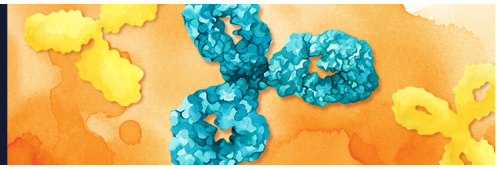


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Plasma Cell Development in Synovial Germinal Centers in Patients with Rheumatoid and Reactive Arthritis¹

Hye-Jung Kim,^{2*} Veit Krenn,[†] Gudrun Steinhauser,^{*} and Claudia Berek^{*}

Plasma cells are found surrounding the inflammatory infiltrates of macrophages, T, and B cells in the synovial tissue of patients with rheumatoid and reactive arthritis. This characteristic arrangement suggests that in the synovial tissue CD20⁺ B cells differentiate into plasma cells. To examine clonal relationships, we have used micromanipulation to separately isolate CD20⁺ B cells and plasma cells from single infiltrates. DNA was extracted, and from both populations the V_H/V_L gene repertoires was determined. The data show that in the inflamed synovial tissue activated B cells are clonally expanded. During proliferation in the network of follicular dendritic cells, V gene variants are generated by the hypermutation mechanism. Surprisingly, we do not find identical rearrangements between CD20⁺ B cells and plasma cells. Nevertheless, the finding of clonally related plasma cells within single infiltrates suggests that these cells underwent terminal differentiation in the synovial tissue. These results indicate that B cell differentiation in the synovial tissue is a dynamic process. Whereas CD20⁺ B cells may turnover rapidly, plasma cells may well be long lived and thus accumulate in the synovial tissue. The analysis of individual B cells recovered from synovial tissue opens a new way to determine the specificity of those cells that take part in the local immune reaction. This will provide new insights into the pathogenesis of chronic inflammatory diseases like rheumatoid or reactive arthritis. *The Journal of Immunology*, 1999, 162: 3053–3062.

In rheumatoid arthritis (RA),³ the synovial membrane is the central site of inflammatory activity. As the disease progresses, the morphology of the synovial tissue (ST) is changed by a massive infiltration of mononuclear cells (1). Proinflammatory cytokines like TNF- α and lymphotoxin α , which play a central role in joint inflammation (2), may help to promote the development of ectopic lymphoid tissue (3, 4). Immunohistochemical examination of the ST shows that lymphocytic aggregates are relatively infrequent in the early stages of the disease; however, they form as the disease progresses (5, 6). The cellular composition of the lymphocyte clusters seems to vary depending on the size. Small aggregates consist mainly of T cells with only few B cells, while large ones contain substantially more B cells (7). Synovial lymphocytic infiltrates often reveal a structure and cellular composition reminiscent of that seen in germinal centers (GC) of the peripheral lymphoid organs (8, 9).

Classical GC arise transiently in the primary B cell follicles of secondary lymphoid organs during immune responses and provide a microenvironment for B cell differentiation (10, 11). Well-studied GCs in human tissue are those found in the tonsil (12, 13). Immunohistochemistry shows that they are composed of a dark and a light zone (14). B cells in the dark zone, which are referred to as centroblasts, undergo massive clonal expansion. Concomitantly, they activate a mechanism that introduces somatic muta-

tions into the rearranged Ig V genes and, thus, can lead to changes in the B cell receptor affinity for its Ag. The light zone of the GC contains a network of follicular dendritic cells (FDC) that have the capacity to retain Ag and present it in the form of immune complexes. B cell variants generated in the dark zone move into the light zone where they are believed to compete for the Ag. Those B cells with receptors of high affinity may receive signals provided by T cells that ensure their survival. The high-affinity B cells selected in this way subsequently differentiate into memory B cells and plasma cells, which have the potential to secrete Abs of high affinity and specificity.

Recent work suggested that an Ag-dependent immune reaction takes place in the ST of patients with RA (15–18). An analysis in which single lymphocytic infiltrates from frozen sections of the ST of patients with RA or with chronic reactive arthritis (ReA) were analyzed has demonstrated that a GC reaction may occur in the ST (19–21). The analysis of the V gene repertoire showed that B cells can proliferate and accumulate somatic mutations in the chronically inflamed ST. The stepwise introduction of somatic point mutations into the rearranged Ig V genes may result in variants that have acquired high affinity to local Ags. In the case of patients with RA, this might be an autoantigen. For example, the analysis of rheumatoid factor-secreting cell lines has suggested that high-affinity B cells with specificity for self-Ig develop in the ST (18). In the case of patients with ReA, it has been suggested that bacterial Ags may drive the chronic immune response (22).

Immunohistological analysis of the lymphocytic infiltrates in the ST showed that plasma cells are organized in concentric rings around the large cellular clusters of CD20⁺ B and T cells (20). These characteristic histological features of the affected tissue have led us to ask whether B cell variants generated in synovial GC are selected to differentiate into plasma cells. If this hypothesis is correct, then it would seem that local Ag in the ST may drive both the diversification of the V gene repertoire and the differentiation of plasma cells.

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³ Abbreviations used in this paper: RA, rheumatoid arthritis; FDC, follicular dendritic cell; GC, germinal center; ReA, reactive arthritis; ST, synovial tissue.

Table I. *V* gene sequences isolated from the ST of patients RA and ReA

	Patient	Infiltrate ^a	Section	CD20 ⁺ B Cells			Plasma Cells		
				Heavy chain	Light chain		Heavy chain	Light chain	
					κ	λ		κ	λ
Group of cells	RA PS	I	56	27/33	6/9	5/8	9/25	5/9	2/2
			56	1/1	14/35	1/5			
			59	10/23					
		VI	61	2/2			2/4		
			56	4/20					
			59	4/15		5/14			
	RA EK	IV	158				1/14		
			160	9/15		5/7	1/6	1/5	
		V	164				11/20	7/14	6/19
			87	4/10			5/16		
AR	III	87	4/10			10/17	8/11	5/7	
ReA TS	II	6	15/23	11/15	6/10	7/17	2/12	7/11	
		7		2/3					
Single cells ^b	RA EK	VIII	65		3		5	1	

^a For infiltrates I to VII, the number of different rearrangements/total sequenced V genes is given.

^b For the single-cell analysis, the number of isolated V genes with a different rearrangement is given.

To address this question, a methodology was employed that allows us to combine immunohistological information with a biochemical analysis. Previous work has demonstrated that through micromanipulation single cells can be isolated from frozen tissue sections and their V gene repertoire can be determined (12). Control experiments have shown that this technique yields specific and reproducible results. For example, it has been of great value for the analysis of tumor cell development (23, 24).

