Antigen-Dependent B Cell Differentiation in the Synovial Tissue of a Patient with Reactive Arthritis

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ABSTRACT

Background: Reactive arthritis (ReA) can develop as a consequence of a bacterial infection with organisms such as *Chlamydia trachomata, Shigella flexneri,* or *Yersinia enterocolitica*. Although the mechanism underlying the induction of a chronic synovitis is unknown, the expression of HLA-B27 seems to play a crucial role in the etiology of the disease. Bacterial antigens induce a humoral immune response, but little is known about the impact of B cells on the inflammatory processes developing in the synovial membrane.

Materials and Methods: Cryostat sections were prepared from the synovial tissue (ST) of patients with ReA and stained with antibodies specific for T, B, and follicular dendritic cells. Lymphoid infiltrates were directly isolated by microdisection and DNA was prepared from them. The rearranged V genes were amplified by polymerase chain reaction (PCR), cloned, and sequenced.

Results: Histological staining showed that germinal, center-like structures develop in the ST of patients with ReA. B cells with a heterogenous repertoire were isolated from these lymphoid infiltrates. The majority of V regions carried somatic mutations indicating that sequences are derived from memory B cells. Genealogical trees demonstrate clonal expansion and diversification of the B cell repertoire in the ST.

Conclusions: The finding of local V-region diversification suggests that in the ST of patients with ReA, an antigen-driven, T cell–dependent differentiation of B cells occurs. This local B cell response may contribute to the progress of the disease. Whether B cells are specific for the bacteria inducing the synovitis or for self-determinants present in the ST remains to be determined.

INTRODUCTION

Reactive arthritis (ReA) is induced by a bacterial infection in the urogenital or the gastrointestinal duct (1). In such patients a chronic inflammation of the synovial tissue (ST) may develop which rarely leads to the destruction of the joint. A primary immune response directed against bacterial antigens may cross-react with normal tissue antigens to trigger an autoimmune reaction.

In general, the primary immune response to infectious agents is of relatively low affinity, although the affinity increases with time—a process known as maturation (2). Immunizations with antigens like phenyl oxazolone or nitrophenyl coupled to carrier proteins have demonstrated that the affinity maturation of the immune response takes place in the microenvironment of the germinal center (3,4). In the first days after immunization, the antigen-activated B cells migrate into the primary follicles of the lymphatic tissue. Strong proliferation of B cells leads to the formation of germinal centers. In this microenvironment a hypermutation mechanism becomes activated which introduces somatic mutations into the V genes of the H and L chains. Numerous B cell variants expressing receptors of different affinity for the antigen are generated. However, only those B cells with high-affinity receptors will differentiate into memory cells and plasma cells. Crucial for antigenic selection are follicular dendritic cells (FDC), which present antigen complexed with antibody on their long dendrites to the B cell receptor (5).

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The pattern of somatic mutations in the V regions indicates whether B cells have been selected for antigen. Whereas the codon usage in V regions predicts an overall ratio for replacement to silent mutations of approximately 3, selection leads to a preferential increase of replacement mutations in those parts of the V region which form the binding site, the complementarity determining regions (CDR) (6). In contrast, silent mutations accumulate mainly in the framework residues (FR). Thus, the ratio of amino acid replacement to silent mutations (R/S value) in the CDR versus the FR can be used as a measurement to determine whether affinity selection has taken place.

As nucleotide exchanges are randomly introduced into the V regions pathogenic autoantigen-specific B cells may be generated during the germinal center reaction. In general, as no T cell help is available, such cells will be deleted (7,8). However, some of these cells may develop into plasma or memory cells and in this way an immune reaction induced through foreign antigen may turn into an autoreactive response (9).

Autoreactive antibodies are found in every individual (10). One such example is the rheumatoid factor (RF) which is specific for self-IgG (11). However, there seems to be no affinity maturation for autoreactive B cells in healthy individuals. A surprisingly low R/S value was observed for the CDRs of H and L chains of autoantibodies, which suggests that there is selection against replacement mutations in the CDR (12).

In autoimmune diseases a maturation of the self-specific immune response does take place (13–17). High-affinity, self-reactive B cells are selected to develop into memory and plasma cells. In Graves' disease, for example, the majority of patients have high-affinity autoantibodies specific for thyroid antigens. These antibodies are thought to play a role in thyroid destruction (15).

In autoimmune diseases the affected tissue is infiltrated by mononuclear cells and germinal, center-like structures often develop (18–21). Using microdissection, such lymphoid follicles have been isolated from the synovial tissue of patients with rheumatoid arthritis and the B cell repertoire has been determined (22,23). Our results demonstrate that an antigen-driven, T cell-dependent immune response takes place in this nonlymphoid tissue (23). Thus, in autoimmune diseases, B cell differentiation may occur locally, directly in the affected tissue.

