Molecular Medicine 4: 240-257, 1998

Molecular Medicine

Clonally-related Immunoglobulin V_H Domains and Nonrandom Use of D_H Gene Segments in Rheumatoid Arthritis Synovium

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Communicated by M. Cooper. Accepted February 20, 1998.

Abstract

Background: Synovia of patients with long-standing rheumatoid arthritis (RA) are typically infiltrated with B lymphocytes and plasma cells that secrete large amounts of immunoglobulin. The CDR3 of an immunoglobulin heavy chain is composed of the V_H-D_H-J_H join, with interposed N region addition, and thus defines clonal relatedness. Furthermore, the CDR3 lies at the center of the antigen binding site, so its length and composition influence antigen binding. We sought definitive evidence of an antigen-driven B cell response (i.e., clones derived from the same V_H, D_H, and J_H gene segments with shared somatic mutations) in RA synovial mRNA transcripts, and to characterize CDR3 intervals at the target of inflammation in this autoimmune disease.

Materials and Methods: We screened a cDNA library generated from unselected cells from the knee joint of a 62-year-old white female with long-standing RA. This technique does not have the potential bias of selecting for antibodies that express a particular reactivity such as rheumatoid factor. Cγ recombinants were sequenced and progenitor V_H, D_H, and J_H gene segments were assigned and somatic mutations determined by comparison to germline sequences. Analyses of D_H reading frame utilization and hydropathy characteristics of CDR3s were performed.

Results: Two of 67 recombinants were derived from the same V_H (V_{3-11}) and J_H gene segments, demonstrated shared mutations, and contained nearly identical V_H-D_H-J_H joins, including N region addition. Three other recombinants contained identical sequence throughout the variable domain. We also found preferential utilization of a limited number of V_H and D_H gene segments and marked preference for a D_H reading frame encoding predominantly hydrophilic residues.

Conclusions: Analysis of expressed heavy chain variable domains strongly supports the hypothesis that the B cell response in RA synovium is at least in part antigen driven and oligoclonal.

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Introduction

Rheumatoid arthritis (RA) is a systemic illness characterized primarily by inflammation and proliferation of the synovial membrane of affected joints (1). B cells and plasma cells are often present in the inflammatory infiltrates in chronically inflamed RA synovial tissue, resulting in secretion of large amounts of immuno-

globulin. This antibody production could result from polyclonal B cell activation in which B lymphocytes proliferate without regard to their antigenic specificity. Alternatively, B cells in the synovium may have been stimulated to proliferate because surface immunoglobulins recognize particular antigens. This would result in oligoclonal expansion of a selected set of B cells bearing particular immunoglobulin variable (V) domains, the regions responsible for antigen recognition. Because the characteristics of antigen receptor repertoires differ in nonspecific versus antigen-driven B cell growth, insight into the mechanisms of B cell proliferation in RA synovium may be gained by analysis of expressed antibody variable domains.

B lymphocytes generate immunoglobulin heavy chain variable domains through sequential DNA rearrangements that result in juxtaposition of variable (V_H), diversity (D_H), and joining (J_H) gene segments, followed by light chain gene rearrangement (2). The human V_H locus consists of ~50 functional V_H gene segments on chromosome 14q32.3 (3–5), grouped into seven V_H families based on the sequence and structure of the framework regions (6–10). There are 27 D_H gene segments grouped into seven families (11–14) and six functional J_H gene segments (15). The V_H-D_H-J_H join forms the third complementarity determining region (CDR3) of the heavy chain, which lies at the center of the antigen-binding site of the immunoglobulin and plays a critical role in defining the antigen specificity of the antibody.

CDR3 sequence is determined by which V_H, D_H, and J_H gene segments are utilized, by variation at the site of gene segment splicing, by somatic hypermutation, and by N region addition (the process of adding non-germline-encoded nucleotides at the time of gene segment rearrangement) (16,17). Diversity contributed by the D_H gene segments is enhanced because each D_H can encode six different peptide sequences, depending on which reading frame occurs at the splice site and whether the rearrangement process involves deletion or inversion (reviewed in ref. 18). The heavy chain CDR3, in addition to its importance in antigen recognition, defines clonal relatedness because V domains derived from a common progenitor B cell share nucleotide sequence identity in the CDR3 interval.

Characteristics suggestive of an oligoclonal, antigen receptor–mediated B cell response include nonrandom utilization of variable domains, high levels of somatic mutation, and high replacement-to-silent (R/S) substitution ratios in the CDRs (19). The most definitive proof of an antigen-driven B cell response is the finding of clonally related V sequences, i.e., those that are derived from the same germline V_H, D_H, and J_H gene segments and contain shared mutations (19).

We have previously published the sequences of 18 immunoglobulin gamma heavy chain constant region (Cγ)-positive clones from an RA synovial cDNA library from this patient (BC) and the results of Southern blot analysis to determine Cγ subclass, V_H family and J_H gene segment utilization of 32 additional Cγ-positive clones from the same library (20). In the present study, we further analyzed this library to determine if clonally related heavy chain V sequences were expressed in this synovial tissue, to characterize utilization of V_H, D_H, and J_H gene segments, and to examine hydropathic characteristics of the CDR3 intervals. By making an unrestricted cDNA library from unselected cells from RA synovium, we avoid potential biases introduced by studying cells with one particular reactivity, such as rheumatoid factor (RF).

We identified three identical clones and two highly mutated clones with highly homologous CDR3 intervals and shared mutations. In addition, we found preferential expression of particular V_H and D_H gene segments. These data support the hypothesis that B lymphocyte expansion in RA synovial tissue is at least in part oligoclonal and antigen driven.

Materials and Methods

Patient Characteristics, Tissue Isolation, and cDNA Library Analysis

The clinical characteristics of the patient and the methods used to process the synovial tissue obtained at the time of joint replacement have been reported previously (21). Patient BC, a 62-year-old white female, had RF-positive RA for 18 years at the time of joint replacement. Two oligo d(T)-primed cDNA libraries were generated in λgt10 from 2-μg aliquots of poly (A)+ RNA from the synovial tissue. We screened approximately 280,000 recombinants from the two cDNA libraries. Filter lifts were screened with a ^{32}P-labeled Cγ (C_H1 domain) oligonucleotide probe designated LB-1 (5′-CTGAATTCCACGACACCG TCACC-3′) at a hybridization stringency of 37°C in SSC-1% SDS. SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0. Each positive plaque was

isolated and the insert cloned into pUC-19 or pBluescript as previously described (20). Nucleotide sequencing was performed by the dideoxy method (22).

We identified a total of 92 Cγ-positive recombinants from these two cDNA libraries from a single RA synovium. As mentioned above, analysis of 50 clones has been previously reported (18 by sequencing, 32 by Southern hybridization) (20). There were 9 artifacts, 2 recombinants that ended in the Cγ domain, and 12 that ended in the CDR3 domain or J_H region, leaving a total of 69 informative V_H-containing clones. We have now analyzed the sequences of the first 58 V_H-containing clones isolated—15 V_H sequences reported previously (20), 14 sequences of V_H-containing clones that had been characterized only by Southern hybridization in the previous report, and 29 newly identified V_H-containing clones.

Analyses of V_H and J_H Gene Segment Utilization

V_H sequences were compared with published sequences and unpublished sequences shared by other investigators using the Windows computer program SAW (Sequence Analysis Workshop) (23). Progenitor germline gene segments were assigned by parsimony. V_H sequences were compared with all known V_H germline gene sequences (4) and all rearranged sequences known to us. Assignment of J_H gene segments were performed as previously described (20).

Analyses of D_H Utilization and Extent of N Region Addition

In previous studies, we and other investigators (24,25) used a criterion of five nucleotides of identity or six nucleotides with one mismatch as the minimum cut-off for identifying the D_H germline sequence within the HCDR3 interval. Recently, however, the entire human immunoglobulin D_H locus has been sequenced and found to contain 27 D_H gene segments belonging to seven D_H families (14). The addition of these new sequences required us to raise the threshold of identity to 10 consecutive nucleotides in order to assign D_H origin with confidence. Taking into account the extensive evidence of somatic mutation detected in both the V_H and J_H gene segments within these class-switched transcripts from synovium, we slightly lowered this threshold and assigned D_H origin on the basis of a minimum of 10 of 11 consecutive nucleotides of identity including a minimum of two consecutive nucleotides of identity at the 5′ or 3′ terminus of the putative D_H.

Analyses of D_H Reading Frames and Hydropathy of CDR3 Regions

D_H gene segments can potentially rearrange by either deletion or inversion, yielding six different peptide sequences, depending on the reading frame used. However, in both the human and the mouse, deletion is greatly favored over inversion, leaving only three commonly used reading frames, termed RF1, RF2, and RF3. In the mouse, RF1 has been assigned as the most commonly used reading frame, RF2 creates a Dμ protein, and RF3 typically contains a termination codon (26). Inspection of the mouse D_H sequences reveals a nonrandom use of codons in each RF, with enrichment for glycines and tyrosines in RF1, hydrophobic residues in RF2, and hydrophobic residues and termination codons in RF3.