To examine whether differentiation of B cells into plasma cells takes place in the ST, both CD20⁺ B cells and plasma cells were isolated directly from single infiltrates by micromanipulation, and their V(D)J rearrangements were analyzed. The results show a stepwise accumulation of somatic mutations in the V genes expressed in B cells proliferating in a network of FDC. In addition, V gene sequences were isolated from plasma cells. We were unable to find clonal relationships between the central CD20⁺ B cells and the surrounding plasma cells. Nevertheless, the data suggest that hypermutation and terminal B cell differentiation take place in the synovial GC-like structures. It seems that the ST supports longevity of plasma cells, leading to their accumulation in the chronically inflamed tissue.

Materials and Methods

Preparation of tissue sections

ST was obtained from two patients who fulfilled the Arthritis and Rheumatism Association criteria for RA (25) (patient EK (20), a 72-year-old seronegative female, from which ST was derived from the wrist, where a vilo-nodular synovitis was diagnosed; and patient PS, a 47-year-old seropositive female with a highly progressive form of definitive RA involving tendon sheath from where the examined ST was derived). In addition, ST was used from one patient with presumptive RA (patient AR, a 18-year-old male) and one patient with established chronic ReA (patient TS (21), a 30-year-old male, who developed ReA after a urethral infection with *Chlamydia trachomatis*; the biopsy was taken 4 years after onset of the disease) (Table I). Frozen tissue was cut into 6- μ m sections, which were dried at room temperature and subsequently treated with acetone.

Immunohistochemical analysis of ST

To identify cell types and their location in the ST, tissue sections were labeled with anti-CD20, anti-CD4, anti-FDC, and anti-plasma cell Abs and visualized using the alkaline phosphatase-anti-alkaline phosphatase (APAAP) technique. Monoclonal mouse anti-human Abs were used as primary Abs (anti-CD20, Dako (High Wycombe, U.K.); anti-CD4, Dako; anti-FDC, Wue 2 (20); anti-plasma cells, Wue 1 (20)). Bridge Abs were rabbit anti-mouse IgG (Dako). Sections were subsequently incubated with

APAAP-conjugated-Ab (Dako) using neufuchsin as the substrate. In addition, some of the sections were labeled with an affinity-purified rabbit Ab specific for the nuclear Ag Ki-67 (Dako). Haematoxylin (Merck, Darmstadt, Germany) staining was used to enhance the contrast between labeled and nonlabeled cells.

Isolation of CD20⁺ B cells and DNA extraction

To define the different areas of the lymphocytic infiltrates, tissue sections were double labeled with Abs specific for FDC and for plasma cells. Small groups of ~50 cells were isolated directly from immunochemically labeled tissue sections using a micromanipulator (Nikon, Tokyo, Japan). Isolated cells were transferred to 500- μ l tubes, and DNA was prepared by incubation at 50°C for 1 h with proteinase K (16.4 mg/ml; Boehringer Mannheim, Mannheim, Germany). Proteinase K was inactivated by heating at 95°C for 10 min.

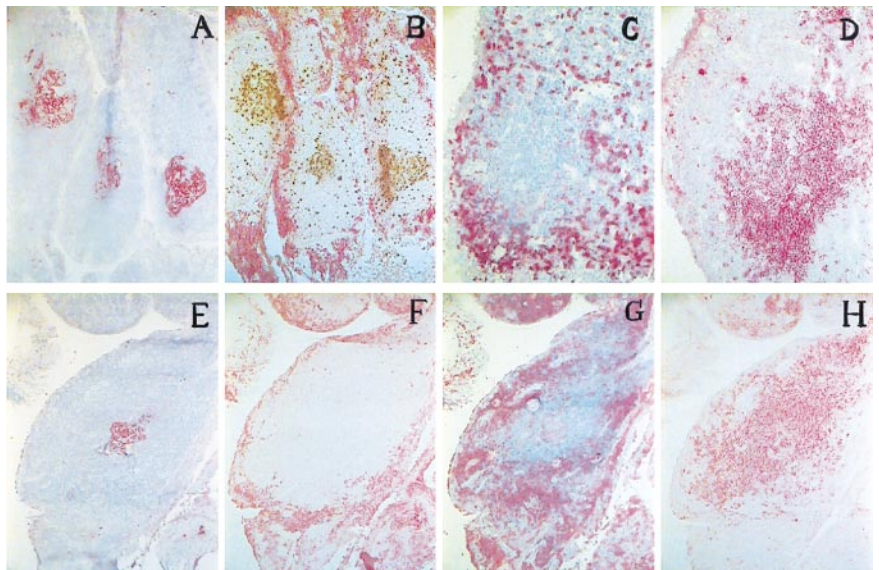
Isolation of plasma cells

To avoid contamination with CD20⁺ B cells, single plasma cells were microdissected from tissue sections labeled with the mAb Wue 1. Approximately 50 cells were pooled, and DNA was extracted as described for CD20⁺ B cells. In only one of the experiments was DNA prepared from single cells. In this case, individual CD20⁺ B cells and plasma cells were microdissected and their expressed V gene repertoire was analyzed. DNA was prepared from single cells in the same way as described for groups of cells.

Amplification of Ig V genes

Rearranged Ig V genes were amplified from CD20⁺ B cells or from plasma cells by PCR. Two steps of amplification were used to obtain appropriate amounts of PCR product. To improve the specificity of the second amplification step, seminested PCR reactions were conducted. In the first step, 35 cycles of amplification with *Taq* polymerase were performed with V_H (20), V κ (20), and V λ 5' primers (V λ 1, 5'-CAGTCTGTGTTGACGCGAGC CGCC; V λ 2a, 5'-CAGTCTGCCCTGACTCAGCCTGC; V λ 2b, 5'-CAGTCTGCCCTGATTACGCTCC; V λ 3a, TCCTATGTGCTGACT CAGCCACC; V λ 3b, 5'-TCTTCTGAGCTGACTCAGGACCC; V λ 4, 5'-CAGTTATACTGACTCAACCGCC; V λ 5, 5'-CAGGCTGTGCTCAC TCAGCCGTC; V λ 6, 5'-AATTTTATGCTGACTCAGCCCA; and V λ 7/8, 5'-CAGACTGTGGTGACGCGAGGAGCC) and external JH (5'-CTCACCTGAGGAGACGGTGACC), J κ (J κ 1-4, 5'-TACTTACGTTT GATCTCCASCTTG; and J κ 5, 5'-GCTTACGTTAATCTCCAGTC GTG), and J λ region (J λ 1, 5'-GAGAGCCACTTACCTAGGACGG; J λ 2, 5'-AGAAGAGACTCACCTAGGACGG) specific 3' primers. In the second round, aliquots were specifically amplified for 39 cycles for either heavy, κ -, or λ -chain genes separately using the same 5' V_H, V κ , and V λ primers but internal JH, J κ , and J λ 3' primers (20). The final concentrations of the reagents were 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 0.1 mM MgCl₂, 200 μ M of each deoxynucleoside triphosphate, 10 pM of each primer, and 2 U *Taq* DNA polymerase. Two different polymerases were used; *Taq* polymerase from Promega (Madison, WI) for the