Bacterial antigens are detectable in both the synovial membrane and the synovial fluid of the inflamed joints of patients with ReA. Although the isolation of live bacteria from the ST is problematic (1), bacterial DNA could be demonstrated (24,25). In addition, preliminary reports indicate the presence of chlamydial mRNA transcripts and rRNA in the ST of patients with ReA (26,27). Thus, the development of chronic inflammatory synovitis may be caused by long-term, persisting bacterial antigen in the joints.

In order to examine the immune response that takes place in the synovial membrane of patients with ReA, we have stained frozen tissue sections with antibodies specific for FDC and T and B cells. As described for patients with rheumatoid arthritis (RA), germinal center-like structures were found. Such cell clusters were isolated from a patient with postchlamydial ReA, DNA was prepared, and the V-gene repertoire of synovial B cells was determined. The analysis of the V-gene diversity shows that certain B cells are clonally expanded and that during proliferation, somatic mutations have accumulated. The pattern of somatic mutations indicates that in the microenvironment of the ST, an antigen-dependent affinity maturation takes place. Thus, after urethral infection with chlamydia in the ST, a local immune response may be induced that is comparable to the one seen in patients RA.

MATERIALS AND METHODS

Patient

Synovial B cells from patient T.S., a 30-year-old, HLA-B27 negative male from India were analyzed. The biopsy was taken 4 years after onset of the disease from the knee. Patient T.S. developed chronic ReA after urethral infection. The urethral swab was positive for *Chlamydia trachomatis*. At the onset of the disease, serum was positive for *Chlamydia*-specific antibodies. In addition, in the ST, *Chlamydia trachomatis* plasmid DNA could be demonstrated by polymerase chain reaction (PCR).

Immunohistochemical Analysis

The preparation of tissue sections was done as previously described (23). Briefly, ST was directly embedded in Tissue-Tek® O.C.T. Compound (Miles, Giessen, Germany) and frozen in liquid nitrogen. Cryostat sections of 6 μm were

thaw mounted onto slides coated with 2% APES (3-aminopropyltriethoxy-silane; Sigma). After drying at room temperature for 1 hr, the sections were heated for 1 min at 50°C and then fixed for 10 min in cold acetone. For histological staining of cells, monoclonal antibodies (MAb) specific for plasma cells (Wue 1 [23]) for surface determinants of B cells (anti CD20, MAb L26 (DAKO), T cells (anti-CD3, MAb T3-4B5, DAKO) and FDC (Wue 2 [23]) were used. Staining was visualized by APAAP technique (DAKO).

Isolation of B Cell Infiltrates and DNA Preparation

B cells were identified in lymphocytic infiltrates using a MAb specific for CD20. On consecutive sections stained with Mayer's hematoxilin only, cell clusters were removed using blood lancets and isolated tissue was digested at 50°C with 20 μl 1 mg/ml proteinase K (Boehringer) (23).

Amplification and Cloning of DNA

The proteinase K–digested material was amplified with a set of primers specific for either rearrangened VH or VL genes (23). Forty cycles of PCR amplification were carried out in 50 μl final volume containing 50 mM KCL; 10 mM Tris-HCL, pH 9.0; 0.1% Triton X-100; 2 mM $MgCl_2$; 200 μM each of dATP, dCTP, dGTP, dTTP; 20 nM of each primer; and 2 units of taq DNA polymerase (Promega). For amplification of VH genes, 40 cycles of PCR with an annealing temperature of 70°C, and for VL genes, 65°C, were used. A 20-μl aliquot of the reaction was analyzed on a 2% low-melting agarose gel (NuSieve) and a region of approximately 350 bp was isolated, although no DNA band was visible.

The agarose piece was melted in 200 μl TE buffer (pH 8.0) and 1 μl was used for 40 cycles of reamplification. In independent reactions, primers specific for the different VH gene families, a primer mix specific for Vλ or a primer mix specific for Vκ were used. The first framework of the H-chain gene families VH1, VH3, VH5, and VH7 differs only by single nucleotides. Therefore, for reamplification only, the VH1 and the VH3 primer were used. Amplification with these two primers gave comparable results showing that there is no preferential amplification with either primer.

The PCR products were cloned into the pCRII vector using the TA cloning system, version 2.3 from Invitrogen (Amsterdam, Netherlands).

DNA Sequence Determination

Variable regions were sequenced using a modified Taq sequencing protocol (USB, Amersham). Two-hundred nanograms dsDNA was amplified for 20 rounds (94°C for 1 min, 45°C for 1.5 min, and 72°C for 2 min). All inserts were sequenced from both sides using a −40 or +40 digoxigenin end-labeled primer (TIB, Mol Biol, Berlin, Germany). Sequencing reaction was applied to a direct blotting machine (MWG Biotech, Ebersberg, Germany) (23).

To identify germ-line genes, searches of the EMBL/GeneBank database were performed. In addition, sequences were compared with the database V BASE (J.M. Tomlinson, Medical Research Council, Center for Protein Engineering, Cambridge, U.K.) by use of the Macintosh program DNA PLOT 1.4 (W. Müller, Institut für Genetik, Köln, Germany). Sequences are available from the EMBL/GenBank nucleotide sequence database accession numbers Z84824–Z84864.

