Primary research

IgVH genes from different anatomical regions, with different histopathological patterns, of a rheumatoid arthritis patient suggest cyclic re-entry of mature synovial B-cells in the hypermutation process

Maria M Souto-Carneiro, Veit Krenn, Ralph Hermann, Achim König, and Hans-Konrad Müller-Hermelink

University of Würzburg, Würzburg, Germany

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Statement of findings

In the present study 55 IgVH genes amplified from three different anatomical regions of a rheumatoid arthritis (RA) patient were analyzed, adding further information on synovial B-cell maturation and recirculation in RA. This analysis demonstrated somatically mutated IgVh genes in all regions studied, with amino acid deletions and mixed IgVh molecules, suggesting the existence of a novel pathway to generate (auto) antibody specificities. Comparison of amino acid sequences of amplified genes that belong to the VH1 family (with predominantly the same germline counterpart) exhibited strong homology, indicating an apparently conserved mutational pattern. This suggests that the number of antigens that activate B cells in different locations is restricted. The most striking result was the finding of clonally related sequences in different anatomical regions, indicating a recirculation of activated B cells between the different affected joints.

Keywords: IgVH genes, rheumatoid arthritis, synovial B-cell recirculation

Synopsis

Introduction: Although IgV genes in rheumatoid B cells have been intensively analyzed, many questions concerning antigen driven B-cell maturation and recirculation remain unanswered. It would be interesting to know whether B-cell maturation in rheumatoid tissue is different from that in secondary lymphatic organs. Moreover, it would be interesting to know whether there exists a restricted number of antigens that act on the lesions of different anatomical sites of the RA patient, and whether B cells recirculate between the different joints.

Methods: RNA and genomic DNA were prepared from tissue sections from three different anatomical sites, with different histopathologies and different onsets (left and right peroneal tendons and cubita synovial membrane), of a RA patient. Genomic DNA was amplified by seminested polymerase chain reaction (PCR), and the cDNA corresponding to the RNA was amplified by PCR using primers specific for each IgVH family. The obtained sequences were compared with their germline counterparts on the V-Base data Bank [1]. An immunohistochemical analysis of the infiltrate and the clinical data of local disease activity were also included.

Results: In the locations with longer disease duration (right peroneal tendon 5 months, left peroneal tendon 2 months) a very intense inflammatory infiltrate with germinal centres containing Ki-M4-positive follicular dendritic cells (FDC) was

CDR = complementarity determining region; FDC = follicular dendritic cells; FR = framework region; PCR = polymerase chain reaction; RA = rheumatoid arthritis; R/S = ratio of replacement mutations to silent mutations.

Figure 1

(a)

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                          CDR1                    CDR2                                                              CDR3
IgHV1-2*02  VSCKASGYTFT GYYMH WVRQAPGQGLEWMG WINPNSGGTNYAQKFQG RVTMTRDTSISTAYMELSRLRSDDTAVYYCAR
k194-88     ----------- --fi- -------------- --d----d--------- -----w----------v-spa------i---- gggtyrnwfdp       wgqgtlvt

IgHV1-3*01  VSCKASGYTFT SYAMH WVRQAPGQRLEWMG WINAGNGNTKYSQKFQG RVTITRDTSASTAYMELSSLRSEDTAVYYCAR
k194-4      ----------- ----- -------------- --d----d--------- -----s-------------------------g avrqllypdh yyyyymdv wgqgttvt

IgHV1-8*01  VSCKASGYTFT SYDIN WVRQATGQGLEWMG WMNPNSGNTGYAQKFQG RVTMTRNTSISTAYMELSSLRSEDTAVYYCAR
k194-774    ----------- n---- -----a------l- ----------------- --a---d--k---------------------- mpaytgdfdp        wgqgtlvt
k194-89     ----------- ---f- ------------i- ---agn---r-s-n--- --ai--dp-an-v-l-----t----------- daegatkaldi       wgqgtlvt
k194-92     ----------- n---- -----a------l- ----------------- --a---d--t---fl--ri-g-d---l-f--- gvpsgtnny         wgqgtlvt
k194-98     ----------- ---f- ------------i- ----------------- -------------------------------- dygdyrv yyyygldv  wgqgtlvt

