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# Evidence for a selected humoral immune response encoded by $V_H4$ family genes in the synovial membrane of a patient with rheumatoid arthritis (RA)

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**SUMMARY**

The analysis of rearranged antibody-encoding genes from B cell foci in rheumatoid synovial tissue has characterized these cells as highly mutated memory B cells with a high proportion of members of the $V_H4$ family. In order to characterize further the $V_H4$ response in one patient, B cell-rich areas from different sections of synovial membrane (SM) were identified by CD20 staining, isolated by microdissection and pooled, in order to analyse highly enriched B cells without selection by *in vitro* culture procedures. From DNA of about $5 \times 10^3$ B cells rearranged $V_H$ genes were amplified by polymerase chain reaction (PCR) and cloned. Sequencing of 11 clones containing rearranged $V_H4$ gene products revealed that seven were potentially functional, and all were mutated with 84–96% homology to known germ-line (gl) genes and $V_H4$ gl genes amplified from the patient's genomic DNA. Analysis of the complementarity determining region (CDR) 3 revealed that two products represented members of one B cell clone which differed by five nucleotide changes. Three of the five mutations encoded amino acid replacements in CDRs indicating antigen-driven expansion of one specific clone. Additional analyses of 25 members of three B cell clones from isolated aggregates showing intraclonal diversity in one of three clones provided further evidence that antigen selection takes place in the SM. Overall, the pattern of mutations and the replacement to silent (R:S) ratios were diverse, with six products indicating antigen selection by their high R:S ratios in CDRs. Although DNA analysis does not allow a characterization of antibody specificities, we can conclude from our analysis of antibody-encoding genes that selection by antigen and expansion of specific clones occur in the SM against the background of polyclonal activation.

**Keywords** rheumatoid arthritis $V_H$ repertoire somatic mutation synovial inflammation B lymphocytes

## INTRODUCTION

RA is a chronic systemic disease mainly involving the joints. The local inflammation of the joints eventually results in their destruction [1,2]. This process is maintained by a pathological immune response triggered by an agent, which is still unknown. Recent investigations concerning the humoral immune response by analysis of immunoglobulin-encoding genes indicate an antigen-driven process in the synovial membrane (SM) [3–8]. A high frequency of somatic mutations and a diverse spectrum of V genes used by RA-derived rheumatoid factors (RF) distinguishes them from other RF induced by hyperimmunization [9] or RF from paraproteins [10].

In longstanding RA, the SM is chronically inflamed. Histologically, there appear infiltrates of mononuclear cells (MNC) containing B and T lymphocytes, and follicular dendritic cells in a formation similar to secondary lymphatic organs [11]. The analysis of rearranged $V_H$ and $V\kappa$ genes from B cell-rich mononuclear infiltrates characterized these cells as highly mutated memory B cells [12,13].

Antibodies are encoded by about 60 $V_H$ gene segments rearranged to a $D_H$ and a $J_H$ segment. The $V_H$ germ-line (gl) repertoire consists of 95 $V_H$ genes on chromosome 14, of which about one third are non-functional [14]. By sequence homology of at least 80% the $V_H$ gene segments are classified into seven families. The $V_H1$ family has 17 (12) members, $V_H2$ four (3), $V_H3$ 51 (28), $V_H4$ 13 (11), $V_H5$ three, $V_H6$ one and $V_H7$ three (2) members, respectively (numbers in parentheses indicate the number of genes with open reading frames). An over-representation of one $V_H4$ gene, the $V_H4.21$, in the autoimmune repertoire has been described [15]. A high representation of members of the $V_H4$ family was also found within hybridomas derived from synovial

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tissue of one patient with RA [16]. Recently, it was postulated that a negative selection of $V_H4$ takes place in the peripheral blood lymphocytes (PBL) of healthy individuals as a means to avoid autoimmunity [17].

As our preliminary investigations of $V_H$ genes in B cell infiltrates in SM indicated a possible over-representation of $V_H4$ genes, we extended the $V_H4$ analysis to a larger B cell sample of one patient's SM and compared it with her own $V_H4$ gl repertoire as well as with other known gl genes.

## MATERIALS AND METHODS

*Material*

The SM of the right knee was obtained from a 78-year-old woman with RA of 15 years duration undergoing total joint replacement. As therapy, the patient was recieving 100 mg azathioprine per day.