RESULTS

Histological Description of ST Samples

The histological analysis of tissue sections derived from the synovial membrane of patients with ReA resulted in a picture comparable to that described for patients with RA. In patient T.S. as well as other patients with ReA the ST was infiltrated by both T and B cells. Often such cell clusters had a germinal center-like structure in which FDC were present. Furthermore, numerous plasma cells can be seen in the ST of patients with ReA. They were found in a circular arrangement around the large cell clusters and they were seen scattered all through the tissue. To determine the V-gene repertoire of synovial B cells two large infiltrates were isolated by micromanipulation directly from the frozen tissue section. In both of these lymphocytic infiltrates the majority of cells were positive for the B cell antigen CD20.

B Cells in ST of a Patient with ReA Express a Diverse V-Gene Repertoire

Cells from two infiltrates, cluster A and cluster B, were isolated from two consecutive sections, sections 65 and 66 (Table 1). A diverse repertoire of

TABLE 1. V-region sequences isolated from the infiltrates A and B

		Cluster A			Cluster B		
		Putative Germ-line Gene		No. of Isolated Sequences	Putative Germ-line Gene		No. of Isolated Sequences
Section 65	H chain	VH1	DP3	1			
			DP4	4			
		VH3	DP47	1[a]			
			DP49	6			
		VH4	DP71	2			
			DP71	1			
			DP71	1			
	L chain	Vλ2	DPL10	1	Vλ1	DPL2	3[b]
			DPL11	2		DPL2	1
		Vλ6	IGLV6S1	1	Vλ3	LV318	1
Section 66	H chain	VH1	DP7	1	VH3	DP53	2
			DP10	2	VH7	VI-41b	1
			DP14	4			
			DP75	1			
		VH3	DP31	1			
			DP35	1			
			DP42	2			
			DP47	1[a]			
			DP47	1			
			DP49	1			
			DP49	1			
			DP49	1			
		VH4	DP65	1			
			DP70	2			
	L chain	Vλ3	LV318	1	Vλ1	DPL2	3[b]
		Vλ4	DPL16	1		DPL3	3
					Vλ3	LV318	1
		Vκ1	O12-O2	1[c]	Vλ4	DPL16	3
			O18-O8	1[c]		DPL16	5
			O18-O8	1	Vλ6	IGLV6S1	1
			O18-O8	1	Vλ5	Unknown	3
		Vκ3	B2	1[c]			

[a] Indicates identical VH rearrangement.
[b] Indicates identical VL rearrangement.
[c] Indicates out-of-frame V-to-J joining.

H and L chain sequences was found in both cell infiltrates.

Altogether, 22 of 39 VH regions had a different V/D/J rearrangement. The deduced amino acid sequences are shown in Fig. 1A. VH regions showed a high degree of diversity in their CDRIII regions. VH regions are joined to various D and JH elements and there is N-region diversity. The length for the CDRIII varied from 7 to up to 20 amino acids. The majority of VH genes belonging