FIGURE 1. Immunohistology of the ST of patients with RA and ReA. Frozen tissue sections (6 μ m) were labeled with mAbs specific for FDC (A, D, and E), plasma cells (B and F), CD4 (C and G), and CD20 (H). A double labeling with Abs specific for Ki-67 (brown color) and plasma cells (red color) is shown (B). A, B, and E–H, Consecutive sections from the ST of patient RA-PS: infiltrate VI is the right hand cluster seen in A and B, infiltrate I is shown in E–H. Infiltrate II from the ST of patient ReA-TS is shown in C and D.



tissue from patients EK, AR, and TS and Ampli Taq Gold (Perkin-Elmer, Norwalk, CT) for patient PS. The cycle program consisted of a denaturation step at 95°C for 2 min followed by 35 or 39 cycles, at 95°C for 1 min, 70°C (for heavy chain genes) or 65°C (for light chain genes) for 1 min 30 s, and 72°C for 2 min. The cycles were followed by a final 15 min incubation at 72°C. Aliquots of the final PCR products were separated by electrophoresis using a 2% low-melting agarose gel, and DNA bands in the range of 350 bp were purified from the agarose gel using Jet sorb DNA extraction (Genomed, Bad Oeynhausen, Germany).

The PCR error rate is negligible under these conditions as shown by the high frequency of unmutated sequences. For example, when CD20⁺ B cells were isolated from the network of FDC, 16 of 36 sequences carried no nucleotide substitution. The average frequency of exchanges introduced by the amplification is less than one exchange/V sequence. Furthermore, to demonstrate the stepwise accumulation of single somatic mutations, two independent PCR reactions were performed with cells isolated from the same synovial infiltrate (see Results and Fig. 2).

Sequence analysis

PCR products were cloned into the TA cloning vector (Invitrogen, San Diego, CA). Randomly picked clones were screened for inserts of ~350 bp and sequenced by the dideoxy chain termination method using Taq polymerase for DNA amplification. Standard sequencing primers were digoxigenin labeled. Sequencing reactions were analyzed as described previously (20). Briefly, DNA bands were blotted directly onto a nylon transfer membrane (Serva, Heidelberg, Germany) and labeled with anti-digoxigenin-Fab coupled to phosphatase (Boehringer Mannheim). Using the DNA plot program (Müller and Althaus, Köln, Germany), putative germline sequences were identified in the VBASE sequence directory (I. Tomlinson, Cambridge, U.K.) and in the IMGT data base (M.-P. Lefranc, Montpellier, France). In addition, searches were performed using GenBank information.

Results

Immunohistology of the ST

Some reports have suggested that lymphocytic infiltrates in the ST resemble tonsillar GCs and that they are compartmentalized in the same way (8, 9). However, in general one does not see a typical dark and light zone (20). The synovial GC is made up of macrophages, T, and B cells and in only about 30% of the cases are FDC found (9). FDC, when present are found in the center of the aggregates (Fig. 1, A, D, and E), and, in contrast to the typical tonsillar light zone, few T cells (Fig. 1, C and G) and no plasma cells are detected in the FDC network (Fig. 1F). Plasma cells are generally found as a halo surrounding the lymphocytic infiltrate (Fig. 1, B and F).

Diversification of the V gene repertoire in the ST of an RA patient

The ST of patient PS is extensively infiltrated by lymphocytes. Multiple large follicle-like structures have developed so that the synovial membrane seems to resemble lymphoid tissue (Fig. 1, A and B). Labeling of tissue sections with Abs specific for the nuclear Ag Ki-67 reveals dense clusters of dividing B cells within the central network of FDC (Fig. 1, A and B), suggesting that B cells are activated by Ag presented on the surface of these cells. To analyze B cell development in the ST of this RA patient, consecutive tissue sections were labeled with mAbs specific for FDC, B cells, and the nuclear Ag Ki-67. Two regions of ~25 Ki-67⁺ B cells were microdissected from the FDC network seen in the right hand infiltrate of Fig. 1A. DNA was extracted and the rearranged V(D)J regions amplified. In the two independent PCR reactions, a total of three different V_H and five different V_λ functional rearrangements were isolated, suggesting an oligoclonal population in this lymphoid infiltrate (Table II). A V_H (VH3–8 (according to Matsuda et al. (26) gene 3–11)/J_H4b) and a V_λ (DPL23/J_λ1) gene rearrangement was recovered several times from both groups of cells, demonstrating expansion of a single B cell clone (Table II). These sequences differ from the putative germline genes by 3–10 nucleotides. The pattern of somatic mutations demonstrates a stepwise accumulation of single nucleotide substitutions into the V genes introduced during B cell proliferation in the ST (Fig. 2).

CD20⁺ B cells and plasma cells are distinct populations

In general, lymphocytic cell clusters in the ST are surrounded by a ring of plasma cells (Fig. 1, B and F). This characteristic distribution suggests that these plasma cells develop from B cells activated in the synovial network of FDC, which then differentiate in the adjacent T cell-rich zone. To demonstrate the process of local plasma cell differentiation, B cell subpopulations were analyzed from three different regions of a single infiltrate: 1) the network of FDC (Fig. 1E), 2) the T cell-rich zone (Fig. 1G), and 3) the ring of plasma cells (Fig. 1F). To distinguish the different B cell subpopulations, tissue sections were double labeled with mAbs specific for FDC and for plasma cells. First, individual plasma cells were microdissected from the tissue section and combined only after isolation. In this way contamination by CD20⁺ B cells could be excluded. In addition, groups of ~50 B cells

Table II. *V* gene repertoire in $CD20^+$ B cells isolated from the network of FDC (patient PS infiltrate VI on section 59)

	Gene Family	Rearrangement	No. of Isolated Sequences: PCR		No. of Somatic Mutations	Length of CDR3	
			1st	2nd			
Heavy chain	VH1	1-2/JH4b		1	1	— ^a	
	VH3	3-11/JH4b	4	3	4 ^b	16	
		3-43/JH4b	5		6	12	
Light λ -chain	VH4	4-30-2/JH4b		1	2	12	
	V λ 1	DPL5/JL1		1	1	13	
	V λ 2	LV2046/JL2.3			2	1	10
		DPL23/JL1	5	3	1 ^b	9	
	V λ 3	DPL23/JL2.3			1	7	11
		IgLV3S6/JL2.3	2			9	8

^a Out of frame rearrangement.

