*. 17:1442–1449.
33. Hajeer, A.H., A.J. MacGregor, A.S. Rigby, W.E. Ollier, D. Carthy, and A.J. Silman. 1994. Influence of previous exposure to human parvovirus B19 infection in explaining susceptibility to rheumatoid arthritis: an analysis of disease discordant twin pairs. *Ann. Rheum. Dis*. 53:137–139.
34. Kouskoff, V., A.S. Korganow, V. Duchatelle, C. Degott, C. Benoist, and D. Mathis. 1996. Organ-specific disease provoked by systemic autoimmunity. *Cell*. 87:811–822.
35. Korganow, A.S., H. Ji, S. Mangialaio, V. Duchatelle, R. Pelanda, T. Martin, C. Degott, H. Kikutani, K. Rajewsky, J.L. Pasquali, C. Benoist, and D. Mathis. 1999. From systemic T cell self-reactivity to organ-specific autoimmune disease via immunoglobulins. *Immunity*. 10:451–461.
36. Matsumoto, I., A. Staub, C. Benoist, and D. Mathis. 1999. Arthritis provoked by linked T and B cell recognition of a glycolytic enzyme. *Science*. 286:1732–1735.
37. Randen, I., O.J. Mellbye, O. Forre, and J.B. Natvig. 1995. The identification of germinal centres and follicular dendritic cell networks in rheumatoid synovial tissue. *Scand. J. Immunol*. 41:481–486.
38. Schroder, A.E., A. Greiner, C. Seyfert, and C. Berek. 1996. Differentiation of B cells in the nonlymphoid tissue of the synovial membrane of patients with rheumatoid arthritis. *Proc. Natl. Acad. Sci. USA*. 93:221–225.
39. Kleinfield, R., R.R. Hardy, D. Tarlinton, J. Dangl, L.A. Herzenberg, and M. Weigert. 1986. Recombination between an expressed immunoglobulin heavy-chain gene and a germline variable gene segment in a Ly 1^+ B-cell lymphoma. *Nature*. 322:843–846.
40. Reth, M., P. Gehrmann, E. Petrac, and P. Wiese. 1986. A novel VH to VHDJH joining mechanism in heavy-chain-negative (null) pre-B cells results in heavy-chain production. *Nature*. 322:840–842.
41. Kleinfield, R.W., and M.G. Weigert. 1989. Analysis of VH gene replacement events in a B cell lymphoma. *J. Immunol*. 142:4475–4482.
42. Covey, L.R., P. Ferrier, and F.W. Alt. 1990. VH to VHDJH rearrangement is mediated by the internal VH heptamer. *Int. Immunol*. 2:579–583.
43. Cascalho, M., J. Wong, and M. Wabl. 1997. VH gene replacement in hyperselected B cells of the quasimonoclonal mouse. *J. Immunol*. 159:5795–5801.
44. Deane, M., and J.D. Norton. 1990. Immunoglobulin heavy chain gene rearrangement involving V-V region recombination. *Nucleic Acids Res*. 18:1652.
45. Brokaw, J.L., S.M. Wetzel, and B.A. Pollok. 1992. Conserved patterns of somatic mutation and secondary VH gene rearrangement create aberrant Ig-encoding genes in Epstein-Barr virus-transformed and normal human B lymphocytes. *Int. Immunol*. 4:197–206.
46. Choi, Y., S.J. Greenberg, T.L. Du, P.M. Ward, P.M. Overturf, M.L. Brecher, and M. Ballow. 1996. Clonal evolution in B-lineage acute lymphoblastic leukemia by contemporaneous VH-VH gene replacements and VH-DJH gene rearrangements. *Blood*. 87:2506–2512.
47. Itoh, K., R. Furie, E. Chartash, R. Jain, and N. Chiorazzi. 1999. Rheumatoid arthritis synovial B cells exhibit novel and unexpected features of the immunoglobulin V gene mutation process. *Arthritis Rheum*. 42:S85.
48. Arnett, F.C., S.M. Edworthy, D.A. Bloch, D.J. McShane, J.F. Fries, N.S. Cooper, L.A. Healey, S.R. Kaplan, M.H. Liang, H.S. Luthra, et al. 1988. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum*. 31:315–324.

49. Fais, F., F. Ghiotto, S. Hashimoto, B. Sellars, A. Valetto, S.L. Allen, P. Schulman, V.P. Vinciguerra, K. Rai, L.Z. Rassenti, et al. 1998. Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. *J. Clin. Invest.* 102:1515–1525.