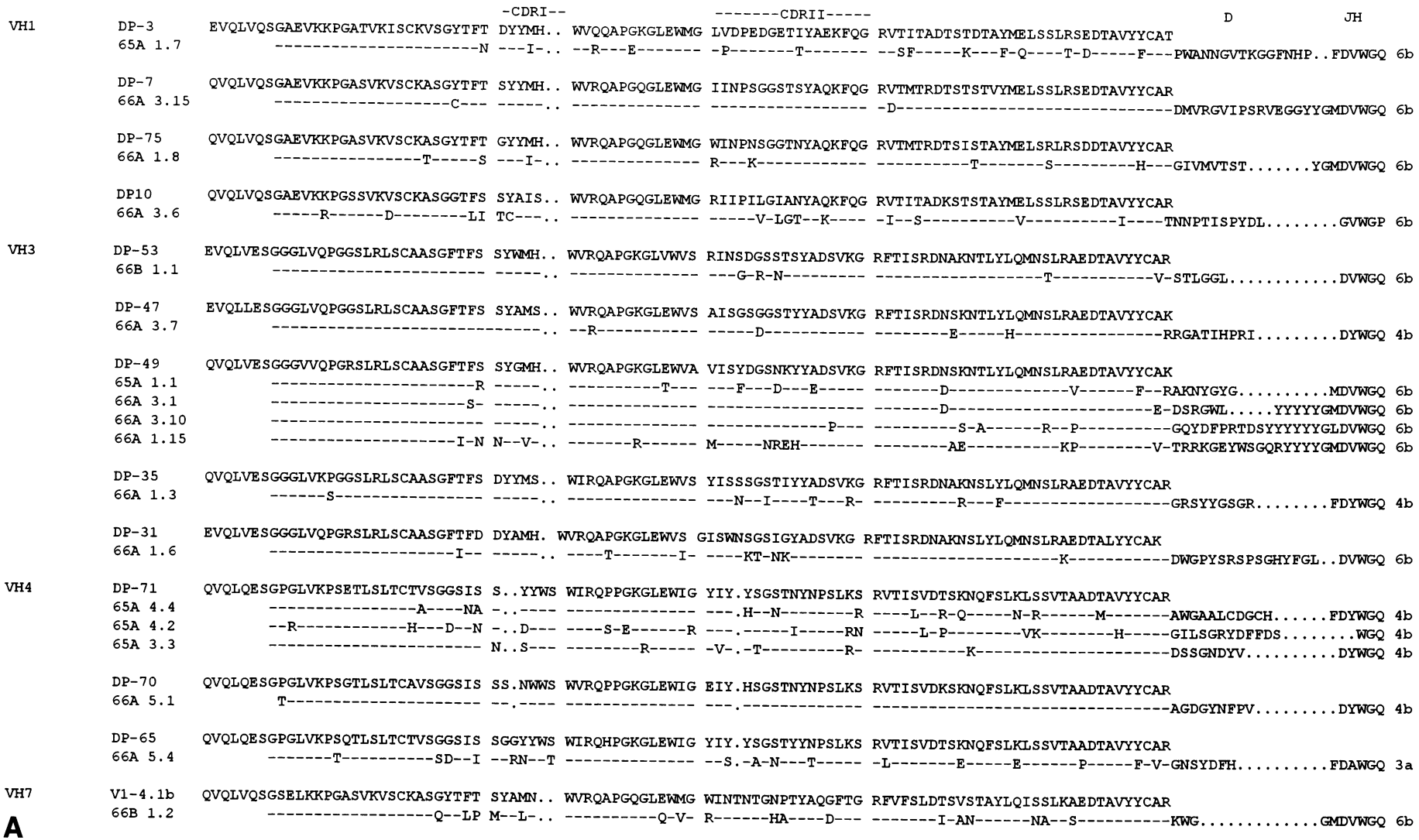

FIG. 1. Amino acid sequences isolated from infiltrates A and B

Sequences are compared with the most homologous germ-line gene. Dashes show sequence identity, to increase homology between different V genes dots have been introduced. CDR and D, and J regions are indicated. (A) Deduced amino acid sequences of VH genes. (B) Deduced amino acid sequences of Vλ and V_κ genes. In the case of the sequence TS-66B 5.8 (Vλ5), the V region is compared with the rearranged Vλ sequences 488.9g.F1 (31), T1 (32), and YM1 (33).