^b Number of common mutations.

were taken from the labeled network of FDC and from the unlabeled area where B cells are in contact with T cells. DNA was extracted from the three isolated B cell subpopulations and the rearranged V_H and V_L genes were amplified by PCR, cloned, and sequenced.

The *V* gene repertoire expressed in $CD20^+$ B cells was comparable to that seen in peripheral blood B cells (Fig. 3, A and B) (27, 28). The majority of H chain sequences belonged to the V_H -3 gene family, and rearrangements of the V_H genes 3–23 (DP47) and 3–30 (COS8, DP46, and DP49) were found frequently (Fig. 3). In contrast, plasma cells seemed to express a more selected repertoire (Fig. 3C). Though the number of different functional V_H gene sequences isolated was rather small ($n = 7$), a shift toward rearrangements of the V_H -4 gene family was evident. Taking only the functional rearrangements into account, 4 of 7 plasma cell sequences (57%) and 3 of 23 $CD20^+$ B cell sequences (13%) be-

longed to the V_H -4 gene family. Thus, $CD20^+$ B cells and plasma cells seem to be two distinct cell populations.

To determine whether clonal relationships between $CD20^+$ B cells and plasma cells exist, we compared the pattern of Ig V(D)J rearrangements seen in the two populations. As shown in Fig. 3, some $CD20^+$ B cells and plasma cells used the same germline *V* segments for their *V* genes; the heavy chain genes 1–18 (DP14), 3–30 (COS8), and 4–59 (DP71) were present in both populations (Fig. 3), as were DPK9 (O2) and DPK24 (B3) (data not shown). However, in each case the heavy and light chain genes were rearranged with different (D)J segments or showed different junctional diversity, demonstrating that they originate from different clones. Thus, there was no evidence of identical V_H and V_L gene rearrangements in the two B cell subsets.

In addition, these B cell subpopulations could be distinguished by their degree of somatic diversity. *V* region sequences isolated

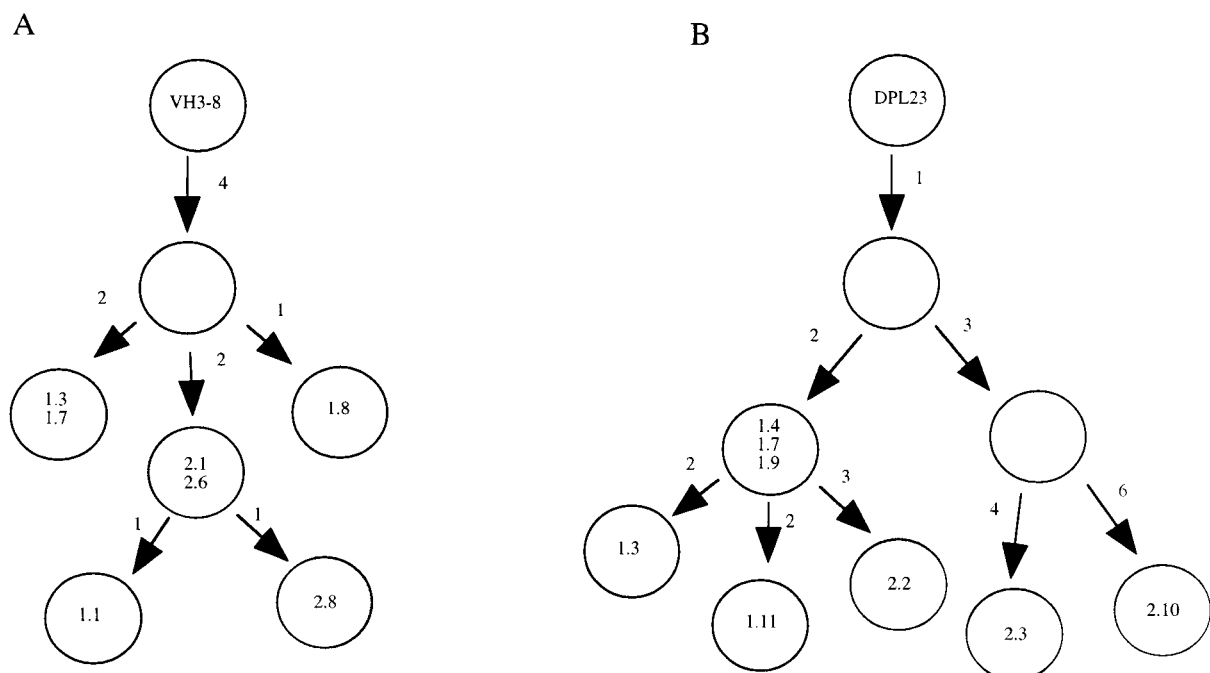


FIGURE 2. Stepwise accumulation of somatic mutations. *V* gene diversification is shown for a heavy chain (VH3–8) (A) and a light chain (DPL23) (B) rearrangement. Numbered circles indicate isolated sequences, empty circles indicate hypothetical intermediates. Numbers besides the arrows refer to the number of nucleotide exchanges that distinguish one sequence from another. Data were obtained with two independent PCR reactions (indicated by open and shaded circles).