In the human, early reports suggest that there is use of all three potential deletional reading frames. As yet, there is no evidence that a Dμ protein influences RF selection. We have found that in humans, as in mouse, each reading frame within a particular gene segment or family has a distinct hydropathic signature (27). One reading frame tends to generate neutral CDR3s rich in glycines and tyrosines (hydrophilic), another typically encodes either hydrophobic or charged residues, and the third commonly contains one or more termination codons with the expressed peptides tending to be either highly hydrophobic or highly hydrophilic. To assign D_H reading frames in the synovial sequences, we inspected the translation products of each D_H gene segment and designated the reading frame Hydrophilic, Hydrophobic, or Stop, as in Corbett et al. (14), or inversional reading frame.

The CDR3 domain was defined as residues 95 to 102 according to Kabat et al. (28). Analysis of the hydropathy of each CDR3 was performed by first assigning each deduced amino acid residue a relative hydropathy value based on the Kyte-Doolittle hydrophobicity index (29). In this index, eight amino acids (Arg, Lys, Asn, Asp, Gln, Glu, His, Tyr) are classified as hydrophilic (hydropathy index −1.3 to −2.7). Five amino acids (Trp, Thr, Ser, Pro, Gly) are classified as neutral (−0.14 to 0.03). Seven amino acids (Ala, Met, Cys, Phe, Leu, Val, Ile) are hydrophobic (0.77 to 1.70). The average hydropathy value per

residue in the CDR3 of each clone was assessed by dividing the sum of the values for all the CDR3 residues by the number of residues.

Results

V_H Family, V_H Gene Segment, and J_H Gene Segment Utilization

Of the 69 V_H-containing clones analyzed by Southern blot hybridization and/or sequencing (ref. 20 and the present report), 68 could be assigned to V_H families. There were 16 V_H1 gene segments, 1 V_H2 gene segment, 42 V_H3 gene segments, 7 V_H4 gene segments, 1 V_H5 gene segment, 1 V_H6 gene segment, and no members of the V_H7 family (Fig. 1A). This analysis corroborates our preliminary finding of predominance of expression of members of the larger V_H3, V_H1, and V_H4 families (20).

Of the 58 V_H-containing clones that were sequenced, 29 (50%) contained the entire coding sequence from framework region (FR) 1 through the FR4 portion of the J_H gene segment. The remaining 29 clones were truncated within the V region (3 in FR1, 1 in CDR1, 1 in FR2, 8 in CDR2, and 16 in FR3). These truncations were likely the result of either incomplete reverse transcription or unintentional restriction endonuclease cleavage due to inadequate methylation of the cDNA, as previously reported (21). Forty-four clones were assignable to progenitor germline gene segments (Table 1).[3–5,9,30–44]

Almost three-quarters of the identifiable JH gene segments were derived from the J_H4 or J_H6 gene segments. J_H3, J_H5, and J_H2 were less frequently expressed and J_H1 was not found among the clones sequenced (Fig. 1B). This pattern is typical of a normal adult repertoire.

Overrepresentation of V_H Gene Segments V_{3-11}, V_{3-23}, and V_{1-69}

Three germline gene segments (V_{3-11}, V_{3-23}, and V_{1-69}) accounted for more than half (23 of 44) of assignable V_H gene segments (Fig. 2 and Table 1). More than 40% (7 of 16) V_H1-containing clones were derived from V_{1-69}, (51p1) a V_H gene segment frequently expressed in paraproteins with rheumatoid factor activity and in patients with chronic lymphocytic leukemia (reviewed in ref. 32). Of the 21 V_H3-containing transcripts, one-third were derived from V_{3-23} (30p1, vh26), a gene segment overrepresented during fetal life and in normal adult repertoires, but 43% (9 clones) were derived from V_{3-11}, a gene segment that has rarely been reported in compilations of V_H rearrangements. The remaining 21 clones were derived from 17 different V_H gene segments (Table 1). Notably, the V_H gene segments $V_{3-30.3}$ (56p1, DP-46), and the closely related gene segments V_{3-30} (1.9III, DP-49) and V_{3-30b} (humhv3005), which have been reported to be frequently represented in adult peripheral blood lymphocyte V_H repertoires and also as components of rheumatoid factors (41), were not found among the clones analyzed.

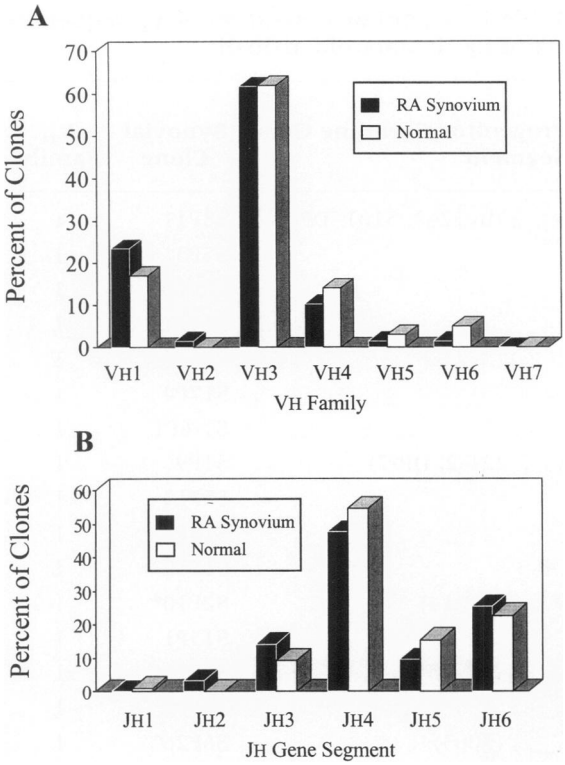


Fig. 1. Analyses of Cγ+ transcripts from synovial tissue of patient with long-standing RA. (A) Utilization of V_H gene segment families. Proportion of gene segments derived from the V_H1 through V_H7 families as assessed by sequence analysis or Southern blot hybridization with family-specific probes from RA synovium (black bars, n = 67 clones) compared with transcripts from an IgG cDNA library from normal adult PBLs [white bars, n = 29 clones (47)]. (B) Relative proportions of expressed J_H gene segments in RA synovial clones (black bars, n = 63 clones) and normal adult PBLs [white bars, n = 97 clones (46)].

D_H Gene Segment and Reading Frame Utilization and N Region Addition

Among the 58 clones with complete V_H-D_H-J_H joins, we were able to assign the progenitor gene

Table 1. Germline derivation of V_H sequences expressed in the synovial tissue of a patient with long-standing rheumatoid arthritis

Progenitor Germline Gene Segment	Synovial Clone	V_H Family	5′ End of Clone	Percent Homology	Association of Progenitor Gene Segment	References
V_{1-69} (hv1263, 51p1, DP-10)	S3P11	1	Complete	81	RF, FL, CLL	4,5,30–33
	S5P3[a]	1	Complete	91		
	S8P10[a]	1	Complete	95		
	S11P17	1	Complete	88		
	S15P6	1	Complete	93		
	S17P4	1	Complete	91		
	S1-6P1	1	Complete	92		
V_{1-46} (21-2, DP-7)	S1P9[a]	1	Complete	92	RF	4,5,9,34
	S5P13	1	Complete	89		
	S15P4	1	CDR2	91		
DP-3	S1P19	1	FR 2	95	—	4
V_{1-18} (DP-14)	S2P10[a]	1	FR 1	94	—	4,5
	S15P1	1	FR 1	95	—	4,5
V_{1-03b}(DP-25)	S2P13	1	Complete	95	Anti-DNA	3,35,36
	S19P2	1	CDR2	93		
V_{1-02} (20p3)	S6P26[a]	1	FR 3	88	RF, FL	3,30,37
V_{2-5} (DP-76)	S14P1	2	Complete	91	RF, CLL	5,38,39
V_{3-11} (DP-35)	S1P15[a]	3	Complete	95	—	4,5
	S4P12[b]	3	Complete	85		
	S5P14[a,b]	3	Complete	82		
	S6P15	3	Complete	80		
	S7P5	3	Complete	94		
	S9P16[a]	3	Complete	93		
	S11P9	3	CDR2	83		
	S17P1	3	CDR2	99		
	S19P3	3	Complete	95		
V_{3-23} (vh26, 30p1, DP-47)	S4P23	3	Complete	86	FL, anti-DNA, RF	4,5,30,35,40,41
	S10P19[a]	3	Complete	93		
	S14P5	3	Complete	94		
	S16P4	3	Complete	92		
	S16P5	3	Complete	95		
	S1-4P4	3	CDR2	98		
	S3P6[a]	3	CDR 2	87	89% homology to anti-TG antibody	42
V_{3-49}	S6P21[a]	3	CDR 2	97	—	5
V_{3-53} (DP-42)	S7P13	3	Complete	86	—	4,5
V_{3-07} (DP-54)	S10P18	3	CDR2	92	—	4,5
V_{3-48} (DP-51)	S17P3	3	FR1	81	—	4,5
V_{3-21} (DP-77)	S20P4	3	Complete	93	—	4,5
V_{4-31} (DP-65)	S1P1[a]	4	Complete	85	CLL	4,5,38
V_{4-04} (DP-70)	S2P1[a]	4	CDR2	80	—	4,5
V_{4-59} (4.11, DP-71, 58p2)	S4P11[a]	4	Complete	91	FL	4,5,30,43