*Tissue preparation*

The SM was snap frozen in liquid nitrogen immediately after surgery and stored at –70°C. Frozen sections of 10 $\mu$m thickness were screened for mononuclear infiltrates by haemalaun staining (Mayer's Haemalaun; Merck, Darmstadt, Germany). When infiltrates were identified, three 10 $\mu$m thick sections were prepared for B cell detection. Then, 30 consecutive sections of 30 $\mu$m thickness were prepared for later B cell isolation. The same procedure was repeated to the end of the tissue block. Sections for B cell detection were stained with anti-CD20 MoAb (L26; Dako, Hamburg, Germany) and developed by the alkaline phosphatase anti-alkaline phosphatase (APAAP) method. Fifty sections of 30 $\mu$m thickness adjacent to CD20-stained sections that contained B cell-rich mononuclear infiltrates were haemalaun-stained and mononuclear infiltrates were isolated with a micromanipulator (Eppendorf, Hamburg, Germany) and collected in 1 ml water in a microtube. Fibrous tissue of the same sections was isolated for amplification of gl genes.

*DNA preparation*

After the addition of 10 $\mu$l Tris (1 M, pH 8·0), 200 $\mu$l EDTA (0·5 M, pH 8·0), 25 $\mu$l SDS 20% and 3 $\mu$l RNAse (10 mg/ml), the tissue was incubated for 1 h at 37°C. Subsequently, 17·5 $\mu$l of Proteinase K (100 $\mu$g/ml; Merck) were added and the sample was incubated overnight at 55°C. After total digestion of proteins, DNA was prepared by repeated phenol and chloroform-isoamyl-alcohol extractions. After precipitation, the DNA was dissolved in 100 $\mu$l TE (Tris–EDTA, pH 7·6).

*Polymerase chain reaction*

Three hundred and fifty nanograms of DNA each were subjected to six different polymerase chain reactions (PCR) of 35 cycles with individual $V_H$-specific and a mix of all $J_H$-specific oligonucleotides. Germ-line genes were amplified with the $V_H4$-specific oligonucleotide and an oligonucleotide ($V_H4$-IS) complementary to the 3′ heptamer nonamer region of known $V_H4$ gl genes. The following oligonucleotides were used:

$J_H1$ 5′CGTCGTCGACCAGGGTGCCCTGGCCCCAGTGC (101–109)
$J_H2$ 5′CGTCGTCGACCAGGGTGCCCTGGCCCCAGTGC (101–109)
$J_H3$ 5′CGTCGTCGACATTGTCCCTTGGCCCCAGACATCA (100–108)
$J_H4$ 5′CGTCGTCAACCACGGTTCCTTGGCCCCAGTAG (101–109)
$J_H5$ 5′CGTCGTCGACGTGACCAGGGTTCCTTGGCCCCAGG(102–110)
$J_H6$ 5′CGTCGTCGACGTGGTCCCTT GCCCCCAGACGTCC (100–108)
$V_H1$ 5′CTACGTCGACCCTCAGTGAAGGTYTCCTGCAAGGC (16–24)
$V_H2$ 5′GCACGTCGACGTCCTGCGCTGGTGAAASCCACACA (16–24)
$V_H3$ 5′GTACGTCGACGGGGTCCCTGAGCTCTCCTGTGCAG (15–24)
$V_H4$ 5′ CGTCGTCGACCCTGTCCCTCACCTGCRCTGTC (16–24)
$V_H5$ 5′CGACGTCGACAAAAAGCCCGGGGAGTCTCTGARGA (12–20)
$V_H6$ 5′CGTCGTCGACCTGTGCCATCTCCGGGGACAGTG (21–29)
$V_H4$-IS 5′GTCTGGGCTCACACTCACCTCCCCT

All oligonucleotides with the exception of the $V_H4$-IS contain a 5′ SalI-restriction site (G/TCGAC). The corresponding amino acid positions are given in parentheses. The $V_H1$ oligonucleotide also matches perfectly to $V_H7$.

Oligonucleotides were used at a final concentration of 0·125 $\mu$M each and nucleotides (Pharmacia, Freiburg, Germany) at 200 $\mu$M in the Taq polymerase manufacturer's reaction buffer (Eurogentec, Seraing, Belgium) with 1·5 mmol $MgCl_2$. GoldStar Taq polymerase (1 U) was added after the first denaturation step. The following conditions were used for the PCR: first cycle, 5 min denaturation at 95°C, 3 min annealing—for $V_H1$, $V_H2$, $V_H5$, $V_H6$ at 64°C, for $V_H3$ and $V_H4$ at 68°C—and 90 s extension at 72°C. Cycles 2–35: 80 s denaturation, 30 s annealing, 90 s extension, with a final extension of 5 min.