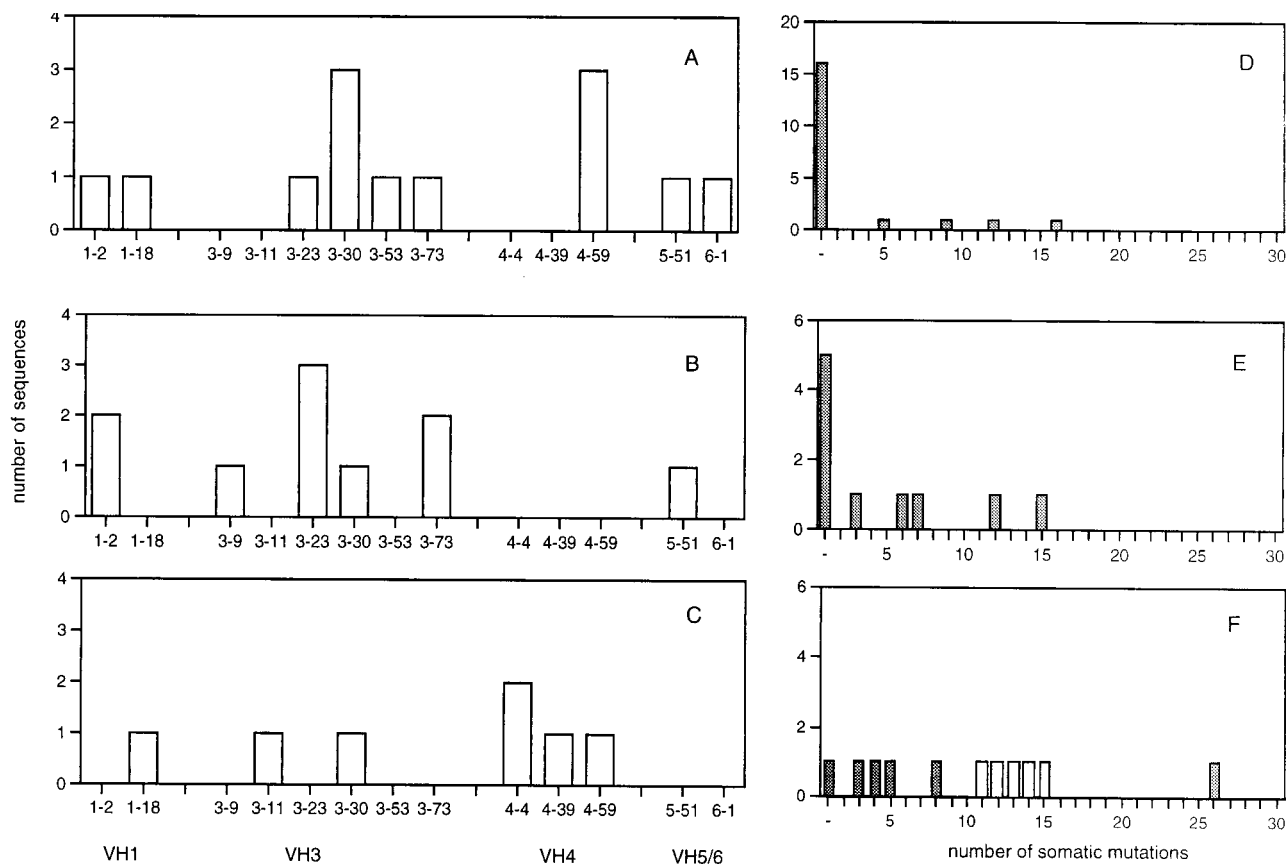


FIGURE 3. Diversity of V gene sequences isolated from infiltrate I of patient RA-PS. The diversity of B cells isolated from the network of FDC (A and D), the T cell-rich zone (B and E), and the ring of plasma cells (C and F) is shown. A–C, The repertoire of productively rearranged V_H genes is shown. D–F, The number of nucleotide differences to the presumptive germline genes is shown. The somatic diversity is given for heavy and light chain sequences. For the two rearrangements where intraclonal diversity was found, the number of nucleotide exchanges is shown by open bars. For V_H genes, the nomenclature of Matsuda et al. is given (26).

from the $CD20^+$ B cells in the network of FDC were almost all unmutated (80% of the sequences), indicating that these B cells were recent immigrants from the peripheral blood (Fig. 3D). In the few sequences isolated from the T cell area, only 50% of the sequences were unmutated (Fig. 3E). This frequency was further reduced to 12.5% in V_H and V_L rearrangements expressed in plasma cells (Fig. 3F). Sequences were ranked according to their degree of somatic diversity. Using a variance analysis (Kruskal-Wallis Test), it was found that B cells taken from the different regions of the synovial GC represent distinct stages of B cell differentiation ($p = 0.002$). Interestingly, plasma cells differ significantly in their frequency of somatic mutations from the $CD20^+$ B cells isolated from the network of FDC (Mann-Whitney test, $p = 0.001$) but not from the $CD20^+$ B cells taken from the T cell area ($p = 0.136$). These results support the interpretation that $CD20^+$ B cells are diversified within the network of FDC and then differentiate in the T cell area into plasma cells.

The analysis of a cellular infiltrate in the ST of a patient with ReA

The immunohistochemical analysis of the ST of patients with ReA has shown that lymphocytic infiltrates have a similar structure to those seen in the ST of RA patients. In addition, a GC reaction takes place in the inflamed tissue (18). To study B cell differentiation in the ST from an established ReA patient, a V gene analysis was performed by isolating $CD20^+$ B and plasma cells of a single lymphocytic cell cluster (infiltrate II from the ST of patient TS, Table I). Fig. 1C shows that the central part of this infiltrate

was practically free of T cells. Here, a dense network of FDC was seen (Fig. 1D). From this part of the infiltrate, $CD20^+$ B cells were isolated and their V gene repertoire was compared with that of the surrounding plasma cells.

The repertoire of V_H genes expressed in both the $CD20^+$ B cells and in the synovial plasma cell population is shown in Fig. 4. Approximately 50 $CD20^+$ B cells from each of two consecutive sections yielded 15 different V_H and 19 V_L rearrangements (13 V_κ and 6 V_λ genes) of which 13 V_H (Fig. 4A), 7 V_κ , and 4 V_λ sequences were productively rearranged. Thus, in this small area there are a minimum of 13 different B cell clones. Plasma cells also showed a high degree of diversity. Here, from the two consecutive sections a total of 14 different V_H (Fig. 4B), 11 different V_κ , and 23 different V_λ genes were isolated.

In cluster II isolated from the ST of the ReA patient TS, the distribution of isolated V_H genes does not differ significantly from that expected on the basis of V gene family size (Fig. 4). For both $CD20^+$ B cells and plasma cells, genes from the largest family, V_H -3, were found most often, followed by genes from the V_H -4 and then V_H -1 and V_H -2 family. However, as described for cluster I from the RA patient PS, a greater use of the V_H -4 family was seen in plasma cells. The only V_H -4 family member isolated from the $CD20^+$ B cells was the V_H gene 4–59 (DP71). In contrast, the V_H -4 genes 4–34 (DP63), 4-OR15–8 (DP69), and 4–39 (DP79) have been found in plasma cells. In addition, differences between the two B cell subsets were seen when the length of the complementarity determining region III (CDRIII) was compared (data not