IgHV1-18*01 VSCKASGYTFT SYGIS WVRQAPGQGLEWMG WISAYNGNTNYAQKLQG RVTMTTDTSTSTAYMELRSLRSDDTAVYYCAR
k194-6      ----------- g-f-h -------------- ----n----h--h---- ----------r----d----------i--gri praagiryqayryrrprggaryp
k194-62     ----------- n---- -------h------ ---tst-y-h---nfh- -----r----n--------------------- grresrhivvvtafdy  wgqgn
k194-76     ------s---- -f--- -------------- ---------d------- -------------------------------- drapycggdcystdy   wgqgtlvt
k194-79     -------d--i nf-f- ------------l- ---n--n---t-pn-r- -ls----p-----fl-----t-n---m----q drdsrgsgtwgrlgp   wgqgtlvt
k194-117    ----d-----s n-v-- -----h-------- -mn--h--------i-- ---k------------------g---e----- dldssswtgeryfdy   wgqgtvv
k194-118    ----------- ghflh ------------v- --npd-------h---- --------------------tpe--s--f-tr tpgdgwpfyf        wgqgtlvs
k194-119    ----------- --d-n -----t-------- ----------------- ------------------n------------- yctstscypgyyhmdv  wgqgtws

IgHV1-46*01 VSCKASGYTFT SYYMH WVRQAPGQGLEWMG IINPSGGSTSYAQKFQG RVTMTRDTSTSTVYMELSSLRSEDTAVYYCAR
k194-58     ------t---m n-ci- -l-------p---- v---t----t------- -----k----n--f--vtr-t-a-----f--- tyfdvwsthrdaqdl   wgqgtlvt
k194-82     ------t---m n-ci- -l-------p---- v---t----t--hn--- -----k----n--f--vtr-t-a-----f--- tyfdvwsthrdalhl   wgqgtlvt