(Continued)

Table 1. (Continued)

Progenitor Germline Gene Segment	Synovial Clone	V_H Family	5′ End of Clone	Percent Homology	Association of Progenitor Gene Segment	References
V_{4-39} (DP-79)	S20P2	4	Complete	99.7	—	4,5
V_{5-51} (DP-73)	S15P5	5	Complete	90	TS Ab	4,5,44
V_{6-01} (DP-74)	S1-3P1	6	FR3	92	FL	4,5,30

Assignments are based on highest percent nucleotide sequence homology. RF, rheumatoid factor; FL, fetal liver; CLL, chronic lymphocytic leukemia; TS Ab, thyroid stimulating antibody from a patient with Graves' disease; anti-TG, anti-thyroglobulin antibody.

[a]Clones previously reported (20) (accession numbers L06910-L06913, L06915-L06924). Accession numbers for remaining sequences are U64432, U64466-U64508.

[b]Clones S5P14 and S4P12 are 95% homologous to each other (Fig. 5B).

segments of 36 (Fig. 3). In one of these 36 sequences, S6P21, a D_H-D_H join was identified. As is typical for such D_H-D_H rearrangements (45), an inverted D_H gene segment (D3-3) had been joined to a D_H gene segment that had rearranged by deletion (D6-13). D_H family and gene segment utilization within these synovial HCDR3 sequences were compared with the analysis of Corbett et al. (14). Members of the D3 family (formerly DXP) were overrepresented in the synovial repertoire, comprising 20 of 58 sequences (35%) versus 177 of 893 sequences (20%, p = 0.01 Chi square), even when the inverted D3-3 gene segment in S6P21 was not included in the analysis. Quantitation of individual D_H utilization revealed that this preference for the D3 family reflected overutilization of the D3-3 (9 of 58 versus 43 of 893, p = 0.001 chi square) and D3-22 (6 of 58 versus 34 of 893, $p < 0.05$ chi square) gene segments, respectively.

The overall pattern of D_H gene family utilization was similar to that seen in normal adult peripheral blood lymphocytes (PBLs), with most frequent representation of members of the D2 and D3 families and rare expression of the D7-27 (D_HQ52) gene segment preferentially expressed during fetal development (16,25,46,47). However, the degree of preference for expression of members of the D3 family was greater than has been reported in normal adult PBLs (46). There was a marked predilection for use of one deletional reading frame that encodes predominantly hydrophilic residues (Figs. 3 and 4C). Thus, in contrast to previous reports of the adult PBL repertoire, there was marked overrepresentation of a single D_H reading frame in this RA synovium.

V_H-D_H and D_H-J_H joins contained variable degrees of N region addition, which contributed to variability in CDR3 lengths, which ranged from 6 amino acid codons (clones S2P10 and S6P26) to 26 amino acid codons (clone S9P16)

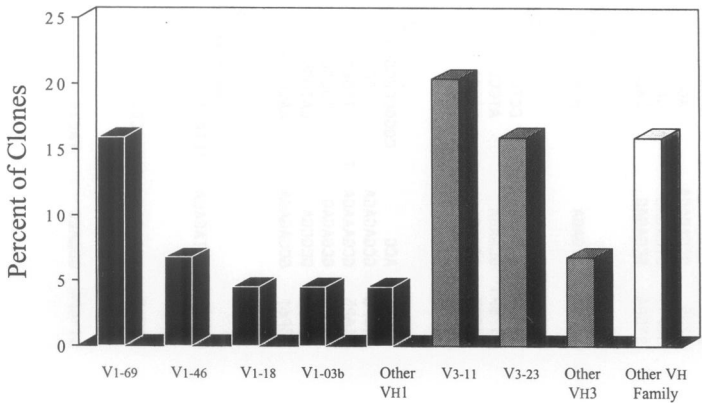


Fig. 2. Utilization of V_H gene segments in RA synovium. Members of the V_H1 family are represented by black bars, members of the V_H3 family by gray bars, and all other V_H families by the white bar. A total of 44 clones were assignable.

A

	VH FR3	P/N Region	DH Gene Segment	RF	N Region	JH	FR4	
			D2-2 AGGATATTGTAGTAGTACCAGCTGCTATACC					
S1-6P2	GTGAGAGA	GGG	..a.....c...t....	Phil	ACTT	...C....C.	.....T..C.....	JH4B
S1P18†	GCGAGA	CATCTTACCGGGTGG	.....t.....g...t..	Phil	GGCCCCGTG	.....	.....	JH6B
S10P19†	GCGAG	GGATGCGTCGGGC	.....g.....	Phil	CC	.....A..T..	.....G.....	JH5B
S11P17	GCGAG		....c...gc...ac.....g...	Phob	ACCAAATTCGTACCCATGTCACA	.....	.....G.....	JH6B
			D2-8 AGGATATTGTACTAATGGTGTATGCTATACC					
S13P2	GTGAGA	TATGGTTTTGACA	.....g.....	Phil	TACTTCATG	....A....C.A	.....G.....C.....C...	JH3B
			D2-8 (Inv) GGTATAGCATACACCATTAGTACAATATCCT					
S14P2	GTGAG	TC	.....t.....	Inv	GTGCC	....A.TCT...	.....	JH4B
			D2-15 AGGATATTGTAGTGGTGGTAGCTGCTACTCC					
S9P16†	GCGAGAG	TGGGGGGCGGGCGGTGGGCCTATAT	.....	Phil	AGTGTTACTCGCGTG	.....C.C.....	.....	JH6B
			D3-3 GTATTACGATTTTTGGAGTGGTTATTATACC					
S14P1	GCACGC	AC	.....c.....	Stop	CGTTA	..G..T	.....T..C.....	JH4B
S14P6	GCGA	CTCCC	.....a...	Phob	C	..T.T.	.....G.....G...	JH4B
S18P1	GTGAAA	GGGGAACAC	.....	Phil	CTTTCACGAAAACCTAAACGAT	.....	.....	JH3A
S5P13	GCGAGAGA	T GTATACGGGT	.....ac.....a.g...	Phob	GGACCT	.....A.....	.....	JH6B
S6P12	GCGAAAGA	CTCCACA	.....a..t....	Phil	CAACCCCTCCCCC	.....	.....	JH4B
S10P18	GCGAGAGA	T GGGAAG	.....	Phil	TTAGTCGGTCT	.....	.....	JH4B
S7P5	GCGAGAGA	AG	.....a...	Phil	CCAACCTCC	..CT..T..T.....	.....	JH6B
S1P1†	GTCAGAG	GA	.....a..	Phil	ATGAAGACGCTCAC	....AC.A.....	.....CT.T.....	JH5B
S15P4	GCGAGAG	CAGT	.....aa..c...c..	Phil	C	.....A..	.....G...	JH6B
			D3-9 GTATTACGATATTTTGACTGGTTATTATAAC					
S4P11†	GCGAGA	CATA	.....t...	Stop	GCAGTTGCG	....G.	.....	JH4B
			D3-10 GTATTACTATGGTTCGGGGAGTTATTATAAC					
S11P9	GCGAGAGA	TC CCTCC	.....t..c..	Stop	CCCACT	.....	.....	JH2
S1-6P1	GCGAGA	ATCCC	.....tc...c.....	Phil	CTACCCTAAG	.....	.....G.....	JH6B
S19P3	GCGAGAGA	TC GGGGA	..c.....	Phil	TAG	....G.....	.....	JH6B
S5P3†	GCGA	CAAGCA	.....c...a.....	Phil	CTCGTCCGGAG	.....C..CT.CA.....	.....C.....	JH6B
			D3-22 GTATTACTATGATAGTAGTGGTTATTACTAC					
S14P3	ACG	CGGGCCCCGGG	...t.....g.....	Phil	CAGGGCATGATGATC	.....C..	.....	JH3A
S15P6	GCGAGAGA	AG	....t.....c..c....	Phil	GGGGACCCCTAT	.....C.T.C.....	.....	JH6B
S16P5	GCGAAAGA	T TTGGCG	.....	Phil	CCCTG	...C.T...	.....	JH4A
S17P3	GCGAGAG	TGGGG	..g.....	Phil		.....C.....	.....	JH6B
S20P2	GCGCGA	CATGGG	.....g..tt...	Phob	GGACGGGT	.....	.....	JH3B
S3P6†	GCGAGAGA	AACCAGA	.....t.tc..c.....	Phil	TGGGG	.....	.....G.....G.....	JH4B
			D3-3 (Inv) GGTATAATAACCACTCCAAAAATCGTAATAC		D6-13 GGGTATAGCAGCAGCTGGTAC			
S6P21†	ACTAGAGA	TCTACACT	..t..... Inv	TC	....g....t.c..... Phil	GGATCCGATTACCCGTAC	.....T.	.....G..A.. JH4B
			D6-13 GGGTATAGCAGCAGCTGGTAC					
S19P2	GCGAGAG	GCCCCCCGCAC	.....	Phob		CCCCTTCCAG	.....G.	..... JH5B
			D4-4 TGACTACAGTAACTAC					
S1-4P4	GCGACC	TACTA	.....c.g....	Phil	TGG	.....	.....	JH5B