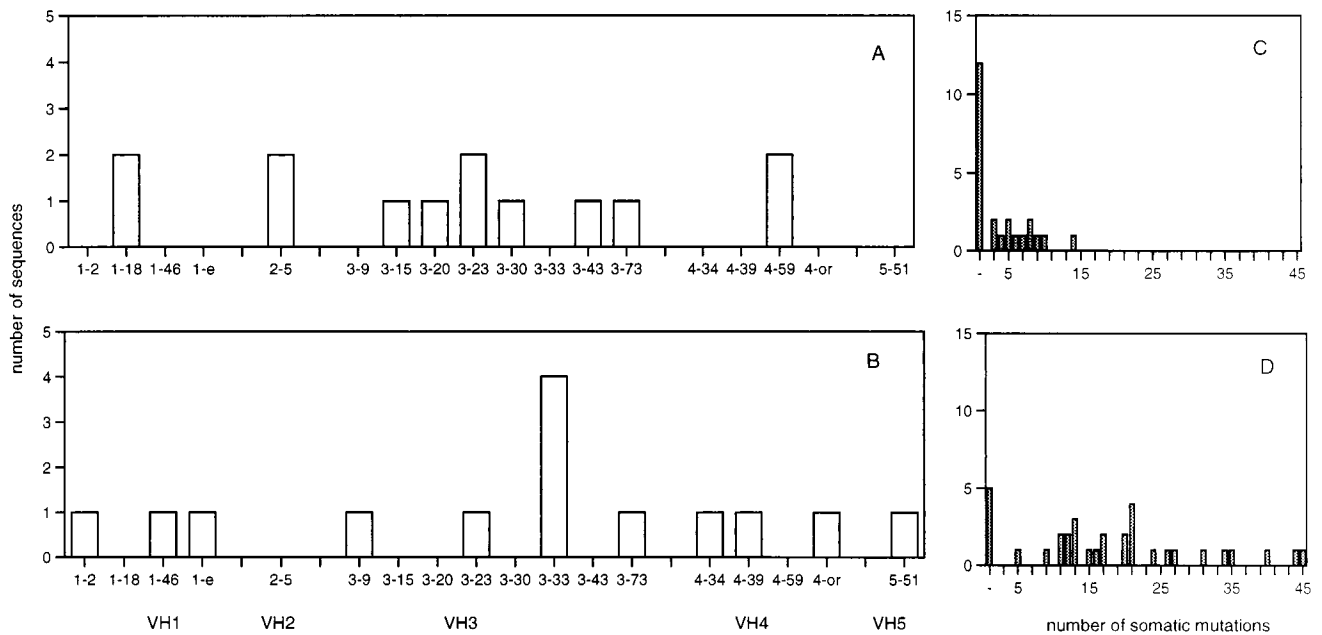


FIGURE 4. Diversity of V gene sequences isolated from infiltrate II of patient ReA-TS. The diversity of B cells isolated from the network of FDC (A and C) and the ring of plasma cells (B and D) is shown. A and B, The repertoire of productively rearranged V_H genes is shown. C and D, The number of nucleotide differences to the presumptive germline genes is shown. Results from two consecutive sections are summarized. 4-Or corresponds to the V_H-4 gene DP69, which is an orphan gene located on chromosome 15 (4-OR15-8; IMGT database, M.-P. Lefranc). For further details, see Fig. 3.

shown). Most of the CD20⁺ B cells expressed V_H/D/J rearrangements with a CDRIII of 10–12 amino acids, whereas plasma cells showed a tendency toward longer CDRIII regions (an average length of 13–15 amino acids). The J_H usage was the same for both populations; most frequently rearrangements to J_H-4 were seen.

Fig. 4, C and D shows the frequency of somatic mutations in CD20⁺ B cells and in plasma cells. A total of 50% of the V gene sequences isolated from CD20⁺ B cells in the network of FDC were unmutated. The other sequences carried low numbers of somatic mutations (Fig. 4C). In the V region sequences isolated from plasma cells, only 12% of the isolated V(D)J rearrangements were unmutated. Using the nonparametric Mann-Whitney test, sequences were ranked according to their degree of somatic diversity. The analysis showed that there is a highly significant difference when the frequency of somatic mutations in the CD20⁺ B cells and plasma cells are compared ($p \leq 0.001$).

The development of plasma cells within the microenvironment of the synovial GC

Altogether from six different cellular infiltrates derived from both patients with RA and ReA, the V gene repertoire of CD20⁺ B cells and plasma cells was compared (Table I). In no case were identical rearrangements seen in the two B cell populations. However, the examination of groups of plasma cells derived from various cell clusters suggests that in the ST CD20⁺ B cells differentiate into plasma cells. From the ST of both, patients with RA and the patient with ReA plasma cell sequences with identical V(D)J rearrangement but different patterns of somatic mutation were isolated. Fig. 4D shows that five sequences derived from plasma cells of ReA patient TS carried no more than two somatic mutations. One of these rearrangements, a joining of a V_κ DPK4 (A20) to J_κ4, showed intraclonal diversity (Fig. 5A). These V genes have one somatic mutation in common but differ by two to seven nucleotides from each other. A genealogical tree shows the accumulation of somatic mutations (Fig. 5A). This result is most readily explained as the consequence of a B cell becoming locally activated and

accumulating somatic mutations during proliferation in the ST. In Fig. 5B, sequences from a V_κ DPK21 (L2)/J_κ1 rearrangement are shown. These three sequences have 13 somatic mutations in common and differ by two to six nucleotides from each other. In this case, it seems that a memory B cell became reactivated in the ST and further diversified during proliferation. Further examples are shown in Fig. 5, C and D. The finding of intraclonal diversity in sequences derived from plasma cells of close physical proximity makes it unlikely that diversification of B cells took place outside of the ST and that descendants of the same B cell clone settled within the same lymphoid structure. Thus, both diversification of the V gene repertoire and differentiation into plasma cells seem to take place within the microenvironment of the ST.

Single-cell analysis of synovial plasma cells

Only the examination of V genes at a single-cell level allows us to show whether sequences with identical rearrangements found in the PCR were in fact amplified from the DNA of distinct cells. For this reason, single cells were isolated from a GC-like cluster in the ST of patient RA-EK. The rearranged V genes were amplified from individual cells and sequenced. Of the 9 single CD20⁺ B cells and 13 plasma cells examined, we isolated 9 V_κ genes (data not shown); 2 from CD20⁺ B cells and 7 from plasma cells. Among the seven sequences isolated from single plasma cells, two showed identical V_κJ_κ rearrangements as well as nearly identical patterns of somatic mutation (Fig. 5E). Both rearrangements used the DPK1 (O18) V gene and the J_κ4 gene and contained the same joining pattern. The sequences have 12 somatic mutations in common. In one of the V_κ regions, two additional somatic mutations were seen. These two mutations are not introduced during PCR amplification, as they were seen in all clones sequenced. These results demonstrate that within the ST a plasma precursor cell not only divided, but in addition accumulated somatic mutations.