IgHV1-69*01 VSCKASGGTFS SYTIS WVRQAPGQGLEWMG RIIPILGIANYAQKFQH RVTITADKSTSTAYMELSSLRSEDTAVYYCAR
k194-3      --------al- n-s-- -------k------ gt--f-nt----kd-kd ---fst-a-mtvv----tnva---a-i----g aqqlgpyrfyygvdv   wgqgtlvt
k194-5      -------df-r tnvfa -l-hv----f---- e-r-vfdvirv-er--- ---li--v-a-------hr-sl---s--f-tr tpggdwpfef        wgqgtpvt
k194-72     ----------- i---- -------------- g----f-t--------- ----st-e--y-v----r-------------- gsswysdyyyyygmdv  wgqgnp
k194-95     -----a-y--t ----- --------ap--v- --t-s-na-dt--r--- --s-s--e--t----s-a--t-d-----f-sk ngkagdffed        wgqgtlvt
```

(b)

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IgHV1-18*01 VSCKASGYTFT SYGIS WVRQAPGQGLEWMG WISAYNGNTNYAQKLQG RVTMTTDTSTSTAYMELRSLRSDDTAVYYCAR
k194-3      -------gals n-s-- -------k------ gtipflnta---kdfkd ---fs--a-mtvv----tnva-e-a-i----g
k194-4      ----------- --amh --------r----- --n-g--d-k-s--f-d ---i-s---a-------s----e--------g
k194-5      -------df-r tnvfa -l-hv----f---- e-rpvfdvirv-erf-- ---lia-v-a-------hr-sle--s--f--t
k194-6      ----------- g-f-h -------------- ----n----h--h---- ----------r----d----------i--gri
k194-58     ------t---m n-c-h -l-------p---- v-nptg-s-t----f-- -----k----n-vf--vtr-t-a-----f---
k194-62     ----------- n---- -------h------ ---tst-y-h---nfh- -----r----n---------------------
k194-72     -------g--s i-t-- -------------- g-ipif-ta-----f-- ---is--e--y-v---------e---------
k194-76     ------s---- -f--- -------------- ---------d------- --------------------------------
k194-77     ----------- n-d-n -----a------l- -mnpns---g----f-- --a--r---k-------s----e---------
k194-79     -------d--i nf-f- ------------l- ---n--n---t-pn-r- -ls----p-----fl-----t-n---m----q
k194-82     ------t---m n-c-h -l-------p---- v-nptg-s-t--hnf-- -----k----n-vf--vtr-t-a-----f---
k194-88     ----------- g-f-h -------------- --dpns-d------f-- -----w---i------vs-pa------i----
k194-89     ----------- --dfn -----t------i- -mn-g----r-s-nf-- --ai-r-p-an-v-l--s--t-e---------
k194-92     ----------- n-d-n -----a------l- -mnpns---g----f-- --a--r-------fl---i-g-----l-f---
k194-95     -----a----- --t-- --------ap--v- r-tpslnaadt--rf-- --sisa-e--t----s-a--t-------f-sk
k194-98     ----------- --dfn -----t------i- -mnpns---g----f-- -----rn--i-------s----e---------
k194-117    ----d-----s n-v-- -----h-------- -mn--h--------i-- ---k------------------g---e-----
k194-118    ----------- ghflh ------------v- --npd-------h---- --------------------tpe--s--f--t
k194-119    ----------- --d-n -----t-------- ----------------- ------------------n-------------
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(a) Comparison of the translated amino acid sequences of 19 VH1 segments with their closest germline counterparts. **(b)** Comparison of the translated amino acid sequences of 19 VH1 segments with germline gene *IGHV1-18*01*. All sequences are deposited in Genbank database with the following accession numbers: AF209875-209902; AF211924-211932; AF241154-241156 and AF241186-241199.

observed. In the location with shorter disease duration (right cubita 2 weeks) a low, diffuse and nonfollicular infiltration with marked oedema was detected. From the 55 analyzed clones seven expressed nonfunctional rearrangements (pseudogenes) with stop codons, and 48 were found to express functional genes. Among the 48 clones that expressed functional genes, there were two that had amino acid deletions on their complementarity determining region (CDR)2 – clones *K194/1* and *K194/111* – similar to the ones described by Wilson *et al* [2] and Goossens *et al* [3]. Two types of mixed molecules were found. Mixed molecules of the first type (*k194/57*, *k194/67* and *k194/109*) are composed of rearrangements of two different IgV genes. Mixed molecules of the second type (*k194/126*, *k194/119*, *k194/30* and *k194/99*) are composed of a IgV gene rearrangement that is fragmented by insertions of small random sequences. These insertions are different from the ones described by Wilson *et al* [2] since they are not duplicates or parts of IgV genes. The ratio of replacement mutations to silent mutations (R/S ratio) increased with disease duration. There was strong heterogeneity among the CDR3 segments.