			D4-23 TGACTACGGTGGTAACTCC							
S15P1	GCGAGAGA	TCT	..g.....	Phil	ATGGGTCGAT	.....	.....	.....G.....		JH3B
			D5-05 GTGGATACAGCTATGGTTAC							
S2P1†	GCG	GGGGGG	.....tcc.....	Phil	TG	.....A.....	.....	.....T.G.....		JH6B
			D5-12 GTGGATATAGTGGCTACGATTAC							
S8P10†	GCG	GGTGTG	.....	Phob	CCGGGC	..CT...T.....C.....	.....	.....C.....		JH6B
			D5-24 GTAGAGATGGCTACAATTAC							
S1P9†	GCGAGAGA	T AAACAG	.....a.c.....	Phil	CCAAGGGTCCAC	.....	.....G...	.....		JH4B
			D6-19 GGGTATAGCAGTGGCTGGTAC							
S1P19	GCA	CAAACC	.....c..	Phil	TC	.....	.....CT.	.....		JH4B
S4P23	GCGA	AAAGTGG	.....a..	Phob	CAGACCTTTTTTT	.....	..T...	.....G....T.....G...		JH5A

	VH FR3	Unassignable N-DH Gene Segment-N	JH	FR4	
S1-3P1	GCAAGA	GACTATTTTGTTGATAGTGGTAGGTTCAAAATC	.....	.....G.C.....	JH4B
S1-4P2	ACGAGA	GCCCATGAGCGGGAACTACCCGGT	.G.....	.....	JH4B
S1-6P2	GTGAGA	GAGGGAGAATATTGTGATAGTACCACCTGTTATGAC	.T...C....C.	.....T..C.....	JH4B
S1P9†	GCGAGAGA	TAAACAGAGAGAAGCCTACAACCAAGGGTCCAC	.....G...	.....	JH4B
S1P15†	GCGAGAG	GGGTTGAGACGGCGGC	....	.....	JH4B
S2P10†	GCGAGAG	CCCTGACACTACCGGT	.	.....	JH4B
S2P13	GCGAGA	GAGGCACTAGGAACTGGGA	.....	.....	JH4B
S2P31†			*.	.....G.....C.G	JH6B
S3P11	GCGAGA	CGGCCCCCCGGGGAACCGGGCGCTTAT	.....CT.	.....	JH4B
S3P15†		*GCCCCAGAGAC	..G.....G...	.....GG.....TGC.....	JH3B
S4P11†	GCGAGA	CATAATTTTGATTGGGCAGTTGCG	....G.	.....	JH4B
S4P12	GCG	ACTGGGACGGAGGGAGGCC	....AA..	.....G...C.....G	JH4B
S5P14†	GCG	GCTGGGGCGAATGGAGGCC	....GA.G	.....G...C.....G	JH4B
S6P15	GCGAGA	GCGTCTGGGACGGAAAGGA	.....	.....A...A.....C..G.....	JH2
S6P26†	GTGA	CTCACTTA	.....	.....C.T.....	JH4B
S7P13	GCGAGAG	GACTCTTGTTGAACACTCATCCTAATGCTCTTTACTCCGATCGAGCTTTT	.....	.....N.G.....C.....	JH3B
S8P5	GCGAGA	ACGCCCAACATAGTAGTACGCTTCGGGGACCGTCT	..T.	.....	JH4B
S13P1	GCGAGA	ACGCCCAACATAGTAGTACGCTTCGGGGACCGTCT	..T.	.....	JH4B
S20P6	GCGAGA	ACGCCCAACATAGTAGTACGCTTCGGGGACCGTCT	..T.	.....	JH4B
S10P2		*ATCC	.....	.....T.....	JH4B
S11P9	GCGAGAGA	TCCCTCCTTCGGTGACTTCCCACT	.....	.....	JH2
S11P17	GCGAG	GGATCTTGGCGTAACACCAGGTGCACCAAATTCGTACCATGTCACA	.....	.....G.....	JH6B
S12P20†			*	.....T...	JH4B
S14P5	GCGAAAGA	TCGGGGATCTGGGGTCGGTGATTAT	....T.C.....A.....	.....G...	JH6B
S15P1	GCGAGAGA	TCTTGGCTACGGTGGTAACTCATGGGTCGAT	.....	.....G.....	JH3B
S15P3	GCGAG	GGGGCCTTTTACGACTGATCTCTACGGGCGC	.....ACT	.....T.....	JH4B
S15P4	GCGAGAG	CAGTGAGTGAATACTATCCCC	.....A..	.....G...	JH6B
S15P5	GCGAGA	CTGGGTGGCAGCTCATTACGATTC	..TC.....	.....G....C.....T.T.	JH4B
S16P4	GCG	GTGTGGCGTGGGGT	...T.....	.....G.....	JH5B
S17P1	GCGAG	GTCCGAGTGGGCGGCAGCACCTC	.....	.....	JH4B
S17P2				*.....	JH3
S17P4	GCGAGA	ACTGAGAGGGAGCTGTTTCATGGTA	.....T..	.....	JH3X
S17P5	GCCACAGAG	TTATTGCCGGGTC	.....C..	.....G.....G.....	JH4B
S19P2	GCGAGAG	GCCCCCCGCACGCAGCAGCTGCCCCTTCC	.G.....G.	.....	JH5B
S20P4	GCGAGA	ATGAGTAGTACGCCTTATGCGGAGTAC	.....	.....	JH4B
S20P5				*.....	JH6B
S20P7				*CCTGGTCACCGTCTCCTCA	JH?

Fig. 3. Sequences of CDR3 intervals of RA synovial sequences. (A) Nucleotide sequences are compared with germline V_H, D_H, or J_H gene segments when assignable; dots indicate sequence homology. Base changes in the putative D_H gene segments are shown in lower case letters. † = clones previously reported (20). P or N nucleotides and D_H reading frames are also shown. Phil = hydrophilic, Stop = termination, Phob = hydrophobic, Inv = inverted (14). (B) Deduced amino acid sequences. † = clones previously reported; * = end of transcript. (Figure continued on next page.)