*Electrophoresis and Southern blot analysis*

PCR products were analysed first by electrophoresis in a 1·5% agarose gel and ethidium bromide staining. Then, products were separated in a 30 cm long 6·6% polyacrylamide gel and analysed by Southern blotting: DNA was transferred electrophoretically from the gel to a nylon membrane (Hybond-N +; Amersham, Aylesbury, UK) in a transblot cell (BioRad, München, Germany). The membrane was hybridized with a $^{32}$P-ATP end-labelled FR3-specific oligonucleotide and autoradiography was performed on Kodak-X OMAT (Eastman-Kodak, Rochester, NY).

*Cloning and sequencing*

$V_H4$ PCR product (1 $\mu$l) was cloned with a TA cloning kit (Invitrogen, Leek, The Netherlands) according to the manufacturer's instructions. Cloning, plasmid isolation and sequencing were done as previously described [8]. Sequences were analysed by homology search with DNASIS and the EMBL database on CD-ROM (Hitachi Europe, Olivet, France).

*Analysis of isolated B cell foci*

Individual B cell aggregates (foci) of about 50–300 cells were isolated by microdissection as previously described [12]. Half of the DNA was amplified by 35 cycles of PCR with a mixture of all $V_H$-specific and $J_H$-specific oligonucleotides. One microlitre of this reaction was amplified in different PCR reactions combining each $V_H$ and each $J_H$ oligonucleotide (36 reactions). Individual bands of the expected length were isolated and cloned as described above. Several bacterial clones were sequenced from each aggregate.

## RESULTS

*$V_H$ gene amplification and family distribution*

B lymphocytes in the SM were identified by CD20 staining [12]. About 50% of mononuclear infiltrates in frozen sections of this SM consisted of B lymphocytes (data not shown). In order to enrich B lymphocytes without selection by tissue culture, MNC were

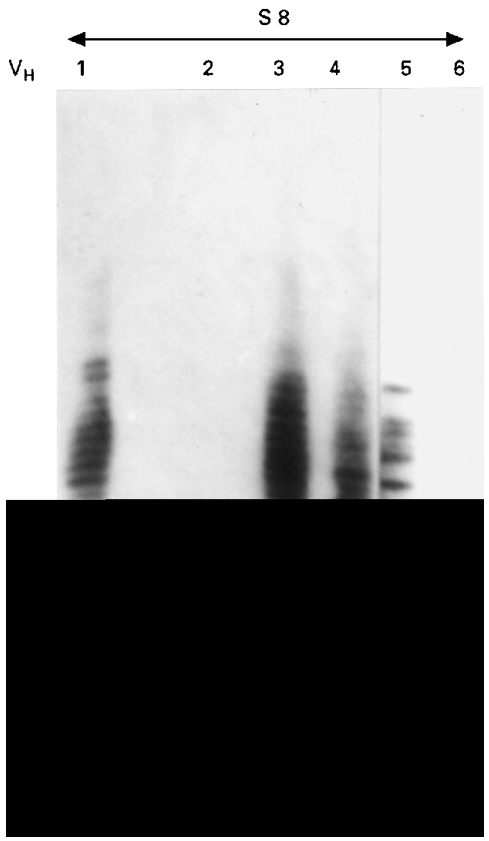


**Fig. 1.** Southern blot of $V_H$ polymerase chain reaction (PCR) products from synovial membrane mononuclear cell DNA, separated on a 6·6% PAA gel, hybridized with a FR3 oligonucleotide probe. Film exposure $V_H5$, $V_H6$, 12 h; all others, 5 h.

collected from adjacent sections by microdissection. DNA was isolated from an estimated number of $10^5$ B cells. Of the total DNA, 350 ng (6·5 $\mu$g) were subjected to 35 cycles of PCR with $V_H$ family-specific oligonucleotides and a mixture of $J_H$-specific oligonucleotides in six different reactions. Products of the expected length of $\approx$ 350 bp were identified for the $V_H1$-, $V_H3$-, $V_H4$-family by electrophoresis in an ethidium bromide-stained 1·5% agarose gel. On a 6·6% acrylamide gel a pattern of 10–15 bands each separated by few bp length difference appeared in each family after transfer to nylon membrane and hybridization with a $^{32}P$ end-labelled FR3-specific oligonucleotide (Fig. 1). $V_H2$, $V_H5$ and $V_H6$ products were not detected on the agarose gel, but on the FR3-hybridized Southern blot a restricted pattern of bands (5) for $V_H5$ and a single band for $V_H6$ were detectable.

Of the 50 $\mu$l volume of PCR product for $V_H4$ rearrangements, 1 $\mu$l was cloned by TA ligation and transformation of *Escherichia coli*. DNA was extracted from 44 bacterial clones and analysed for the appropriate inserts: 11 clones contained products of 350 bp and were sequenced.