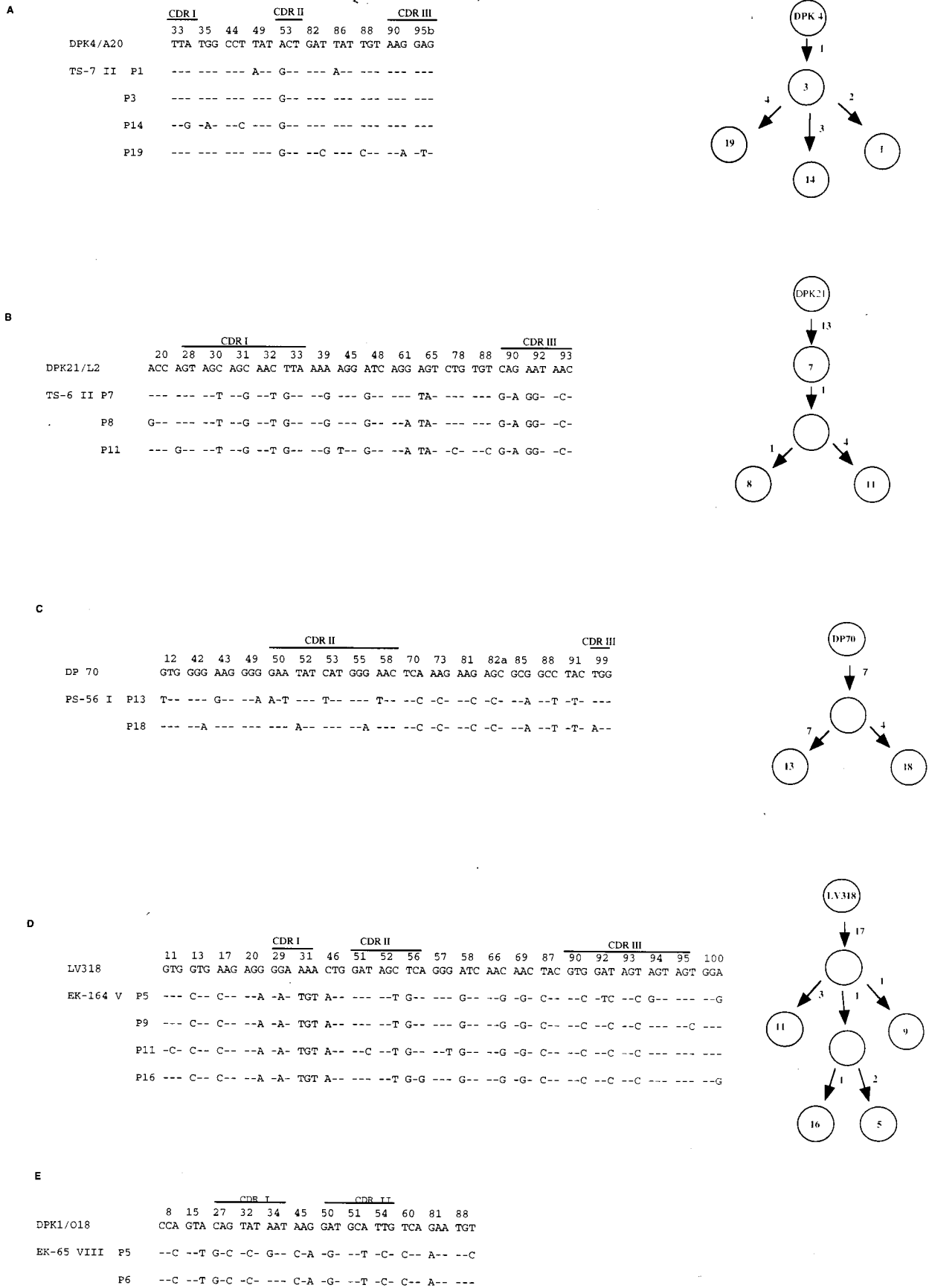


FIGURE 5. Intracлонаl diversity of plasma cell V genes. Heavy and light chain sequences are compared with the most homologous germline gene. Only nucleotide differences are shown, CDR are indicated, and codons are numbered according to Kabat (41). Sequences were isolated from plasma cells of two patients with RA (infiltrate I from patient RA-PS and infiltrate V from patient RA-EK) and one patient with ReA (infiltrate II from patient ReA-TS). Genealogical relationship indicating the clonal V gene diversification is shown. For further details, see Fig. 2. E, The results of a single-cell analysis is shown.

Discussion

Recent results demonstrate that in patients with autoimmune diseases such as RA or Sjögren's syndrome the GC reaction may take place in the affected tissue (19, 21, 29). Here, we show that in such ectopic GC CD20⁺ B cells proliferate in the network of FDC and that in this microenvironment somatic mutations accumulate in the expressed V genes.

The analysis provided no direct evidence for a clonal relationship between differentiating CD20⁺ B cells in the GC-like structures and the surrounding plasma cells. However, sequences with an identical rearrangement but a different pattern of somatic mutations were isolated from neighboring plasma cells of single lymphocytic infiltrates (Fig. 5). Thus, we are left with the puzzling observation that within these infiltrates we find sets of clonally related CD20⁺ B cells and sets of related plasma cells, but there is no detectable lineage relationship between the CD20⁺ B cell and plasma cell populations.

Clearly, the V gene repertoire of CD20⁺ B cells is diversified in the ST, but do they then differentiate into plasma cells in these ectopic GC structures? Both immunohistology and the molecular analysis suggest that indeed they do. The increasing somatic diversity seen in V genes expressed in B cells from the network of FDC, from the T cell-rich zone, and from the ring of plasma cells suggests a stepwise differentiation of B cells in the ST (Fig. 3, D–F). We would like to speculate that, in analogy to the GC in the peripheral lymphoid organs, a range of somatic variants are generated from which only a few are selected to differentiate into plasma cells.

In tonsillar GC, B cell proliferation is concentrated in the dark zone and only a few dividing centroblasts are observed in the network of the FDC in the light zone (12). The situation is different in the synovial GC, where B cell proliferation is mainly seen within the network of FDC (Fig. 1B). The analysis of GC in mouse spleen has demonstrated that GC have a dynamic architecture that changes over the course of an immune response, and B cell proliferation in the network of FDC is observed only in the early stages (30). In view of this, the GC isolated from the ST of the RA patient PS probably represent newly induced GC. This interpretation is supported by the repertoire analysis. B cells taken from the network of FDC expressed a random repertoire characteristic of the peripheral blood B cells, and, as one might expect for naive cells, the majority of V genes were unmutated (Fig. 3D).

If an Ag-dependent diversification of the repertoire and selective differentiation of B cells take place in the ST, why then is it so difficult to find sequences with identical rearrangements in the B cells of different developmental stages? Two factors probably explain this point. The first is the high degree of B cell diversity in the synovial GC. When a single section from the infiltrate I of RA-PS was analyzed, 13 different functional V_H rearrangements were found in the CD20⁺ B cell population (Fig. 3A). It is likely that only a few of these cells are activated to take part in a GC reaction. For example, the V gene analysis of proliferating B cells isolated from the network of FDC (Fig. 1, A and B and Table II) showed expansion of a single B cell clone (Table II). In addition, only few of the activated B cells will be selected to differentiate into plasma cells. Second, the B cell population that influxes to the ST may turn over rapidly. In contrast, plasma cells may well be long-lived in the ST and accumulate here (31). This means that the plasma cells and the CD20⁺ B cells within a synovial GC are not only spatially but also temporally distinct populations. Taken together, these two points explain the fact that we fail to recover CD20⁺ B cells and plasma cells of the same clonal origin.