B

	FR3	N-DH-N-(DH-N)-JH	FR4
S1-3P1	AR	DYFVDSGRFKIDY	WGQGTRLTVSS
S1-4P2	TR	AHERELPGCDY	WGQGTLVTVSS
S1-4P4	AT	YYDYTDYGDP	WGQGTLVTVSS
S1-6P1	AR	IPYYFGPGSSTLRDV	WGQGTTVTVSS
S1-6P2	VR	EGEYCDSTTCYDFFDS	WGQGIPVTVSS
S1P1†	VR	GYYDFWNDEDAHNYYDP	WGQGLLVTVSS
S1P9†	ARD	KQREAYNQGSTFEY	WGQGTLVTVSS
S1P15†	AR	GVETAAY	WGQGTLVTVSS
S1P18†	AR	HLTGWYCISSSWAPCYYYGMDV	WGQGTTVTVSS
S1P19	A	ETGYSSGSFYFDL	WGEGTLVTVSS
S2P1†	A	GGGYSPGYDYYYGMDV	WGQGIAVTVSS
S2P10†	AR	ALTLPV	WGQGTLVTVSS
S2P13	AR	EALGTGNFDY	WGEGTLVTVSS
S2P31†			*WGQGTAVTVSP
S3P6†	ARE	TRYYFDSSGYYLGFDY	WGQGALVTVSS
S3P11	AR	RPPGEPGAYYFDLWG	WGQGTLVTVSS
S3P15†		*PRDAFEI	WGRGTAVTVSS
S4P11†	AR	HNFDWAVADC	WGQGTLVTVSS
S4P12	A	TGTEGGLEN	WGRGTLVTVSS
S4P23	A	KSGAVAGRPFFDS	WGQGILVTVSS
S5P3†	AT	SNYHDSGSSRPEYYSSAMDV	WGQGTPVTVSS
S5P13	ARD	VYGSIYGVVSMDLYYGMDV	WGQGTTVTVSS
S5P14†	A	AGANGGLEK	WGRGTLVTVSS
S6P12	AKD	STYYDFWSDFYEPLFLDY	WGQGTLVTVSS
S6P15	AR	ASGTERNL	WGHGTLVTVSS
S6P21†	TRD	LHSSPKSYGSTWYGSDYPYYFDF	WGQGTLVTVAT
S6P26†	V	THLFDY	WGQGTLVPVSS
S7P5	ARE	DYDFWSDYQPPSYYGMDV	WGQGTTVTVSS
S7P13	AR	GLLLNTHPNALYSDRAFDI	WGRGTLVTVSS
S8P5	AR	TPNIVVRFGDRLF	WGQGTLVTVSS
S8P10†	A	GVVATTGHLYYYGLDV	WGQGTPVTVSS
S9P16†	AR	VGGGRWAYICSGGKCYSRDYYYALDV	WGQGTTVTVSS
S10P2		*SYFDY	WGLGTLVTVSS
S10P18	ARD	GKYDFWSGYYLVGLFDY	WGQGTLVTVSS
S10P19†	AR	DASGSSASPNWFNS	WGQGTLVTVSS
S11P9	ARD	PSFGDFPLWYFDL	WGRGTLVTVSS
S11P17	AR	DLGVTPGAPNSYHVTMDV	WGQGTTVTVSS
S12P20†			*WGQGTLVTVSS
S13P1	AR	TPNIVVRFGDRLF	WGQGTLVTVSS
S13P2	VR	YGFDSTDGYFMAYDL	WGQGTLVTVSS
S14P1	AR	TLEWLLSLEY	WGQGIPVTVSS
S14P2	VS	HTFSSAYYSY	WGQGTLVTVSS
S14P3	T	RAPGYYDGSGYSGHDDLDL	WGQGTMVTVSS
S14P5	AKD	RGSGVGDYYFHYYGMDV	WGQGTTVTVSS
S14P6	A	TPFGEVDF	WGRGTLVTVST
S15P1	ARD	LGYGGNSWVDAFDI	WGQGTVVTVSS
S15P3	AR	GPFTTDLYGRYFDT	WGQGILVTVSS
S15P4	AR	AVSEYYPHGMDI	WGQGTTVTVSS
S15P5	AR	LGGSSLRFYLDY	WGQGTPVTVSL
S15P6	ARE	DYYDSSASYGDPYYYAFDV	WGQGTTVTVSS
S16P4	A	VWRGVFDP	WGQGTLVTVSS
S16P5	AK	DLADSSGYSLFHY	WGQGTLVTVSS
S17P1	AR	SEWAAAPLDY	WGQGTLVTVSS
S17P2			*TMVTVSS
S17P3	AR	VGESSGYGMDV	WGQGTTVTVSS
S17P4	AR	TERELFHGTFDF	WGQGTMVTVSS
S17P5	ATE	LLPGLDH	WGQGALVTVAS
S18P1	VK	GEHYDFWSGYFHENLNDAFDV	WGQGTMVTVSS
S19P2	AR	GPPHAAAAPSSWFDR	WGQGTLVTVSS
S19P3	ARD	RGYGSGSLDGMDV	WGQGTTVTVSS
S20P2	AR	HGITMMVLGTGAFDI	WGQGTMVTVSS
S20P4	AR	MSSTPYAEYYFDY	WGQGTLVTVSS
S20P6	AR	TPNIVVRFGDRLF	WGQGTLVTVSS
S20P5			*GQGTTVTVSS
S20P7			*LVTVSS

Fig. 3. (Continued)

(Fig. 3). The average amount of N addition between V and D gene segments was slightly less in synovium (6.3 ± 4.7 nucleotides) than in the extensive database of 893 immunoglobulin sequences analyzed by Corbett et al. (mean 7.3 nucleotides) (14). Conversely, the average N addition between D and J was slightly greater in the synovial transcripts (8.2 ± 5.7 nucleotides) than in the analysis of Corbett et al. (mean 6.3 nucleotides).

Analysis of CDR3 Lengths and Hydropathy

The distribution of CDR3 lengths resembled a normal curve, with 46 of the 58 clones (79%) containing CDR3s between 8 and 19 codons. In general, the degree of variability in CDR3 lengths was similar to that reported in repertoires expressed in normal adult PBLs (16,25,46,47).

Of 58 clones, the CDR3s of the vast majority (86%) demonstrated an overall mean hydropathy index value per residue of between −0.20 and 0.30. Clone S2P10, in addition to having the shortest CDR3, was also the most hydrophobic (AlaLeuThrLeuProVal). S1P9 and S1P1 were the most hydrophilic of the clones, with mean index value per residue of −0.48 and −0.43, respectively. The identical clones S8P5, S13P1, and S20P6 had mean index value per residue of 0.29, while related clones S4P12 and S5P14 had values of −0.18 and −0.01, respectively. We also analyzed the hydropathy index value per CDR3 residue according to the deletional D_H reading frame used (Fig. 4D). As expected from their definition, the Hydrophilic D_HRF tended to generate CDR3s that were relatively neutral, whereas use of the Stop and Hydrophobic D_HRFs generally led to more hydrophobic CDR3 intervals. Thus, although the majority of CDR3 intervals had an overall mean hydropathy index value that was in the neutral range, the D_HRF did appear to influence the hydropathic characteristics of the CDR3 interval in the final antibody molecule.

Evidence of Oligoclonality and Antigen-Driven B Lymphocyte Expansion

Clones S13P1 and S20P6 were found to contain an identical sequence consisting of a V_H3 gene segment truncated in FR3, most likely D3-10, and J_H4B (Fig. 5A). We generated an oligonucleotide probe designated BC-3 (5-ACGCCCAACAT AGTAGTACGCTTCGGGGACCGTCT-3') that was based on the unique sequence of the CDR3 region of clones S13P1 and S20P6. This probe encompassed the N region at the V_H-D_H join, the D_H gene segment, and the N region at the D_H-J_H join. The BC-3 oligonucleotide probe was used to screen phage DNA of V_H clones previously analyzed only by Southern blot (20). Clone S8P5