Figure 2

(a)

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IgHV4-30-1/4-31*02          AGACCCTGTCCCTCACCTGCACTGTCTCTGGTGGCTCCATCAGCAGTGGTGGTTACTACTGGAGCTGGATCCGCCAGCACCCAGGGAAGGGCCTGGAGTG
k194-100                    ------------------------------------------------------------------------------------------------------------
k194-101                    ------------------------------------------------------------------------------------------t---------------
k194-23                     ------------------------------------------------------------------------------------------------------------
k194-103                    ------------------------------------------------------------------------------------------------------------
k194-102                    ------------------------------------------------------------------------------------------------------------

IgHV4-30-1/4-31*02          GATTGGGTACATCTATTACAGTGGGAGCACCTACTACAACCCGTCCCTCAAGAGTCGAGTTACCATATCAGTAGACACGTCTAAGAACCAGTTCTCCCTG
k194-100                    --------------------------a-----t------------------t------------------------g-----------------------
k194-101                    ---------------------------------c----------------------tt---t-----------------------------------------
k194-23                     ------------------------------------------------------------------------------------------------------------
k194-103                    ---------------------------------c----------------------------------------------------------------------
k194-102                    ---------------------------------c---------------------t------------------------------------------------

IgHV4-30-1/4-31*02          AAGCTGAGCTCTGTGACTGCCGCGGACACGGCCGTGTATTACTGTGCG
k194-100                    ------------------------------------------------gggactacggtgactacgagagtacttaactgg...t...tcgaccc ctggg
k194-101                    ------------------------------------------------gggactacggtgactacgagagtacttaactgg...t...tcgaccc ctggg
k194-23                     ------------------------------------------------agagagggtcctattatagtagtggttaccccgaacgagattgacta ctggg
k194-103                    ------------------c-----------------------------agagagccgaccgatatagcagctcgtacttgggcct...ttgacta ctggg
k194-102                    ------------------c-----------------------------agagagccgaccgatatatcagctcgtacttgggcct...ttgacta ctggg

IgHV4-30-1/4-31*02
k194-100                    gccaaggaaccctggtcaca
k194-101                    gccaaggaaccctggtcaca
k194-23                     gccaaggtaccctggtcaca
k194-103                    gccaaggtaccctggtcaca
k194-102                    gccaaggaaccctggtcaca
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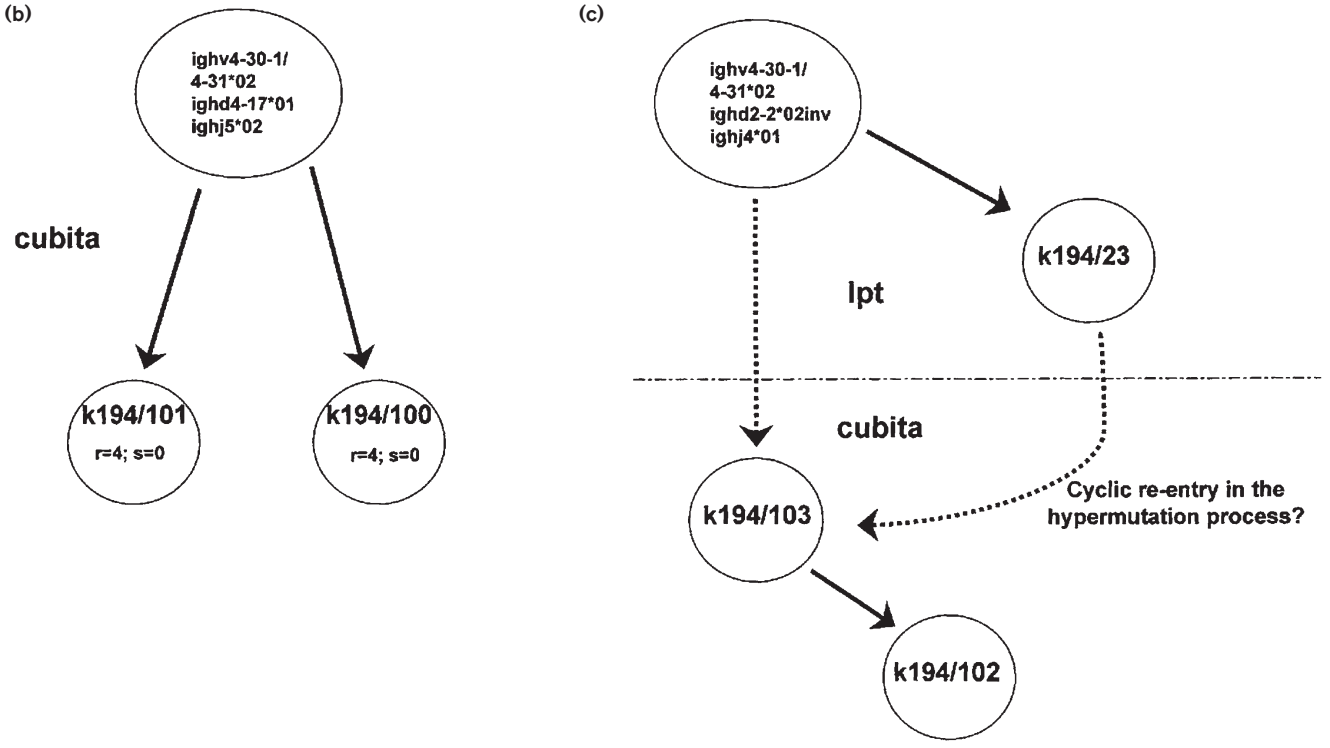


Analyses of the VH4 family amplificates. **(a)** Comparison of the five nucleotide VH4 sequences with their germline counterpart IgHV4-30-1/4-31*02. **(b)** Clonal relation between sequences *k194/100* and *k194/101*; replacement (r) and silent (s) mutations on the VH segment when compared with the germline. **(c)** Clonal relation between sequence *k194/23* amplified from the left peroneal tendon (lpt) and sequences *k194/102* and *k194/103* amplified from the cubita. Dashed arrows indicate the possibility of cyclic re-entry of the mature B cells into the hypermutation process.

The amino acid sequences that belonged to the VH1-family obtained from the three anatomical regions were primarily compared with the amino acid sequences of their closest germline counterparts (Fig. 1a). One result from this comparison was the heterogeneity in the CDR3 rearrangements. Moreover, sequences *k194/58* and *k194/82* are clonally related (confirmed at nucleotide level). Then, the 21 amino acid sequences were compared with the most widely used germline counterpart *IgHV1-18*01* (Fig. 1b). All of these VH1 sequences had mainly conservative mutations in the framework