*$V_H4$ gl gene analysis*

From the 11 known $V_H4$ gl genes with open reading frames (ORF) on chromosome 14 [14], nine were identified in 15 different gl clones (total number 21) from the patient's genomic DNA (Table 1). Eight of the 15 clones were identical to known gl genes, the remaining seven had a homology of 98·7–99·6% to known gl genes. Six of the latter represent polymorphic alleles, because they were also found as identical copies of known gl genes.

*Analysis of $V_H4$ rearrangements*

Ten of the 14 $V_H4$ rearrangements (11 from collected DNA, three from foci) were potentially functional, while four were non-productive (Table 2). Twelve of the 14 $V_H4$ products were > 90%, one 88% and one only 85% homologous to previously described gl genes (Table 2).

Of the 14 rearranged $V_H4$ genes, four were most homologous to $V_H4.34$ ($V_H4$-GL2/8), three to $V_H4.22$/DP67 ($V_H4$-GL9), two each to $V_H4.33$/DP65 ($V_H4$-GL6) and $V_H4.11$/DP71 ($V_H4$-GL7) and one each to V71-2/DP66 ($V_H4$-GL1), V12G-1/DP68 ($V_H4$-GL5), and $V_H4.21$/DP63 ($V_H4$-GL4) (Tables 1 and 2).

*Analysis of mutations*

The rearranged $V_H4$ genes had a minimal number of four mutations (S8VH47) and a maximal number of 28 mutations (8F2B01). Within the CDR1 and 2, mutations ranged from zero (S8VH47) to eight (S8VH410), within the FR1–3 they ranged from four (S8VH45 + 47) to 12 (S8VH49) (Table 2, Fig. 2).

Ratios of replacement (R) to silent (S) mutations varied in their level as well as in their location in CDR and FR (Table 2, Fig. 2). For S8VH410 the R:S ratio was 7 in the CDR and 0·8 in the FR. Comparable R:S ratios were found in 8F1B02 and 8F1C01 with R:S ratios of infinity for the CDR, and 1·5, respectively, 3·5 for the FR. A similar pattern was obvious in S8VH48 and S8VH411. For S8VH412 the R:S ratio was 6 in the CDR as well as in the FR. Three products showed a strikingly lower R:S ratio in the CDR than in the FR: S8VH47 had no mutation at all within the CDR and a R:S ratio of 3 within the FR. S8VH45 and S8VH46 had similar R:S ratios of 2 and 1·3 in the CDRs, and 3 and 5 in the FRs, respectively. S8VH414 and 8F2B01 harboured a high number of nucleotide exchanges (15/28) with relatively low R:S ratios in both the CDR (0·5/3·5) and FR (0·8/1·3).

*The CDR3*

The CDR3 was determined according to Kabat from amino acid position 95–102 [18]. CDR3 lengths of $V_H4$ rearrangements ranged from 30 bp to 54 bp with a mean of 37·2 (Table 2). All products contained at least one D segment homologous to a known gl D segment [19] and N additions between V and D as well as between D and J (Table 3). In S8VH46 two D segments were aligned, interspersed by N-nucleotides; this product was potentially functional. Three products (S8VH44, -48 and -411) had rearranged the DLR2. The gl segment D21-9 as well as DIR1 appeared twice (DIR1 in one case inverted). DN1, DA1, DA5, DK4, DXP′1, DLR3, DLR4, and D21-10 were used once each.

Five of 14 products were rearranged to the $J_H4$ segment, three to $J_H5$, two each to $J_H1$ and $J_H6$, and one each to $J_H2$ and $J_H3$ (Table 3).

*$V_H4$ genes from B cell foci*

Three $V_H4$ rearrangements were derived from isolated B cell aggregates of the same SM by independent PCR reactions (Table 2, 8F1B02, 8F1C01, 8F2B01). Twenty-five bacterial clones were sequenced, in order to look for intraclonal diversity. Fourteen independent isolates from 8F1C revealed 21 identical nucleotide