In the murine system, it has been shown that in the T cell-dependent immune response early plasma cell development takes place extrafollicularly. These plasma cells secrete mainly low-affinity Abs of the IgM class, and V genes expressed in these plasma cells are unmutated (32). In contrast, high-affinity plasma cells expressing mutated V gene sequences develop in GC. Interestingly, the majority of plasma cells isolated from the ST V genes carried multiple somatic mutations. To which extent these plasma cells were generated in synovial GC-like structures cannot be answered. However, it is most likely that those rearrangements, which showed intraclonal diversity, developed within the same infiltrate.

Plasma blasts developing in GC of the human tonsil or murine splenic tissue are believed to leave this microenvironment and to accumulate in the bone marrow (33–36). The ST may, like bone marrow, provide an environment that supports longevity of plasma cells (31, 37). Thus, in contrast to the lymphoid tissue, plasma cells may stay in the ST and accumulate in the vicinity of the GC microenvironment. This may explain the high numbers of plasma cells often seen in the ST of patients with a long-term chronic synovitis.

While it is certainly possible that synovial plasma blasts are still dividing, there is no evidence so far available that somatic mutations accumulate once plasma blasts have left the microenvironment of the GC (38). However, even in the absence of hypermutation affinity maturation through selection may continue. Recent data suggest that the bone marrow supports a long-term protective high-affinity humoral immune response (38). If the same is true for the ST the consequences for autoimmune diseases may be considerable.

The analysis of plasma cells in the cluster I from the ST of the RA patient PS indicated a prevalence of rearrangements of the V_H-4 gene family (Fig. 3). When we pool the data from six different infiltrates together (62 different rearrangements isolated from CD20⁺ B cells and 41 different rearrangements isolated from plasma cells), the shift toward V_H-4 in the plasma cell population is confirmed (Fig. 6) (χ^2 test, $p = 0.076$). These results may point to selection by Ags, self or foreign, that preferentially interact with V_H-4 gene products or alternatively to the activation of B cells through superantigens, which have been shown to play a role in shaping the B cell repertoire. For example, staphylococcal enterotoxin D specifically activates B cells expressing rearrangements of the V_H-4 gene family (39). Ags other than superantigens may be involved in the selective differentiation of synovial plasma cells because we see both a shift toward V_H-4 and in addition a selection for longer CDRIII. Interestingly, the tendency toward rearrangements of the V_H-4 gene family is seen in plasma cells from patients with different rheumatic diseases. However, the sample size is rather limited as only two patients with defined RA and one with ReA were examined.

The comparison of RA and ReA is complicated by the fact that the pathogenesis of these two disorders is not well defined. Thus, it is unclear what Ag(s) drive the immune response in the ST. The isolation of clonally related rheumatoid factor-secreting cell lines from the ST of a patient with RA suggested that autoreactive cells undergo affinity maturation within the ST (18). The selected repertoire expressed in the isolated plasma cells would support this interpretation. On the other hand, B cells may be activated in the ST by infectious agents or by self-Ags that mimic them. However, so far only ReA has been shown to be triggered by a bacterial infection (22, 40).

We do not know why B cells migrate into the ST. Multiple factors, like the cytokine milieu, chemokine pattern, or the presence of activated CD4 Th cells, may support B cell immigration. Proinflammatory cytokines like TNF- α and lymphotoxin α may

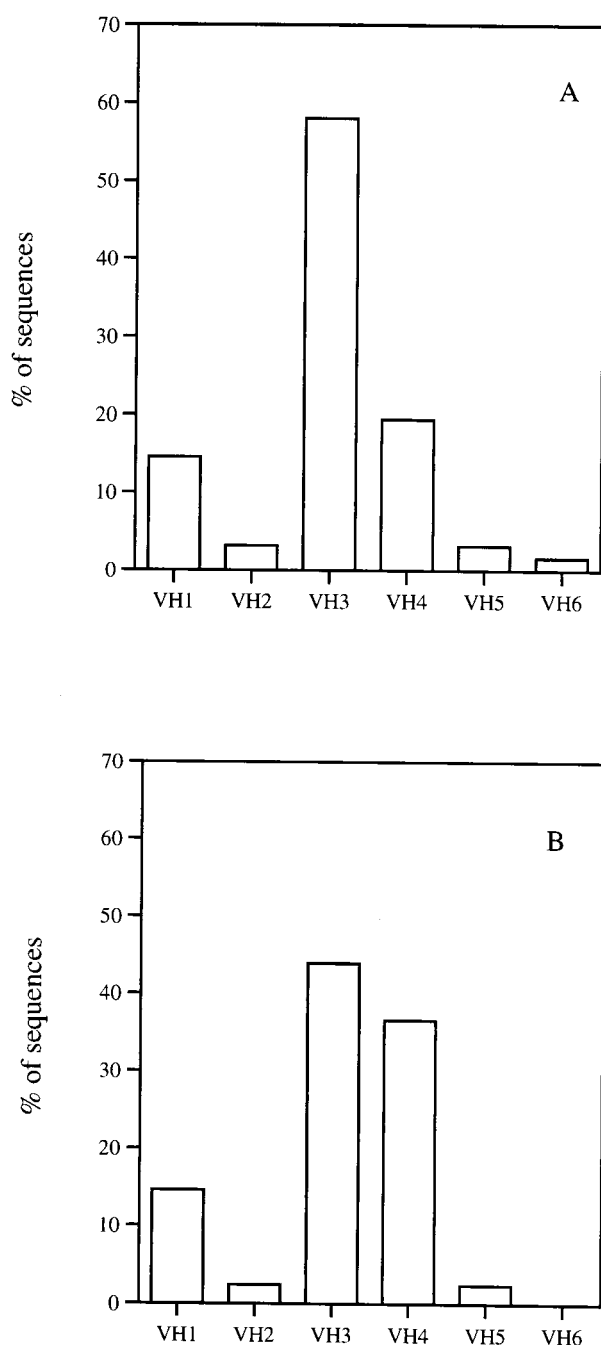


FIGURE 6. A comparison of the V_H gene repertoire expressed in $CD20^+$ B (A) and plasma cells (B). Results from four different patients with RA and ReA are summarized. A total of 62 different rearrangements from $CD20^+$ B cells and a total of 41 from plasma cells were isolated.

support the development of a network of FDC, and this may be the first step in the development of GC-like structures (3). Thus, in the ST a microenvironment may build up that allows a GC reaction to take place. Microdissection makes it possible to identify those B cells in the ST that go through an Ag-dependent process of affinity maturation. Thus, we have a tool to analyze the V genes expressed in B cells that are selected in the ST to differentiate into plasma cells. Through the expression of heavy and light chain V genes it should be possible to determine the specificity of those cells which take part in the local immune response.

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