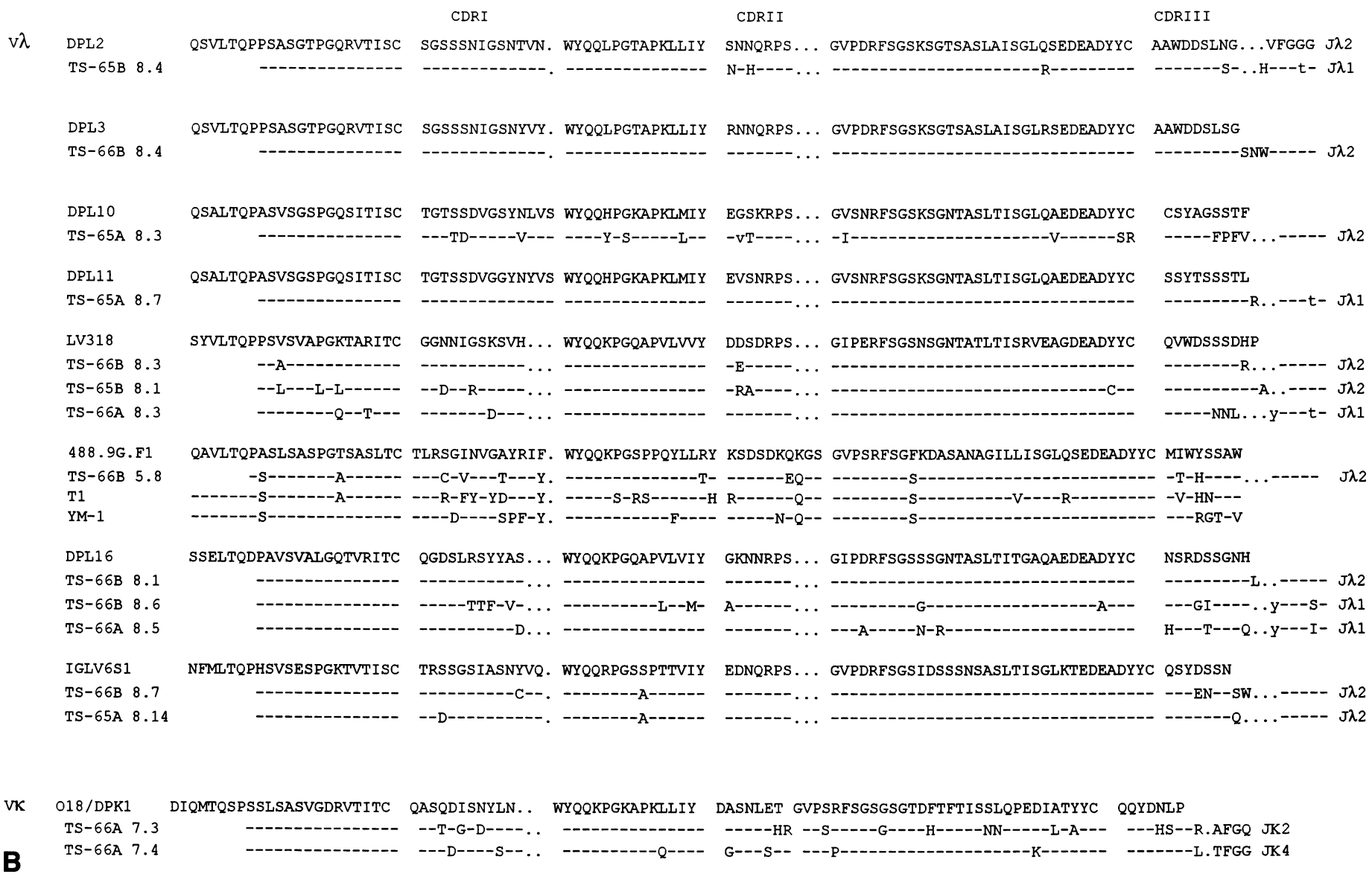

FIG. 1. *continued*

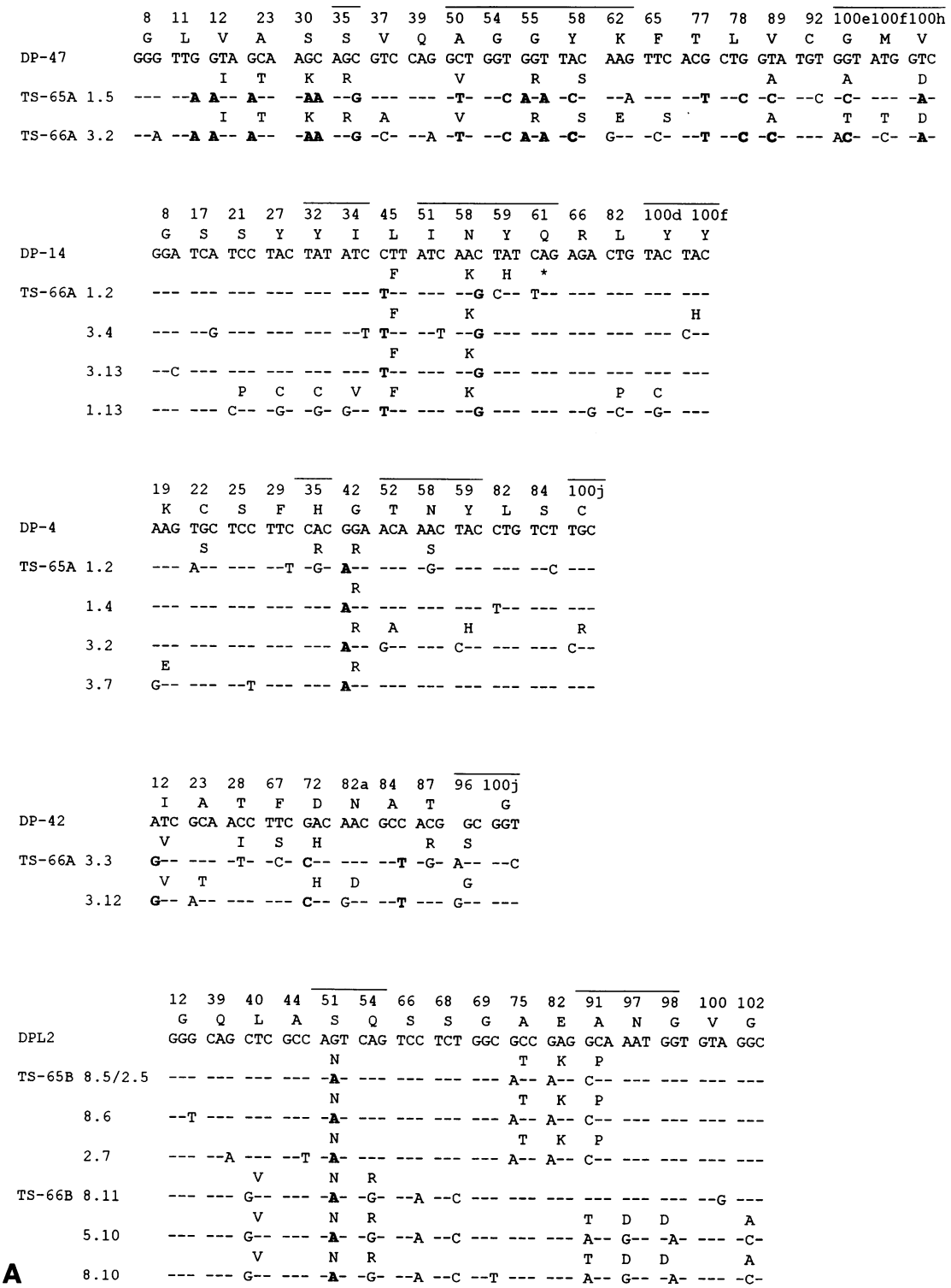

FIG. 2. Diversification of the V-gene repertoire in the ST

(A) Clonally related sequences differing in their number of somatic mutations. Sequences are compared with their most homologous germ-line gene; only nucleotide differences are shown; CDR are indicated, codons are numbered according to Kabat et al. (34). Bold letters show somatic mutations common to clonal-related sequences. (B) Genealogical trees showing the step-wise accumulation of somatic mutations in the ST. Results are given for the sequences shown in Fig. 2A. Numbered circles indicate isolated sequences, empty circles, presumptive cells. Numbers