**Table 1.** List of $V_H4$ germ-line (gl) genes

| S8 | Tomlinson/Cook | Sanz[a],Matsuda[b] Lee[c], Weng[d] | v.d. Maarel | Homologous S8 |
|---|---|---|---|---|
| $V_H4$–GL20 | 100% DP63/3–34 | 100% $V_H4.21$[a] | 100% 4d76 | |
| $V_H4$–GL4 | 99·5% DP63/4–34 | 99·5% $V_H4.21$[a] | 99·6% 4d76 | |
| $V_H4$–GL12 | 100% DP64/4–30.2 | 94·9% V4–31X[b] | 100% 3d216d | 100% $V_H4$–GL13 |
| $V_H4$–GL17 | 98·6% DP64/4–30.2 | 95·7% V4–31X[b] | 98·7% 3d216d | |
| $V_H4$–GL6 | 100% DP65/4–31 | 100% $V_H4.33$[d] | 100% 3d75d | |
| $V_H4$–GL8 | 97·7% DP65/4–31 | 99·6% $V_H4.34$[d] | 99·6% 3d230d | 99·6% $V_H4$–GL2, 100% $V_H4$–GL14 |
| $V_H4$–GL2 | 97·3% DP65/4–31 | 99·6% $V_H4.34$[d] | 99·6% 3d230d | |
| $V_H4$–GL1 | 100% DP66/4–61 | 100% V71–2[c] | | 100% $V_H4$–GL11 |
| $V_H4$–GL10 | 99·1% DP66/4–61 | 99·1% V71–2[c] | | |
| $V_H4$–GL19 | 93·7% DP66/4–61 | 100% $V_H4.18$[a] | 100% 4d154 | |
| $V_H4$–GL9 | 99·1% DP67 | 99·1% $V_H4.22$[a] | | |
| $V_H4$–GL5 | 100% DP68/4–28 | 100% V12G–1[c] | 100% 3d28d | 100% $V_H4$–GL16, 100% $V_H4$–GL18, 100% $V_H4$–GL21 |
| $V_H4$–GL3 | 98·2% DP69 | 98·7% V79[c] | 99·1% 4d68 | |
| $V_H4$–GL15 | 100% DP70/4–4 | | 100% 4d68 | |
| $V_H4$–GL7 | 100% DP71/4–59 | 100% $V_H4.11$[a] | 100% 3d197d | |

Homologies between gl genes derived from our RA patient (S8) to other published $V_H4$ segments. The order of rank is following the numeric order of DP segments published by Tomlinson *et al*. [39], second column. The last column shows identical isolates from the same experiment. Most homologous segments to a respective S8VH4-gl gene are underlined. References to the other gl genes are Cook [14], Sanz [24], Matsuda [40], Weng [23], Lee [25], Van der Maarel [27].

**Table 2.** List of rearranged $V_H4$ genes analysed, their most homologous germ-line (gl) gene and percentage of nucleotide homology to their respective gene, absolute number of mutations and R : S ratio in CDR and FR, nucleotide length of the CDR3, $D_H$ and $J_H$ gl segments used, functionality of the rearrangement

| Rearranged gene | Germ-line gene | Homology (%) | Mutations CDR | FR | R : S CDR | FR | CDR3 length (bp) | $D_H$ | $J_H$ |
|---|---|---|---|---|---|---|---|---|---|
| S8VH44 | GL8 | 91·0 | 7 | 5 | 5 : 2 | 3 : 2 | 44 | DLR2 | 4 nf |
| S8VH45 | GL7 | 94·1 | 3 | 4 | 2 : 1 | 3 : 1 | 47 | DA5 | 4 nf |
| S8VH46 | GL1 | 92·5 | 7 | 6 | 4 : 3 | 5 : 1 | 42 | D21–10, DIR1 | 2 pf |
| S8VH47 | GL5 | 96·5 | 0 | 4 | 0 : 0 | 3 : 1 | 33 | DN1 | 4 pf |
| S8VH48 | GL2 | 94·7 | 3 | 8 | 3 : 0 | 5 : 3 | 33 | DLR2 | 5 pf |
| S8VH49 | GL6 | 93 | 2 | 12 | 1 : 1 | 8 : 4 | 30 | DK4 | 4 pf |
| S8VH410 | $V_H4.22$ | 90.1 | 8 | 11 | 7 : 1 | 5 : 6 | 35 | DIR1 | 4 nf |
| S8VH411 | GL2 | 93·8 | 4 | 8 | 4 : 0 | 5 : 3 | 33 | DLR2 | 5 pf |
| S8VH412 | GL6 | 93·4 | 6 | 7 | 6 : 0 | 6 : 1 | 42 | DXP 1 | 5 pf |
| S8VH414 | GL9 | 90·2 | 6 | 9 | 2 : 4 | 4 : 5 | 30 | D21–9 | 1 pf |
| S8VH413 | GL4 | 93·8 | 5 | 9 | 4 : 1 | 5 : 4 | 41 | DA1 | 1 nf |
| 8F1B02 | GL2 | 92·6 | 8 | 5 | 8 : 0 | 3 : 2 | 45 | D21–9 | 6 pf |
| 8F1C01 | GL7 | 92·3 | 7 | 9 | 7 : 0 | 7 : 2 | 54 | DLR3 | 3 pf |
| 8F2B01 | GL9 | 85·3 | 9 | 19 | 7 : 2 | 11 : 8 | 42 | DLR4 | 6 pf |