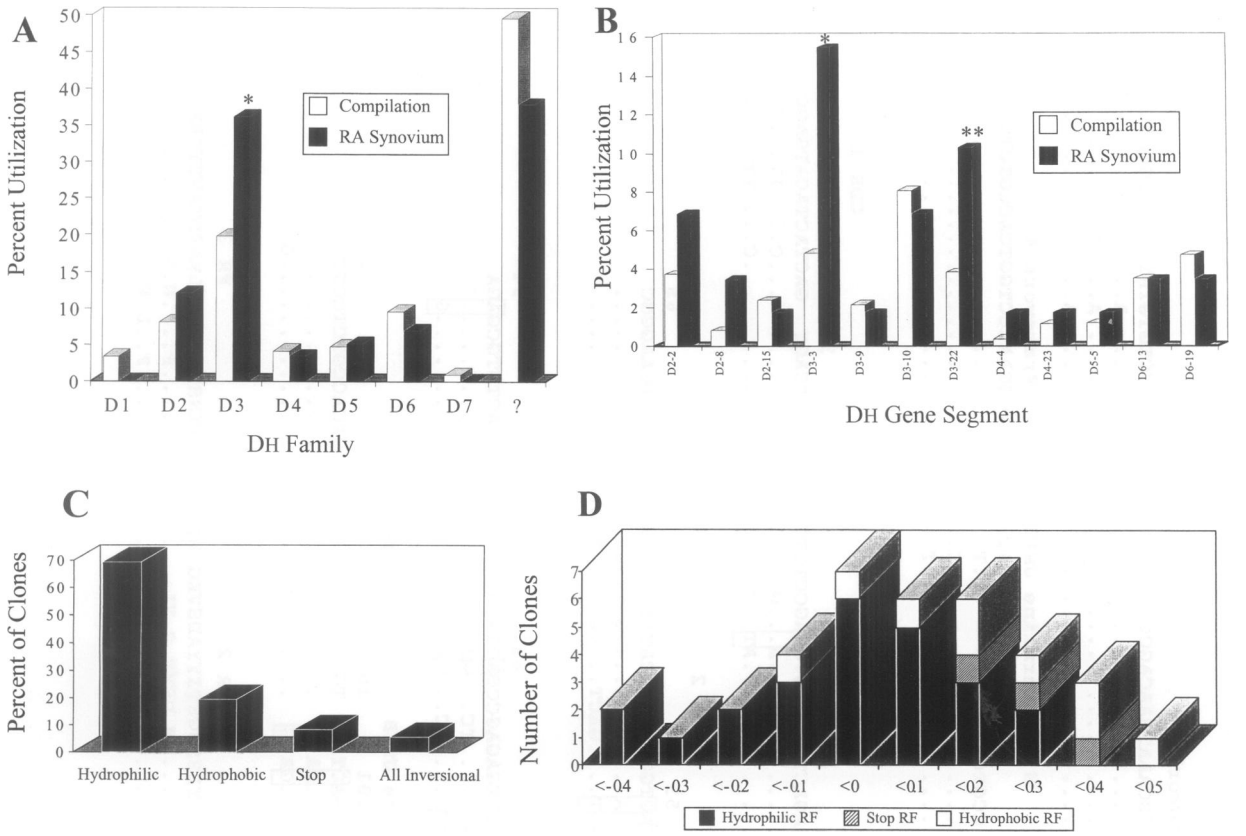
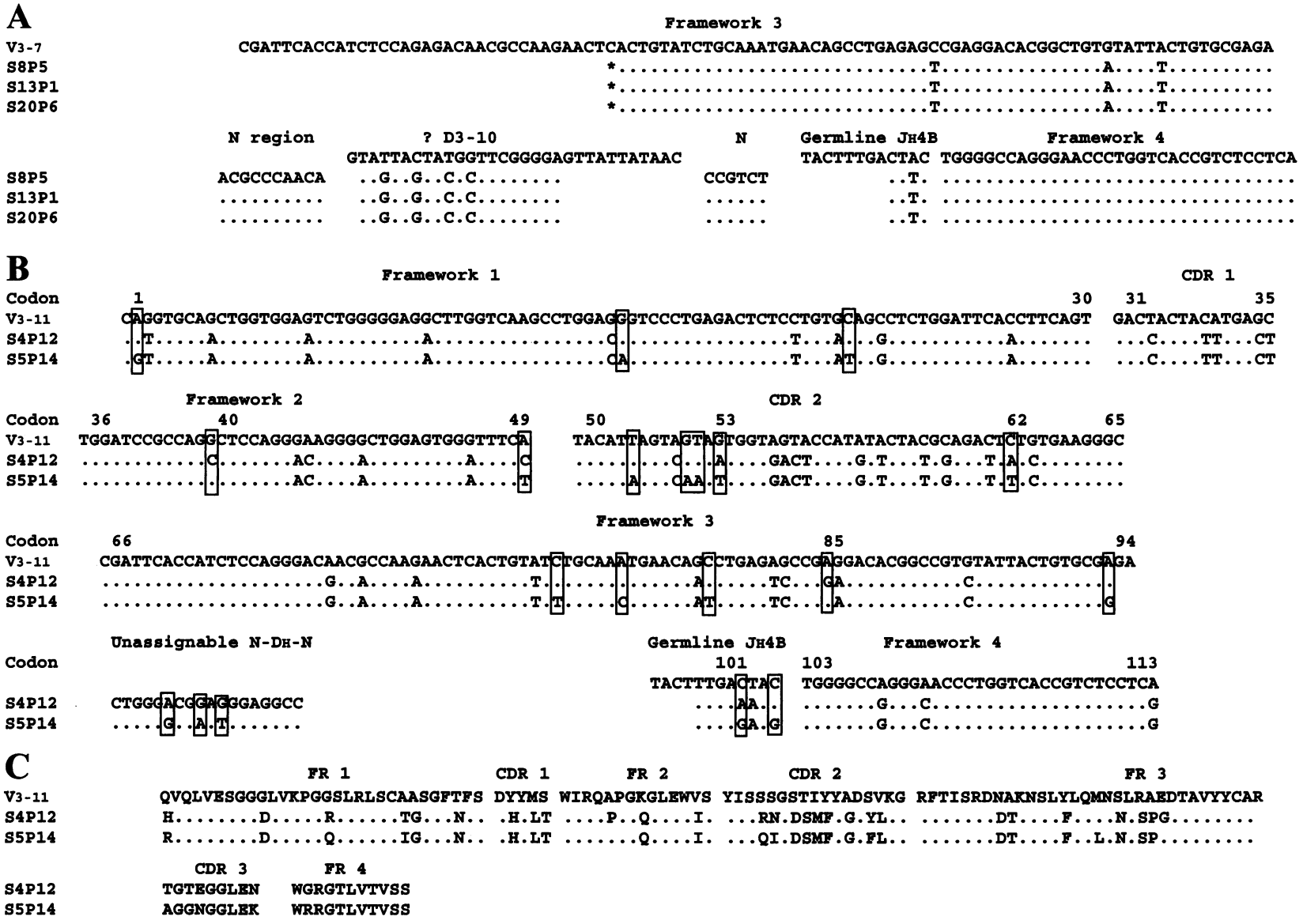


Fig. 4. Utilization of D_H families, gene segments, and reading frames, and CDR3 hydropathy. (A) Utilization of D_H families D1 through D7 in RA synovial clones (black bars) and in the compilation by Corbett et al. [white bars (14)]. ? = unassignable D_H gene segments. The RA synovial clones were significantly enriched for members of the D3 family (*p = 0.01, Chi-square). (B) Utilization of D_H gene segments in RA synovial clones (black bars) and in the compilation by Corbett et al. [white bars (14)]. Only D_H gene segments found to be expressed in the RA synovial sample are shown. *For RA versus control, p = 0.001, Chi-square; **for RA versus control, $p < 0.05$, Chi-square. (C) Use of deletional D_H reading frames resulting in enrichment for hydrophilic residues, hydrophobic residues, and stop codons, and inversional reading frames of 36 RA synovial clones shown in Figure 3. (D) Hydropathy characteristics of the CDR3 intervals of RA synovial clones. The clones are analyzed according to which deletional RF is used: hydrophilic D_HRF (black bars), stop D_HRF2 (gray bars), and hydrophobic D_HRF3 (white bars). Clones using inversional reading frames or containing more than one D_H gene segment are not included.

hybridized to this oligonucleotide. Sequence analysis revealed that S8P5 was identical to clones S13P1 and S20P6 (Fig. 5A). Thus, three of 58 sequences (4%) were oligoclonal, i.e., derived from the same expanded B cell or plasma cell clone.

To rule out contamination during subcloning as an explanation for the finding of identical sequences S13P1 and S20P6, we probed the original filters containing DNA from the S13P1 and S20P6 phage plaques with probe BC-3 and found that both recombinants were positive. Clone S13P1 was found to contain a poly (A) tail 112 nucleotides longer than clone S20P6. These findings, in conjunction with the fact that PCR was not used in the generation of this library, reasonably excludes contamination during subcloning as an explanation for the finding of three identical sequences.

Clones S4P12 and S5P14 appear to be genealogically related, as they have identical CDR3 lengths with similar unassignable N region-D_H-N region, and they have shared mutations in the V_H and J_H gene segments (Fig. 5B). To determine if any of the V_H clones previously analyzed only by Southern blot were clonally related to S4P12 or S5P14, we probed with oligonucleotide probes specific for the N-D_H-N portion of the CDR3 in-


A
Framework 3
V3-7 CGATTCACCATCTCCAGAGACAACGCCAAGAACTCACTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCTGTGTATTACTGTGCGAGA
S8P5 *.....T.....A....T.....
S13P1 *.....T.....A....T.....
S20P6 *.....T.....A....T.....
N region
? D3-10
N
Germline JH4B
Framework 4
GTATTACTATGGTTCGGGGAGTTATTATAACT
TACTTTGACTAC TGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
S8P5 ACGCCCAACA ..G..G..C.C..... CCGTCT ..T. .....
S13P1 ....G..G..C.C..... ....T. .....
S20P6 ....G..G..C.C..... ....T. .....
B
Framework 1
CDR 1
Codon 1 30 31 35
V3-11 CAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTGGTCAAGCCTGGAGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTCAGT GACTACTACATGAGC
S4P12 ..T.....A.....A.....A.....C.....T...A...G.....A..... ...C....TT...CT
S5P14 .GT.....A.....A.....A.....CA.....T...AT..G.....A..... ...C....TT...CT
Framework 2
CDR 2
Codon 36 40 49 50 53 62 65
V3-11 TGGATCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTTTCA TACATTAGTAGTAGTGGTAGTACCATATACTACGCAGACTCTGTGAAGGGC
S4P12 .....C.....AC....A.....A....C .....C...A....GACT....G.T...T.G...T.A.C.....
S5P14 .....A...CAA.T....GACT....G.T...T.G...T.T.C.....
Framework 3
Codon 66 85 94
V3-11 CGATTCACCATCTCCAGGGACAACGCCAAGAACTCACTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTGTATTACTGTGCGAGA
S4P12 .....G..A....A.....T.....A.....TC...GA.....C.....
S5P14 .....G..A....A.....T.T....C.....AT.....TC....A.....C.....G
Unassignable N-DH-N
Germline JH4B
Framework 4
Codon 101 103 113
TACTTTGACTAC TGGGGCCAGGGAACCCTGGTCACCGTCTCCTCA
S4P12 CTGGGACGGAGGGAGGCC ....AA.. .....G...C.....G
S5P14 ....G..A.T..... ....GA.G .....G...C.....G
C
FR 1 CDR 1 FR 2 CDR 2 FR 3
V3-11 QVQLVESGGGLVKPGGSLRLSCAASGFTFS DYYMS WIRQAPGKGLEWVS YISSSGSTIYYADSVKG RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR
S4P12 H.....D.....R.....TG...N.. .H.LT ....P..Q....I. ...RN.DSMF.G.YL.. .....DT....F....N.SPG.....
S5P14 R.....D.....Q.....IG...N.. .H.LT .....Q....I. ...QI.DSMF.G.FL.. .....DT....F..L.N.SP.....
CDR 3 FR 4
S4P12 TGTEGGLEN WGRGTLVTVSS
S5P14 AGGNGGLEK WRRGTLVTVSS