beside the arrows refer to the number of nucleotide exchanges that distinguish one sequence from another.

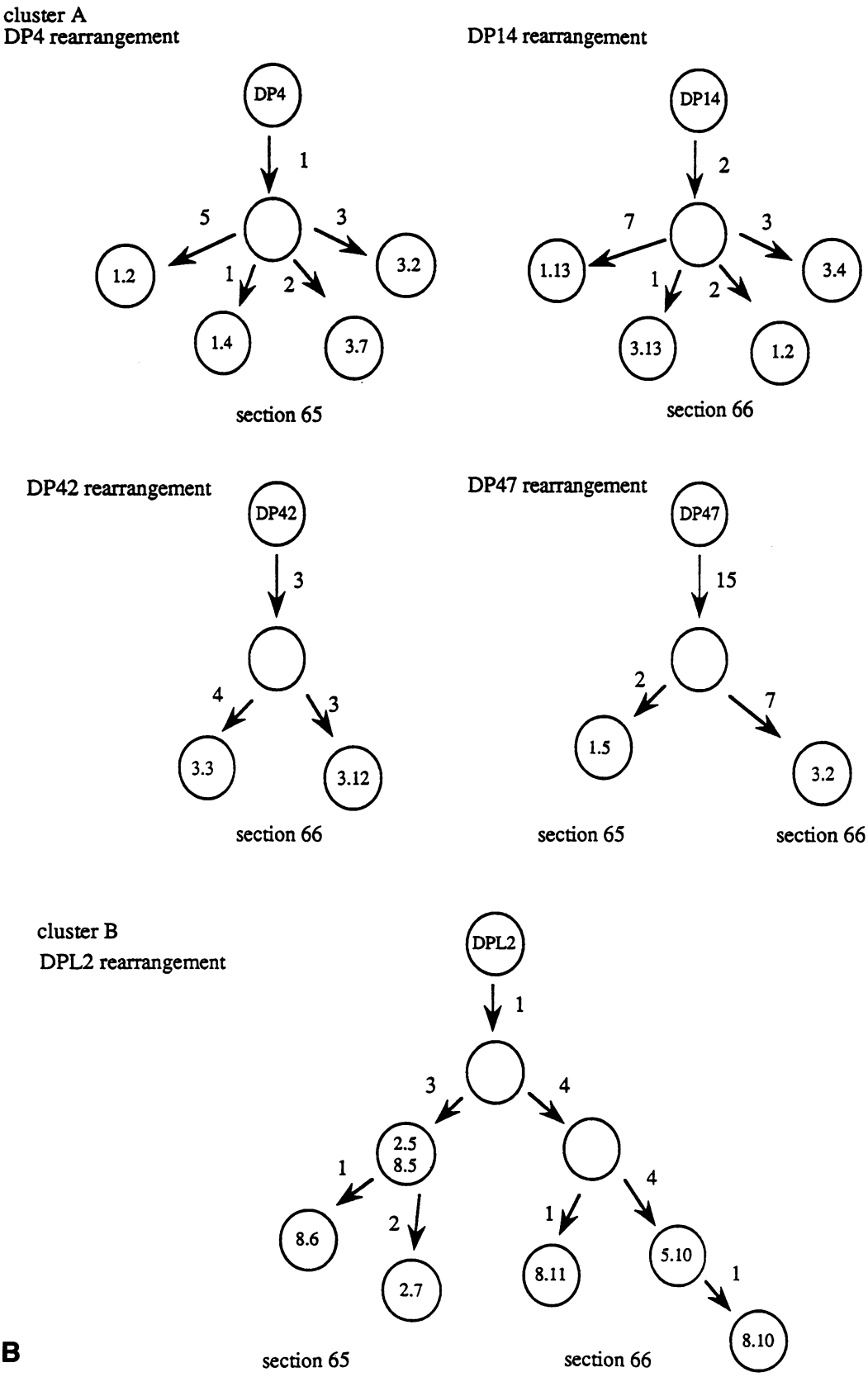

FIG. 2. *continued*

to the gene families VH1, VH3, and VH7 were associated with JH6. In contrast, four out of five VH4 genes were joined to the JH4 gene.

A comparable diverse repertoire was seen for the L chains (Table 1). From the 35 L-chain sequences determined, 19 had a different V-to-J joining. Whereas lambda L chains had a functional rearrangement, three out of five Vκ sequences had an out-of-frame V-to-J joining. These nonproductive Vκ regions may be derived from a Vλ expressing B cells. The sequences with in-frame rearrangements are shown in Fig. 1B.