S8VH44–414: Sequences from individual bacterial colonies derived from $5 \times 10^3$ B cells amplified by 35 cycles of polymerase chain reaction (PCR); 8F1B02, 8F1C01, 8F2B01: individual sequences from three different B cell clones derived from individual aggregates (8F1B=3, 8F1C=14, and 8F2B=8 bacterial colonies, respectively). pf, Potentially functional; nf, non-functional rearrangement. Homology is compared within the $V_H$ segments from amino acid position 25 (FR1) up to 94 (end of FR3), length of CDR3 is measured between amino acid position 95 and 102 according to Kabat *et al*. [18]. $J_H$ segment numbers are according to reference segments published by Ravetch *et al*. [38].

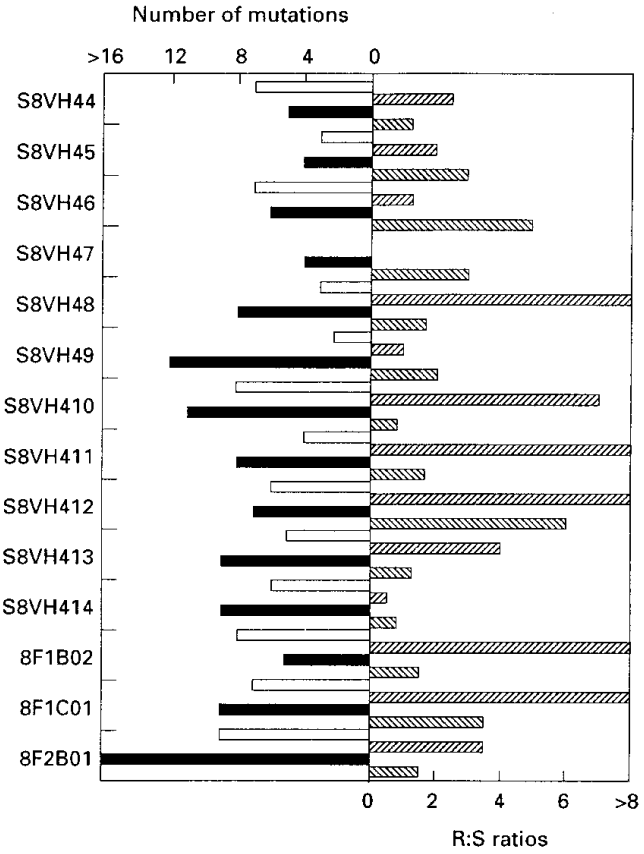


**Fig. 2.** Numbers of mutations and R:S ratios of 14 synovial membrane-derived rearranged $V_H$ genes in comparison, separated between CDR and FR. □, Mutations CDR; ▨, R:S ratio CDR; ▧, R:S ratio FR; ■, mutations FR. The R:S ratio is the quotient of replacement mutations and silent mutations. In all cases where R:S is > 8 the denominator (S) is 0 so the quotient is of infinity.

changes compared with the corresponding gl gene. In addition there were 17 single point mutations in the 3579 base pairs sequenced, resulting in a frequency of 0·47%. This frequency is 3·4-fold the frequency expected from the error rate of the Taq polymerase (0·14% for 70 cycles, data not shown) (Fig. 3).

## DISCUSSION

Analysis of $V_H$ gene rearrangements by amplifying DNA allows a characterization of B cell infiltrates for clonality and mutations irrespective of different mRNA levels in resting cells compared with activated cells and plasma cells. A relatively high quantity of DNA (350 ng representing a minimal number of $5 \times 10^3$ cells, since $1 \times 10^5$ B cells were collected in 6500 ng DNA) was used for each PCR reaction and amplified in only 35 cycles, in order to analyse a representative sample of synovial B cells.

The intensity and number of bands of the $V_H4$ and $V_H3$ family on the autoradiography of amplified $V_H$ genes was similar and revealed a relative over-representation of $V_H4$ genes compared with their representation on chromosome 14 (18%) [14]. This indicated a relative predominance of $V_H4$ genes in rheumatic SM, as has previously been shown for hybridomas from rheumatoid synovial tissue [16] and the autoimmune repertoire in general [15]. Also in transcripts from PBL of two healthy donors an over-representation of $V_H4$ genes has been described [20], while other studies reported a representation according to the gl complexity for transcripts [21] as well as for genomic DNA [17] from PBL of a healthy donor. Fifteen percent of $V_H4$ genes in c$\gamma$ transcripts from synovial membrane and 20% in c$\gamma$ transcrips from PBL of RA patients as described by Bridges *et al.* [22] were in contrast to our results. However, differences in composition of the cDNA libraries may be explained by cell selection and contamination with plasma cells in synovial tissue.