region (FR) and nonconservative mutations in the CDR. Also, there was an almost overall conservation of the mutational cold spots and 'structural cold spots' [4] among the 19 VH1 segments. The replacement (11 from 19 replacements resulted in a proline residue) in position 34 of CDR2 could be interpreted as an antigen-selected mutational hotspot.

The comparison of the five sequences belonging to *IgHV4-30-1/4-31*02* resulted in two types of clonal relation (Fig. 2a). The first type of clonal relation, between sequences *k194/100* and *k194/101* (Fig. 2b), suggests that both sequences are derived from a single progenitor cell. The second type of clonal relation is between sequences *k194/23*, *k194/102* and *k194/103* (Fig. 2c). It suggests that an unmutated progenitor cell gave rise to *k194/23* (left peroneal tendon), from which *k194/103* (right cubita) derived and later generated *k194/102* (right cubita).

Discussion: The analysis of the 55 IgVH sequences corroborates the findings of other groups that studied a single location and RA B-cell hybridomas [5–10] and adds further information on B-cell distribution and activation in RA. First, amino acid deletions and mixed molecules could be interpreted as novel pathways to generate antibody specificities, leading, for instance, to autoreactive antibodies that could contribute to the local and systemic tissue destruction. Second, an apparently conserved mutational pattern among the 19 amino acid VH1 segments suggests that in all three RA lesions of this patient the synovial B cells are dealing with a restricted number of antigens. Third, the existence of clonally related B cells in the cubita and left peroneal tendon leaves no doubt that in this patient there is a cyclic re-entry of mutated B cells in the hypermutation process [11]. The already mutated B cells from the early RA lesions sequentially colonize new germinal centers in secondary lymphatic organs as proposed by Kepler *et al* [12]. These reactivated B-cells then invade new anatomical regions, leading to the perpetuation of the chronic inflammation in RA.

Full article

Introduction

Molecular analysis of synovial tissue and B-cell hybridomas [5–7,13] has demonstrated that synovial B cells, which are a characteristic feature of RA [7,14,15], are expanded in an antigen-dependent manner [16]. Because germinal centres may be detected primarily in synovial tissue of severely affected joints of RA patients [15,17], this is very suggestive that antigens that drive local B-cell expansion are directly involved in the pathogenesis of RA.

Although IgV genes in rheumatoid B cells have been intensively analyzed, many questions concerning the antigen driven B-cell maturation and recirculation remain unanswered. It would be interesting to know whether B-cell maturation in rheumatoid tissue is different from that in secondary lymphatic organs. Moreover, it would be interesting to know whether there exists a restricted number of antigens that act on the lesions of different anatomical sites of the RA patient, and whether B cells recirculate between the different joints.

Therefore, in the present study IgVH genes from synovial tissue B cells of different anatomical regions (with different times of disease onset) from a RA patient were analyzed. Furthermore, we included a histopathological analysis and clinical data of local disease activity, which can give a more complete picture of the role of B cells in the pathogenesis of RA.

Patient and methods

Patient, disease activity and tissue samples