Although a diverse repertoire of V gene sequences was isolated from these infiltrates, there is evidence that certain B cell clones are expanded in the ST. An identical VH rearrangement was found in the two sections 65 and 66 from infiltrate A. Similarly, an identical Vλ rearrangement was found on consecutive sections of infiltrate B (Table 1, indicated by *a*, *b*). Furthermore, a number of V(D)J rearrangements were isolated many times from one section (Table 1). For example, a VH DP14 sequence was found four times in section 66 of cluster A. The V(D)J rearrangement shows that these V genes are derived from one B cell clone. However, because they differ in their pattern of somatic mutations, they originate from different cells (Fig. 2A).

Somatic Diversity in H- and L-Chain Sequences

Figure 1 shows a comparison of the isolated H- and L-chain sequences with the putative germ-line genes. The majority of synovial V regions differ in their amino acid sequence from the presumptive germ-line gene. Only few sequences were unmutated. Approximately half of the V genes carried low numbers of somatic mutations (Fig. 3). The frequency of nucleotide substitutions was comparable to the diversity seen in peripheral blood B cells (28). The other half of sequences were highly mutated (Fig. 3). Up to 28 exchanges per V region were seen.

It was impossible to determine the number of somatic mutations, in only one case, a Vλ5 rearrangement. A comparison with published cDNA sequences indicated that this sequence is derived from a yet undescribed germ-line gene (Fig. 1B).

Frequency of Replacement Mutations in the CDR of Memory B Cells

Table 2 gives the R/S values for the CDR of those sequences that are highly mutated and have more than 10 substitutions per V region. The average frequency in the overall number of somatic mutations is 19 exchanges per variable region. However, sequences differ in their ratio of replacement to silent mutation in the CDR. In approximately half of the sequences there is a high frequency of replacement mutations, which suggests that they are derived from B cells that have been positively selected for their affinity. In the other half of the sequences there is a surprisingly low frequency of replacement mutations. For the CDR an R/S value of 2 was found, which is even less than one would expect with a random distribution of somatic mutations. These V regions show that there must be a negative selection for replacement mutations. Hence, in the ST memory, B cells accumulate and the pattern of somatic mutations suggests that the affinity maturation has taken place.

Antigen-Dependent Differentiation of B Cells in the ST

Sequences with identical V(D)J rearrangements but different numbers of somatic mutations were isolated from both lymphoytic infiltrates (Fig. 2). These V genes suggest that an antigen-driven and T cell–dependent immune response takes place in the ST of this patient with ReA. For example, sequences with a Vλ DPL2 rearrangement had one somatic mutation in common but differed by up to 14 nucleotides from each other (Fig. 2B). Thus, the somatic diversity seen in this B cell clone has been introduced during local proliferation in the ST.

Sequences with a VH DP47 rearrangement had 16 somatic mutations in common but differed at nine positions (Fig. 2B). These V regions show that B cells that have gone through multiple cycles of division and hypermutation become reactivated in the ST and accumulate locally further somatic mutations. Thus, in the ST, both newly activated and memory B cells are going through an antigen-dependent differentiation.

Maturation of the Immune Response in the ST

The pattern of somatic mutations indicates whether B cells are selected for affinity (6). An analysis of the nucleotide exchanges that have been introduced during proliferation in the ST shows that these somatic mutations are nonrandomly distributed. Twenty-four out of the 57 substitutions are in the CDR and the majority of

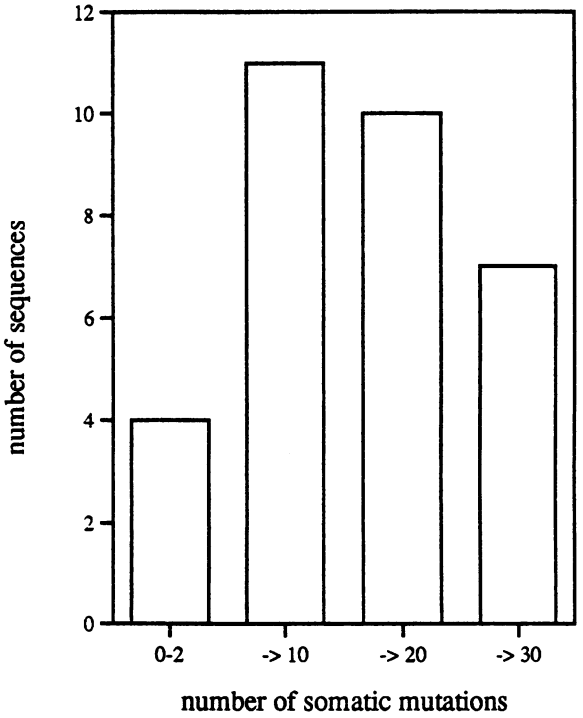

FIG. 3. V-gene diversity in synovial B cells

them are replacement mutations (Fig. 2A). The R/S value for the CDR is 7.3 and suggests that these B cells are positively selected for their affinity. This provides evidence for an antigen-dependent maturation of the immune response through hypermutation and selection for high-affinity variants in the ST from a patient with ReA.