As the gl sequences were derived from cloned PCR products, sequences with 99·5% homology corresponding to one nucleotide difference per sequence may be due to infidelity of the Taq polymerase. The collection of gl genes may not be complete, because highly mutated genes may be missed by our approach of PCR cloning (oligonucleotides complementary to known gl sequences). For example, product 8F2B01 (85·3% homologous to GL9) had a high number of mutations leading to amino acid replacements within the FR and may be derived from a different gl gene. S8VH410 showed higher homology to $V_H4.22$ (90·1%) than to the most homologous gl gene derived from the same SM ($V_H4$-GL9: 88·3%). The fact that eight $V_H4$ gl genes were identical to reported gl genes demonstrates a conservation of gl genes within the established polymorphism of $V_H4$ genes [23–27].

Only a limited number of $V_H$ gl segments contributed disproportionately to the B cell repertoire of this SM, because seven of the 14 $V_H4$ rearrangements were encoded by two of the eight different gl segments (four $V_H4.34$, three $V_H4.22$). This finding corresponds to results from PBL analyses of healthy donors [28,29] as well as to analyses of the autoimmune repertoire [3,15]. In accordance with the findings of Brezinschek *et al.* [17] we did not find a dominance of $V_H4.21$/DP63 which was demonstrated for the autoimmune repertoire by Pascual *et al.* [15,30] as well as for the normal repertoire by Kraj *et al.* [20]. Instead, there was an obvious over-representation of $V_H4.34$/3d230d, a gene that has so far rarely been identified in rearranged as well as in gl gene analyses [29].

All rearranged genes analysed here were highly mutated except S8VH47, confirming the results of other studies on antibody-encoding genes in RA [12,13,22,31].

The amino acid composition of immunoglobulin V genes is such that random mutations of CDRs result in higher R:S ratios than in FRs [32]. Instead of the R:S ratio 2·9 for a random composition of amino acids, the expected R:S ratios for $V_H4$ genes are between 4·5 and 5·1 for CDRs and about 2·6 for FRs. Following these criteria six of the $V_H4$ rearrangements from this investigation showed a pattern of mutations that signifies antigenic selection by R:S ratios higher than 5 in CDRs. However, our collection of rearranged $V_H4$ genes is too small and too diverse to discriminate between intrinsic and antigen-selected mutational hotspots in these $V_H4$ genes [33].

Two rearranged genes showed low R:S ratios in the CDRs as well as in the FRs and a high number of mutations. This pattern almost fits into the model of selection against replacement mutations which has been described for rearrangements encoding RF [34] and cold agglutinins [35]. This mechanism is thought to prevent the generation of high-affinity autoantibodies in healthy individuals.

Eight of the 14 genes did not show evidence of antigen-specific selection, so that antigen-independent expansion and polyclonal activation must have made a significant contribution to the synovial B cell repertoire.

**Table 3.** List of CDR3 regions of 14 rearranged $V_H4$ genes compared with their most homologous published germ-line (gl) D segment

| Gene | 95 | N | D | N | JH | 101 | 102 | |
|---|---|---|---|---|---|---|---|---|
| DLR2 | | | AGGATATTGTAGTGGTGGTAGCTGCTACTCC | | | | | |
| S8VH44 | GCA | GTGAGACTGC | ----G---------AT--------- | GCC | TTT | GAC | TAC | (J-$_H$4) |
| S8VH48 | AGA | GGTATGGA | ---------- | | CCCAC | TTC | GAC | CCC ($J_H$5) |
| S8VH411 | AGA | GGTATGGA | ---------- | | CCCTC | CTC | GAC | CCC ($J_H$5) |
| D21–10 | | ...(15) TTTGGG (22)... | | | | | | |
| D1R1 | | | ...(117)CCCAGCCCCCACC(130)... | | | | | |
| S8VH46 | AGA | AA ------ | CCGGC ---------- | GTGG | TACTTC | GAT | GTC | (J-$_H$2) |
| DIR1 (inv.) | | ...(101)CTTCAGGGGCTCTTGA(81)... | | | | | | |
| S8VH410 | AGA | GATCG | ----G----G--C--- | CTCCT | TTA | GAC | TAC | (J-$_H$4) |
| DN1 | | | GGGTATAGCAGCAGCTGGTAC | | | | | |
| S8VH47 | AGC | T | ---------- | GGAACGGGGACTA | TTT | GAC | TAC | (J-$_H$4) |
| DA1 | | | TGACTACAGTAACTAC | | | | | |
| S8VH413 | AGA | CCTTACGGCGA | ----A------- | GGCCGA | TACTTC | CAG | AAC | (J-$_H$1) |
| DA5 | | | TGACTATGGTGCTAACTAC | | | | | |
| S8VH45 | AGA | GCGCGGGNGTT | ---------C- | CGGCCCTACCTAC | TACTTT | GAA | TTC | (J-$_H$4) |
| DK4 | | | GTGGATACAGCTATGGTTAC | | | | | |
| S8VH49 | AGA | GTG | -C----------G---A- | CTA | | GAC | TAC | (J-$_H$4) |
| DXP′1 | | | GTATTACTATGGTTCGGGGAGTTATTATAAC | | | | | |
| S8VH412 | AGA | GATGGGTG | -------TA--A----C--- | CGTCG | ATC | GAC | CCC | (J-$_H$5) |
| D21–9 | | | GTATTACTATGATAGTAGTGGTTATTACTAC | | | | | |
| S8VH414 | AGA | CAGC | ------T------- | TACTAC | | TAC | CTC | (J- |