50. Fais, F., B. Sellars, F. Ghiotto, X.J. Yan, M. Dono, S.L. Allen, D. Budman, K. Dittmar, J. Kolitz, S.M. Lichtman, et al. 1996. Examples of in vivo isotype class switching in IgM^+ chronic lymphocytic leukemia B cells. *J. Clin. Invest.* 98: 1659–1666.
51. Deane, M., and J.D. Norton. 1991. Immunoglobulin gene 'fingerprinting': an approach to analysis of B lymphoid clonality in lymphoproliferative disorders. *Br. J. Haematol.* 77: 274–281.
52. Itoh, K., and N. Chiorazzi. 2000. Clonal expansion is a characteristic feature of the B-cell repertoire of patients with rheumatoid arthritis. *Arthritis Res.* 2:50–58.
53. Pearse, M., P. Gallagher, A. Wilson, L. Wu, N. Fisicaro, J.F. Miller, R. Scollay, and K. Shortman. 1988. Molecular characterization of T-cell antigen receptor expression by subsets of $CD4^-$ $CD8^-$ murine thymocytes. *Proc. Natl. Acad. Sci. USA.* 85:6082–6086.
54. Papavasiliou, F., R. Casellas, H. Suh, X.-F. Qin, E. Besmer, R. Pelanda, D. Nemazee, K. Rajewsky, and M. Nussenzweig. 1997. V(D)J recombination in mature B cells: a mechanism for altering antibody responses. *Science.* 278:298–300.
55. Schlissel, M., A. Constantinescu, T. Morrow, M. Baxter, and A. Peng. 1993. Double-strand signal sequence breaks in V(D)J recombination are blunt, 5′-phosphorylated, RAG-dependent, and cell cycle regulated. *Genes Dev.* 7:2520–2532.
56. Meffre, E., F. Papavasiliou, P. Cohen, O. de Bouteiller, D. Bell, H. Karasuyama, C. Schiff, J. Banchereau, Y.J. Liu, and M.C. Nussenzweig. 1998. Antigen receptor engagement turns off the V(D)J recombination machinery in human tonsil B cells. *J. Exp. Med.* 188:765–772.
57. Bridges, S.L., Jr., S.K. Lee, W.J. Koopman, and H.W. Schroeder, Jr. 1993. Analysis of immunoglobulin gamma heavy chain expression in synovial tissue of a patient with rheumatoid arthritis. *Arthritis Rheum.* 36:631–641.
58. Berek, C., G.M. Griffiths, and C. Milstein. 1985. Molecular events during maturation of the immune response to oxazolone. *Nature.* 316:412–418.
59. Bridges, S.L., Jr., B.E. Clausen, J.C. Lavelle, P.G. Fowler, W.J. Koopman, and H.W. Schroeder, Jr. 1995. Analysis of immunoglobulin gamma heavy chains from rheumatoid arthritis synovium. Evidence of antigen-driven selection. *Ann. NY Acad. Sci.* 764:450–452.
60. Radic, M.Z., and M. Zouali. 1996. Receptor editing, immune diversification, and self-tolerance. *Immunity.* 5:505–511.
61. Usuda, S., T. Takemori, M. Matsuoka, T. Shirasawa, K. Yoshida, A. Mori, K. Ishizaka, and H. Sakano. 1992. Immunoglobulin V gene replacement is caused by the intramolecular DNA deletion mechanism. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:611–618.
62. Conley, M.E., L. Rapalus, E.C. Boylin, J. Rohrer, and Y. Minegishi. 1999. Gene conversion events contribute to the polymorphic variation of the surrogate light chain gene lambda 5/14.1. *Clin. Immunol.* 93:162–167.
63. Reynaud, C.A., V. Anquez, H. Grimal, and J.C. Weill. 1987. A hyperconversion mechanism generates the chicken light chain preimmune repertoire. *Cell.* 48:379–388.
64. Thompson, C.B., and P.E. Neiman. 1987. Somatic diversification of the chicken immunoglobulin light chain gene is limited to the rearranged variable gene segment. *Cell.* 48: 369–378.
65. Becker, R.S., and K.L. Knight. 1990. Somatic diversification of immunoglobulin heavy chain VDJ genes: evidence for somatic gene conversion in rabbits. *Cel.* 63:987–997.
66. Schiaffella, E., D. Sehgal, A.O. Anderson, and R.G. Mage. 1999. Gene conversion and hypermutation during diversification of VH sequences in developing splenic germinal centers of immunized rabbits. *J. Immunol.* 162:3984–3995.