DISCUSSION

Large infiltrates were isolated from the ST of a patient with ReA and the expressed V-gene repertoire of the synovial B cells was determined. In both cell clusters a diverse repertoire was found showing that these infiltrates are polyclonal. Practically all sequences carry somatic mutations, although many of them have only a few nucleotide exchanges when compared with the putative germ-line genes (Fig. 3). This diverse repertoire and the high frequency of sequences with low numbers of somatic mutations suggests that there is a constant influx of resting or newly activated B cells from the peripheral blood into the ST.

However, there are in addition sequences with high numbers of somatic mutations (Fig. 3). These V genes, differing in more than 20 nucleotides from the closest germ-line gene, may represent chronically activated memory cells. The sequence data indicate, however, that only a few B cell clones have been expanded. In the infiltrates isolated from the ST of this patient with ReA there is a much higher clonal diversity than seen in the infiltrates isolated from patients with RA (23). What is the specificity of B cells that accumulate in the inflammed tissue of patients with ReA? Memory synovial B cells might be directed against bacterial antigens or alternatively, they may be specific for autoantigens expressed on the ST.

TABLE 2. Somatic mutations in synovial memory B cells

VH/VL Sequences	No. of Mutations	Mutations in CDR		R/S
		R	S	
65 A 1.1	17	3	2	1.5
1.7	17	4	—	≥4
3.3	14	5	1	5
4.4	22	3	2	1.5
4.2	25	4	1	4
8.3	18	6	1	6
66 A 1.6	15	4	1	4
1.8	18	3	2	1.5
1.15	25	7	3	2.5
3.6	21	7	2	3.5
5.4	25	8	3	2.7
7.3	21	9	2	4.5
7.4	11	5	1	5
8.5	14	4	2	2
65 B 8.1	17	6	2	3
66 B 1.2	28	7	—	≥7
8.6	19	7	5	1.4

The ratio of replacement to silent mutations in the CDR suggests that sequences are derived from B cells that have gone through an affinity maturation process (Table 2). This antigen-driven and T cell–dependent immune reaction might have taken place in the ST. The fact that sequences with identical V(D)J rearrangements have different numbers of somatic mutations demonstrates that B cell diversification occurs locally. The preferential accumulation of substitutions in the CDR, together with the high R/S values, is indicative of an antigen-dependent immune response.

These B cell clones activated in the ST may be specific for the foreign chlamydial antigen. Data suggest that even years after infection bacterial antigens are present in the ST (29). Although it has not been possible to isolate viable bacteria from the ST of patients with ReA, the persistence of bacteria in the joint tissue was suggested by PCR where both chlamydial DNA and RNA could be demonstrated (1,25–27). Furthermore, it has been shown that antigen can be preserved for long periods of time on FDC in the form of antigen–antibody complexes (30). The presence of these cells in the germinal, center-like infiltrates in the ST of patients with ReA may suggest that FDC play a role in mediating a chronic activation of B cells. Chlamydial antigenic debris presented on FDC may select for high-affinity B cell clones which may help to control the chlamydial infection. One may speculate whether the synovial inflammation is a bystander effect.

On the other hand, sequences may represent pathogenic self-specific B cell clones. Thus, the original immune response against the bacterial antigens may have resulted in a cross-reactive, autoreactive response. Self-antigens may be responsible for maintaining the chronic inflammatory processes seen in the ST of this patient. Clearly, the specificity of B cells activated in the ST has to be determined before one can distinguish between these different interpretations of the results.

An analysis of the mutational pattern showed that from the ST, two different types of sequences were isolated. In both infiltrates we found V regions that seemed to be positively selected and some that seemed to be negatively selected for replacement mutations in the CDR (Table 2). The high ratio of replacement to silent mutations in the CDR suggests positive selection for high affinity (6). Although a low R/S value is normally taken of negative selection, a low R/S value may also be derived from high-affinity B cells. Thus, in sequences where a further accumulation of replacement mutations in the CDR is disadvantagous, silent mutations may accumulate. Comparable results have only rarely been reported; however, when autoantibodies of healthy individuals were sequenced, a population of memory cells was obtained that appeared to be negatively selected for replacement mutations (12). In this case, it was suggested that a mechanism may be operating that opposes the affinity maturation of self-reactive B cells.

RA is thought to be an autoimmune disease, although the self-antigen(s) driving the B cell response has not been identified. In contrast, ReA is induced by a bacterial infection. Nevertheless, the histology of the inflamed ST is strikingly similar in both situations (22,23). In both cases massive infiltrations of lymphocytes are seen and germinal, center-like structures often develop. In the nonlymphoid tissue a microenvironment is built up that allows the antigen-dependent differentiation of B cells. The specificity of the synovial B cells will give insight into the etiology of these rheumatic diseases.

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