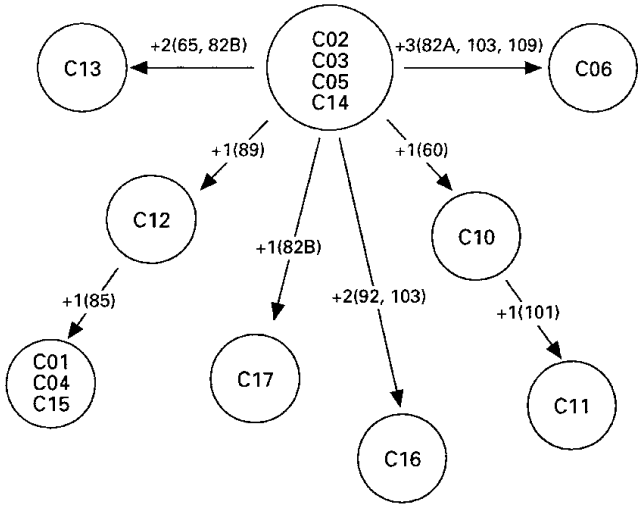


**Fig. 3.** Hypothetical development of the rearranged clone 8F1C01. Fourteen individual bacterial clones, which were sequenced, are designated C01 to C06 and C10 to C17 and outlined as a cell. C02, C03, C05 and C14 have 17 identical nucleotide exchanges compared with GL7. The other products aquired additional mutations. Numbers of point mutations and mutated codon are given in parentheses above or beside the arrow.

While the CDR3 of $V_H$ genes in general vary extensively in length and structure [36], the gene products described in our study had almost homogeneous CDR3 lengths of intermediate size and conserved D segments. These findings correspond well to studies by Borretzen *et al.*, who had similar results for RF from RA patients as well as for RF from immunized donors [37]. In contrast, an extraordinary diversity in length and composition of the CDR3 was described for a set of RA-derived polyreactive antibodies and RF [30] and rearranged $V_H$ genes from PBL of a healthy donor [17]. For the $J_H$ segments we can confirm a predominant use of $J_H4$ [17,19].

The detection of two clonally related products within 11 independently isolated $V_H4$ rearrangements indicated the expansion of a common progenitor clone. The five nucleotides that differ between the products excluded an amplification of DNA from the same cell and exceeded the frequency of mismatches caused by infidelity of the Taq polymerase (< 0·07% for 35 cycles, 0·14% for 70 cycles, data not shown). S8VH48 and S8VH411 differed from their hypothetical progenitor by five point mutations, of which three resulted in amino acid replacements in the CDR and probably represented somatically mutated subclones generated during affinity maturation of B cells in SM, as previously suggested by

analyses of SM-derived hybridomas [4,7]. Their infinitesimal R:S ratios within the CDR (3:0 and 4:0, respectively) and a low R:S ratio within the FR (1·7 both) were consistent with these conclusions. Somatic mutation in one clone was also demonstrated by the analysis of the three individual B cell aggregates, supporting the hypothesis that in the synovial membrane some B lymphocyte clones expanded and probably differentiated by affinity maturation [12,13].

Taken together, our results illustrate the important role of $V_H4$ family genes besides the DP63/$V_H$4.21 gene in RA and the role of the synovial membrane as micro-environment for B cell expansion in one patient. Further investigations concerning B cell repertoires in different compartments of patients with RA in comparison with healthy individuals may help to identify the agents and conditions contributing to the immune process in the joints.

*Note added in proof*

The nucleotide seqeunce data reported in this paper will appear in the EMBL, Genbank and DDBJ Nucleotide Sequence databases under the accesion numbers X97779 to X97787 and Z75333 to Z75361.

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