tervals these two clones. Probe LB-79 (5'-CTGG GACGGAGGGAGGCCTTGA-3') was used to probe for clones related to S4P12 and LB-80 (5'-CTGGGGCGAATGGAGGCCTTGA-3') was used to probe for clones related to S5P14. No clones hybridized to these probes. S4P12 and S5P14 share 95% nucleotide sequence homology to each other. Of all germline V_H gene segments, both S4P12 and S5P14 were most homologous to V_{3-11} (85% for S4P12 and 82% for S5P14) (5). The use of the same germline V_H, D_H, and J_H gene segments and the high degree of nucleotide sequence homology and shared somatic mutations in these two clones is similar to that seen in B cells subjected to clonal selection (19,48).

The pattern of somatic mutations in S4P12 and S5P14 is consistent with antigen-driven clonal selection, as evidenced by disproportionately high replacement mutations in the CDRs compared with the FRs. The number of replacement mutations, silent mutations, and R/S ratios for each domain of the two clones combined (in comparison to the germline sequence of V_{3-11}) were: FR1 (12, 6, 2.0), CDR1 (6, 2, 3.0), FR2 (5, 6, 0.83), CDR2 (18, 5, 3.6), and FR3 (14, 6, 2.33).

The S5P14 and S4P12 sequences appear to be derived from a previously mutated B cell progenitor. Clone S4P12 contains 48 mutations from germline in FR1-FR3 and the J_H gene segment; 42 of these mutations are also present in S5P14. There are 20 nucleotide differences between S5P14 and S4P12 (Fig. 5B). Fourteen of these are mutations from the germline V_{3-11} sequence that were not present in S4P12. Four of the six remaining differences represent mutations that encode nucleotides different from both S4P12 and the germline V_{3-11} sequence (C → T at codon 49 in FR2, A → T at codons 53 and 62 in CDR2, and A → G at codon 101 in J_H4B). The two remaining differences likely represent a retained V_{3-11} germline nucleotide sequence (G instead of C at codon 40 in FR2 and A instead of G at codon 85 in FR3). The mutational patterns of these two clones are consistent with antigen-driven selection.

Discussion

V_H Family, D_H Gene Segment, and J_H Gene Segment Utilization in Normal Adults

The distribution of V_H family utilization in RA synovium in this report resembles that seen in normal adult PBLs as assessed by Southern hybridization analysis of B cell lines (49), by in situ hybridization using V_H family–specific probes (50), and by single-cell PCR (25). The V_H family distribution in the present report is remarkably similar to that in 29 V_H-containing clones from an IgG cDNA library prepared from normal adult PBLs [Fig. 1A, (47)]. The pattern of J_H gene segment utilization, with predominance of J_H4 and J_H6, parallels that seen in normal adult tissues (16,46,47).

Compared with results of a study of D_H utilization in PBLs from normal adults (46) and to a compilation of D_H use (14), the RA synovial sample appears to be enriched for expression of members of the D3 family. There was a lower proportion of unassignable D_H gene segments in our synovial sample (~38%) than in the compilation by Corbett et al. (~49%) (Fig. 4A). Difficulty in D_H assignment is a common problem. Now that presumably all D_H gene segments have been reported, it is unlikely that there is frequent use of a previously unreported D_H gene segment. The most likely reasons for difficulty in assigning progenitor D_H gene segments in some of the clones include the presence of a large number of somatic mutations and the inability to definitively assign N regions at the V_H-D_H and D_H-J_H joins. In fact, difficulty in assignment of D_H gene segments appears to be closely correlated with CDR3 length. The sequences with a minimum of ten nucleotides of identity to a D_H gene segment

Fig. 5. Clonally expanded gamma heavy chain V domains expressed in synovial tissue of a patient with long-standing RA. (A) Nucleotide sequences of three identical truncated clones. Clones are compared with the germline V_H3 sequence V_{3-23} (30p1), D_H D3-10, and J_H4 gene segments; dots denote germline identity. Nucleotides at the V_H-D_H and D_H-J_H junctions that could not be assigned to the V_H, D_H, or J_H gene segments are identified as the likely products of N region addition. * = end of transcript. For deduced amino acid sequence of CDR3 domains, see Figure 4. (B) Nucleotide sequences of two clonally related, nonidentical clones, compared with the germline V_H3 sequence V_{3-11} and the J_H4 gene segment. The D_H gene segment could not be assigned. Nucleotide differences between clones S4P12 and S5P14 are boxed. (C) Deduced amino acid sequences of clones S4P12 and S5P14.

averaged 17.6 ± 4.0 codons, whereas the sequences in which D_H identity could not be assigned averaged 12.9 ± 3.6 codons. The 608 nucleotides that encode the 27 D_H gene segments of the human D_H locus have a guanidine or cytidine (GC) content of 43% (14). We analyzed the GC content of the putative N additions in the 36 sequences in which we identified a D_H gene segment. Of the 639 nucleotides attributable to D_H gene segments (either germline or mutated), the GC content was 41%, virtually indistinguishable from germline. However, of the 526 nucleotides attributable to N region addition, GC content was 60% and compatible with the known preference for GC addition by TdT in N addition (51,52). Of the 498 nucleotides contained within the CDR3 intervals that did not meet the criteria for assignment to a D_H segment, GC content was 59%, supporting the hypothesis that the majority of this sequence was N addition.

V_{3-11} Is Not Commonly Expressed in Antibodies from Normal Individuals or in Autoantibodies

In most reported fetal and adult V_H repertoires, there is expression of a relatively small subset of germline V_H gene segments (53). Of 95 germline gene segments, only 51 have open reading frames and have been found to rearrange (54). In our RA synovial sample, more than half of the assignable clones were derived from three functional V_H gene segments: V_{3-23}, V_{3-11}, and V_{1-69}.

V_{1-69} (Humhv1263, 51p1) was first described in a study of V_H gene segments expressed during fetal development (30,31). V_{1-69} has been reported to encode RF from patients with RA (33,41,55,56), an anti-cardiolipin/anti-DNA antibody from a patient with systemic lupus erythematosus (SLE) (57), and is frequently expressed in B cells of patients with chronic lymphocytic leukemia (CLL) (32,58). V_{1-69} is thus thought to be overrepresented in the autoimmune or CD5+ B cell population. V_{3-23} (vh26, 30p1) is also frequently expressed during fetal development of the antibody repertoire (30) and is found in RF and in 16/6 cross-reactive idiotype (CRI) positive anti-DNA antibodies from patients with SLE (35,41,59). V_{3-23} has been reported to be present in 4–10% of J_H-positive transcripts expressed in PBLs of two normal adults and 28% of V_H3 transcripts from tonsil (60). In a single-cell PCR analysis of PBLs of a normal adult, V_{3-23} was found in ~17% of functional rearrangements (25). Susuki et al. found that V_{3-23} accounted for 17% of rearrangements in PBLs of two normal adults (61). V_{3-23} is thought to be a promiscuous V_H gene segment, able to contribute to many antigenic specificities.

Synovial clones assigned to the V_{3-11} gene segment demonstrated a wide range of sequence homologies to the germline V_{3-11} sequence (80–99%). This could result either from variation in the amount of somatic mutation or from the contribution of previously unknown V_H progenitors. Given the high degree of polymorphism and the presence of alleles in the human V_H3 family, there is a slight possibility that some of the synovial clones assigned to V_{3-11}, including S5P14 and S4P12, may represent V_H gene segments derived from other germline gene segments rather than somatic mutation. However, after extensive analysis of the V_H locus by several investigators, there are no germline gene segments more homologous to S5P14 and S4P12 than the V_{3-11} gene segment. Moreover, except for clones S5P14 and S4P12, none of the sequences assigned to V_{3-11} shared base pair changes from the germline V_{3-11} sequence. The absence of such shared changes argues against derivation of these clones from an unknown germline gene segment. S5P14 and S4P12 are ~95% homologous to each other, but only 82–85% homologous to the closest known germline gene, V_{3-11}. In this synovial sample we found no clones without somatic mutation, so it appears very unlikely that either of these two clones is in unmutated germline form. Furthermore, the CDR3 sequences of these two clones are highly similar and they use the same J_H4B gene segment with shared mutations. The J_H4 gene segments, compared with germline gene segments that are definitively known, were highly mutated [6 of 41 nucleotides (14.6%) in S5P14 and 5 of 41 nucleotides (12.2%) in S4P12]. Thus, it is clear that these two clones are derived from B lineage cells that derived from a common progenitor cell which underwent somatic hypermutation.