67. Hesse, J.E., M.R. Lieber, K. Mizuuchi, and M. Gellert. 1989. V(D)J recombination: a functional definition of the joining signals. *Genes Dev.* 3:1053–1061.
68. Bogue, M.A., C. Wang, C. Zhu, and D.B. Roth. 1997. V(D)J recombination in Ku86-deficient mice: distinct effects on coding, signal, and hybrid joint formation. *Immunity.* 7:37–47.
69. Han, J.O., S.B. Steen, and D.B. Roth. 1997. Ku86 is not required for protection of signal ends or for formation of nonstandard V(D)J recombination products. *Mol. Cell. Biol.* 17: 2226–2234.
70. Brezinschek, H.P., S.J. Foster, R.I. Brezinschek, T. Dorner, R. Domiati-Saad, and P.E. Lipsky. 1997. Analysis of the human VH gene repertoire. Differential effects of selection and somatic hypermutation on human peripheral $CD5^+/IgM^+$ and $CD5^-/IgM^+$ B cells. *J. Clin. Invest.* 99:2488–2501.
71. Komori, T., Y. Minami, N. Sakato, and H. Sugiyama. 1993. Biased usage of two restricted VH gene segments in VH replacement. *Eur. J. Immunol.* 23:517–522.
72. Arpin, C., O. de Bouteiller, D. Razanajaona, I. Fugier-Vivier, F. Briere, J. Banchereau, S. Lebecque, and Y.J. Liu. 1998. The normal counterpart of IgD myeloma cells in germinal center displays extensively mutated IgVH gene, Cmu-Cdelta switch, and lambda light chain expression. *J. Exp. Med.* 187:1169–1178.
73. Lam, K.P., R. Kuhn, and K. Rajewsky. 1997. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell.* 90:1073–1083.
74. Meffre, E., E. Davis, C. Schiff, C. Cunningham-Rundles, L.B. Ivashkiv, L.M. Staudt, J.W. Young, and M.C. Nussenzweig. 2000. Circulating human B cells that express surrogate light chains and edited receptors. *Nat. Immunol.* 1:207–214.
75. Randen, I., D. Brown, K.M. Thompson, N. Hughes-Jones, V. Pascual, K. Victor, J.D. Capra, O. Forre, and J.B. Natvig. 1992. Clonally related IgM rheumatoid factors undergo affinity maturation in the rheumatoid synovial tissue. *J. Immunol.* 148:3296–3301.
76. Tuomi, T., K. Aho, T. Palosuo, K. Kaarela, R. von Essen, H. Isomaki, M. Leirisalo-Repo, and S. Sarna. 1988. Significance of rheumatoid factors in an eight-year longitudinal study on arthritis. *Rheumatol. Int.* 8:21–26.
77. Munthe, E., and J.B. Natvig. 1972. Immunoglobulin classes, subclasses and complexes of IgG rheumatoid factor in rheumatoid plasma cells. *Clin. Exp. Immunol.* 12:55–70.
78. Hertz, M., V. Kouskoff, T. Nakamura, and D. Nemazee. 1998. V(D)J recombinase induction in splenic B lymphocytes is inhibited by antigen-receptor signalling. *Nature.* 394:292–295.
79. Dorner, T., S.J. Foster, N.L. Farner, and P.E. Lipsky. 1998. Immunoglobulin kappa chain receptor editing in systemic lupus erythematosus. *J. Clin. Invest.* 102:688–694.
80. Chen, P.P., G.J. Silverman, M.F. Liu, and D.A. Carson.

1990. Idiotypic and molecular characterization of human rheumatoid factors. *Chem. Immunol.* 48:63–81.
81. Jukes, T.H., and J.L. King. 1979. Evolutionary nucleotide replacements in DNA. *Nature.* 281:605–606.
82. Shlomchik, M.J., A. Marshak-Rothstein, C.B. Wolfowicz, T.L. Rothstein, and M.G. Weigert. 1987. The role of clonal selection and somatic mutation in autoimmunity. *Nature.* 328:805–811.
83. Rogozin, I.B., N.E. Sredneva, and N.A. Kolchanov. 1996. Somatic hypermutagenesis in immunoglobulin genes. III. Somatic mutations in the chicken light chain locus. *Biochim. Biophys. Acta.* 1306:171–178.
84. Manheimer-Lory, A.J., G. Zandman-Goddard, A. Davidson, C. Aranow, and B. Diamond. 1997. Lupus-specific antibodies reveal an altered pattern of somatic mutation. *J. Clin. Invest.* 100:2538–2546.