V_{3-11}, unlike V_{1-69} and V_{3-23}, has not been frequently reported to be expressed in normal individuals or in those with autoimmune diseases or lymphoid malignancies. In a recent study of V_H utilization in normal individuals, V_{3-11} represented only ~4% of V_H3-containing rearrangements from CD19+ cells from peripheral blood of one individual, and was apparently not expressed among IgM+ cells from the peripheral blood of a second individual (61,62). In a study of IgD+ circulating B cells (presumably recent bone marrow emigrants), only ~8% of

the expressed V_H3 repertoire contained V_{3-11}, but V_{3-23} was found in 29% of the V_H3-containing rearrangements (63). In a single-cell PCR analysis of PBLs from a normal adult, V_{3-11} represented only 2 of 71 rearrangements; both of these were nonproductive (25). Thus, B cells and plasma cells in this RA synovial sample frequently express a V_H gene segment that appears to be rarely expressed in most normal human repertoires studied to date.

Several mechanisms have been postulated to explain the overrepresentation of particular V_H gene segments in the expressed human V_H repertoire, such as the presence of more than one copy of the gene segment in the germline, biased rearrangement as a result of a more favorable position on the chromosome, more favorable accessibility to or recognition by recombinase, the presence of gene-specific promoter sequences, or positive selection of B cells expressing V_H gene segments (60). V_{3-23} and V_{1-69} are frequently found in normal repertoires, but no identity in the CDR3 sequences of clones containing V_{3-23} or V_{1-69} was found in this synovial sample, suggesting their overrepresentation may be due to factors other than their antigen-binding characteristics as classically defined. Such factors could include overrepresentation of these gene segments in the genome (64), the effect of selection by binding to superantigen (65), or preferential activation of specific B cell subpopulations that are enriched for the use of these V_H gene segments, e.g., CD5+ B cells. The overrepresentation of V_{3-11} in this RA synovial sample compared with normal adult repertoires suggests that its presence may reflect antigen selection. The finding that V_{3-11}-derived clones S4P12 and S5P14 contain highly mutated, clonally related, class-switched V_H domains and that the somatic mutations of these two clones follows the pattern seen in antigen selection strengthens this interpretation.

In studies of V_H domains expressed in B cell subsets from human tonsil and spleen, other investigators have found clonally related V_H sequences among IgG-expressing B cells, but not in IgM transcripts, suggesting that evidence of clonal expansion may be commonly found in IgG repertoires (66). The presence of clones S5P14 and S4P12 lends further support to the hypothesis that the IgG repertoire in this RA synovium may be heavily influenced by clonal expansion as a result of antigen-driven B cell proliferation.

Comparison of the Extent of Oligoclonality in Gamma Heavy Chain and Kappa Light Chain Repertoires in This RA Synovium

The present finding that 3 of 58 (5.2%) of the Cγ-positive recombinants expressed in this RA patient's synovium are identical closely parallels the results of our analysis of the immunoglobulin Vκ repertoire in this same sample, in which three (4.7%) of 64 Cκ-positive recombinants were identical (67). The present report, however, provides more definitive proof of antigen-driven expansion in RA synovium. Two nonidentical V_H domains derived from the same V_H, D_H, and J_H gene segments with shared mutations were found. No Vκ domains with similar Vκ-Jκ joins and shared mutations in the Vκ or Jκ gene segments were isolated in the previous analysis, possibly because of the smaller number of clones analyzed (24 sequences).

The immunoglobulin light chains paired with the V_{3-11}-derived clones, and consequently, the antigenic specificities of the antibodies expressed in vivo are unknown, a difficulty inherent to analyzing cDNA libraries from unselected cells. Because of the marked differences in somatic mutations and CDR3 characteristics of the synovial clones derived from V_{3-11}, it is possible that these clones have very different reactivities. However, if the two clonally related, nonidentical clones S4P12 and S5P14 are present as a result of antigenic selection for increased affinity for the antigen, we predict that these antibodies will have similar reactivities and that the more mutated clone may have a higher affinity for the cognate antigen. Because this patient had long-standing disease, the relevance of this antigen with regard to the initiation of the disease is open to question, but it may be important in the propagation of the abnormal B cell response in synovium.

Potential Mechanisms by which Clonally Related B Lymphocytes Are Present in RA Synovial Tissue

There are two possible mechanisms by which clonally related B cells may be present in RA synovial tissue: migration of clonally related B cells into RA synovium or generation of a T cell–dependent B cell response within the synovial lymphocytic infiltrates. Random or nondirected extravasation of clonally related circulating B cells into inflamed RA synovial tissue appears to be unlikely from a statistical point of view. Using probes specific for V_H CDR3 domains, Yamada et al. estimated the frequency of

specific B cell clones in peripheral blood of normal individuals as not greater than 1 per 20,000 cells (46). Using this ratio of 1 specific B cell clone per 20,000 circulating B cells, our finding that 2 of 64 synovial clones were genealogically related is highly improbable by chance alone ($p < 0.007$, Fisher's exact, two-tailed). Furthermore, identification of 5 clonal transcripts out of 58 in a cDNA library generated from 22 grams of inflammatory synovial tissue further emphasizes the oligoclonality of the response and the fact that this oligoclonality is likely antigen driven.

We cannot exclude the possibility that the three identical V_H-containing clones were all secreted by the same B cell or plasma cell. However, in this synovial sample, ELISPOT analysis revealed that $\sim 1.6 \times 10^6$ cells were able to secrete immunoglobulin in culture (data not shown). Thus, it seems statistically unlikely (because of the large number of cells from which mRNA was obtained) that multiple mRNAs from one particular cell would be repeatedly isolated from this non-PCR-amplified cDNA library.

Inflamed RA Synovium Can Function as a Secondary Lymphoid Organ

Nodular lymphocytic infiltrates that are histologically similar to the germinal centers of normal lymphoid organs have long been noted to occur in the subsynovial layer of some patients with long-standing RA (68–71). Some of these synovial cellular aggregates contain plasma cells and plasmablasts (72), networks of CD23+ follicular dendritic cells (71), and high endothelial venules (HEVs) (69,73). RA synoviocytes have been reported to be capable of allowing resting B cells to differentiate into plasma cells secreting large amounts of immunoglobulin (74). A recent study documented sequences with shared mutations from within a germinal center-like structure in RA synovium (75). These data strongly support the hypothesis that positive antigenic selection and clonal outgrowth of B cells expressing antigen receptors with high affinity for particular antigens may occur in RA synovial tissue, i.e., the synovium may function in a manner similar to normal secondary lymphoid organs.

The presence of germinal center-like structures has been reported in inflamed nonlymphoid tissues from individuals with chronic infectious/inflammatory diseases, such as chronic hepatitis B and C (76–78), Lyme disease (79), and reactive arthritis (80). Oligoclonal B cell expansion has been shown in germinal center-like structures in synovial tissue of a patient with reactive arthritis (81), a disease associated with particular infectious organisms. The histologic similarities between these infectious diseases and chronic inflammatory diseases suggests that an infectious organism may be involved in the pathogenesis of RA. The identity of antigens capable of driving the B cell response in RA synovium remains elusive, but analysis of the specificity of clonally related antibodies may provide important clues.

In summary, we have found nonrandom utilization of V_H and D_H gene segments, and D_H reading frame in RA synovium. There was frequent utilization and clonal expansion of the V_H gene segment V_{3-11}, which is not frequently expressed in normal human antibody repertoires. Oligoclonal B lymphocyte response in chronically inflamed RA synovial tissue has important pathogenetic implications, as local antigens present in synovium could potentially propagate the chronic inflammatory response.

Acknowledgments

We thank Jennifer Collins for excellent technical assistance and Dr. John Volanakis for reviewing the manuscript. This work was supported in part by a VA Merit Award (W.J.K.) and NIH grants AR01867 (S.L.B.), AR03555 (H.W.S. and W.J.K.), AI18958 (W.J.K.), AI18745 (W.J.K.), AR20614 (W.J.K.), DK40117 (W.J.K.), AI23694 (H.W.S.), and AI30879 (H.W.S.). B.E.C. was supported by the World Health Organization.

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