Tissue samples (one from the right and another from the left tendon of musculus peroneus longus and one from the cubita synovial tissue) from a 48-year-old female patient with confirmed seropositive RA [18] were obtained at synovectomy and were snap frozen. The patient was receiving antirheumatic medication (gold, methotrexate and sulphasalazine). In the present investigation, the degree of local disease activity was scored according to the method of Fuchs *et al* [19] and Krenn *et al* [20], on the basis of warmth, effusion and swelling. The patient was seropositive for rheumatoid factors. All tissues (approximately 50%) were fixed in formalin and embedded in paraffin (Giemsa, haematoxylin and eosin staining) to be used for diagnosis and scoring of the degree of inflammation. The only material used for immunohistochemical analyses was tissue that exhibited macroscopic signs of inflammation, taken from at least three different regions of the resected synovial membrane.

Immunohistochemistry

For immunohistochemical staining, 7-µm cryosections (mounted on poly-L-lysine-coated slides) were used. Immediately before staining, the cryosections were treated with acetone for 10 min, air dried at room temperature (10–20 min) and double immunohistochemical staining was performed, as described by Krenn *et al* [7]. Briefly, the indirect immunoperoxidase technique (Ki-M4; DAKO, Hamburg, Germany) was combined with the alkaline phosphatase/antialkaline phosphatase technique (CD20; DAKO). No counterstaining was performed. In all cases, control staining was performed and single stainings were compared with double stainings in order to ensure that the pattern of immunohistochemical reaction remained unaltered.

Histopathological score of inflammatory infiltration

A portion of tissue (approximately 50%) was fixed in formalin and paraffin-embedded (Giemsa, haematoxylin and eosin staining) for use in diagnosis and scoring of the degree of the inflammatory infiltration, which in the present study was performed according to the method of Krenn *et al* [7] on a semiquantitative 1–5 scale. Very low inflammatory infiltration was indicated by 1 on the scale: the synovial intima is slightly enlarged (two to three cell layers thick); the degree of lymphocytic infiltration is very low, showing a diffuse pattern; and the subsynovial region exhibits chronic tissue granulation with slight fibrosis. Low inflammatory infiltration was indicated by 2 on the scale: the synovial intima is slightly enlarged (two to three cell layers thick), and the degree of inflammatory infiltration is low, with a diffuse perivascular lymphocytic and plasma cell infiltration; and the subsynovial region shows chronic tissue granulation with moderate fibrosis. Moderate inflammatory infiltration was indicated by 3 on the scale: the synovial intima is moderately enlarged (three to five cell layers thick), and the degree of lymphocytic infiltration is moderate, with small follicle-like aggregates near small blood vessels; and there is moderate cellularity of the subsynovial region, which exhibits slight fibrosis. Strong inflammatory infiltration was indicated by 4 on the scale: the synovial intima is extensively enlarged (five to 10 cell layers), and lymphocytes exhibit a dense follicle-like pattern; and the 'interfollicular' area exhibits very high cellularity without fibrosis. Very strong inflammatory infiltration was indicated by 5 on the scale: the synovial intima is extensively enlarged, and the distribution of lymphocytes exhibits a dense follicle-like pattern with formation of germinal centres; and granulomas and hemigranulomas can be seen in the subsynovial region. In each histopathological analysis, 10 fields were examined, and the most prominent finding in a given field determined the score.

cDNA synthesis and polymerase chain reaction amplification

Total RNA from about 50 tissue sections of 20 μm was prepared using the method of Chomczynski and Sacchi [21]. cDNA synthesis was performed with 5 μg RNA using Gibco BRL (Karlsruhe, Germany) M-MLV reverse transcriptase according to the supplier's manual. The amplification of the VH genes was carried out in a 25 μl volume containing 1.75 mmol/l $MgCl_2$, 0.4 pmol/l primer, 1U Taq polymerase (MBI Fermentas, St Leon-Rot, Germany) and 200 μmol/l of each dNTP. The cycle profile for amplification consisted of DNA denaturation at 95°C for 2 min, followed by 45 cycles of 94°C for 30 s, primer annealing at 65°C for 30 s for VH3 and VH4 primers (60°C for VH1, VH2 and VH5), and extension at 72°C for 80 s. Primer sequences were described previously [22], and are located from codons 17 to 24 (according to V-Base [1] sequence comparison). In brief, the following primers, given in the 5′–3′direction, were used:

V_H1	5′ CCT CAG TGA AGT YTC CTG CAA GGC 3′
V_H2	5′ GTC CTG CGC TGG TGA AAS CCA CAC A 3′
V_H3	5′ GGG GTC CCT GAG ACT CTC CTG TGC AG 3′
V_H4	5′ GAC CCT GTC CCT CAC CTG CRC TGT C 3′
V_H5	5′ AAA AAG CCC GGG GAG TCT CTG ARG A 3′
V_H6	5′ ACC TGT GCC ATC TCC GGG GAC AGT G 3′
J_H1-5	5′ GGT GAC CAG GGT BCC YTG GCC CCA G 3′
J_H6	5′ GGT GAC CGT GGT CCC TTG CCC CCA G 3′

DNA extraction and amplification of IgV_H genes by nested polymerase chain reaction

DNA extraction and amplification of IgV_H genes was performed according to the method of Kim *et al* [16] with minor modifications. In short, DNA was prepared by incubating 10 × 5 μm tissue sections at 50°C for 1 h with proteinase K (Boehringer Mannheim, Mannheim, Germany), which was inactivated by heating at 95°C. To improve the specificity of the PCR amplification, seminested PCR reactions were carried out as follows. In the first step, amplification with Taq polymerase was performed with V_H 5′ primers and external J_H region-specific 3′ primers [16]. In the second round, aliquots were specifically amplified for the heavy-chain genes using the same 5′ V_H region primers, but internal J_H region primers (seminested PCR). In brief, the following primers, given in the 5′–3′ direction, were used:

V_H1	5′ CCA TGG ACT GGA CCT GGA 3′
V_H2	5′ ATG GAC ATA CTT TGT TCC AC 3′
V_H3	5′ CCA TGG AGT TTG GGC TGA GC 3′
V_H4	5′ ATG AAA CAC CTG TGG TTC TT 3′
V_H5	5′ ATG GGG TCA ACC GCC ATC CT 3′
V_H6	5′ ATG TCT GTC TCC TTC CTG AT 3′
$J_H$external	5′ CTC ACC TGA GGA GAC GGT GAC C 3′
$J_H$internal	5′ TGA (AG)GA GAC GGT GAC C(AG)(GT) GT(GCT) CC 3′

The final concentrations of the reagents were 0.1 mmol/l $MgCl_2$, 200 μmol/l of each dNTP, 10 pmol/l of each primer and 2 U Taq DNA polymerase. The cycle program consisted of a denaturation step at 95°C for 5 min followed by five cycles at 95°C for 40 s, 65°C for 40 s and 72°C for 1 min and 50 s; five cycles at 95°C for 40 s, 60°C for 40 s and 72°C for 1 min and 50 s; and 25 cycles at 95°C for 40 s, 55°C for 40 s and 72°C for 1 min and 50 s. The cycles were followed by a final 10-min incubation at 72°C.

Sequence analysis

Aliquots of the final PCR products were separated by electrophoresis using a 2% low melting agarose gel (Roth, Karlsruhe, Germany), and DNA bands in the range of 350 bp were purified from the agarose gel using High-Pure DNA gel extraction kit (Boehringer Mannheim). Cloning of PCR fragments was performed using the pCR-Script Amp SK(+) cloning kit (Stratagene, Heidelberg, Germany). Positive clones were sequenced using the DyeDeoxy Termination Cycle Sequencing Kit (Applied BioSystems

Table 1

Comparison between local disease activity and molecular data

Location	Disease duration (weeks)	Local disease activity		Inflammation score	Pattern of infiltration	Presence of FDCs	ΣR/ΣS	
		Swelling	Tenderness				CDR	FR
Right peroneal tendon	20	(+++)	(+++)	5	Follicular	(+)	7.50	1.48
Left peroneal tendon	8	(+++)	(+++)	5	Follicular	(+)	3.65	1.70
Right cubita	2	(+++)	(+++)	2	Diffuse	(–)	3.00	1.39

Inc, Weiterstadt, Germany), and analyzed using an automated DNA sequencer ABIPrism373 (Applied BioSystems Inc). Both strands were sequenced using T3 and T7 primers. The sequences were analyzed using DNAman for Windows software (Lynon BioSoft, Vaudreuil, Quebec, Canada), Genebank and v-Base databases [1].

Results

Local disease activity and duration of local disease

The female patient, who suffered from a confirmed seropositive RA with involvement of tendon sheaths, exhibited severe signs of local disease activity, with tenosynovitis of the right distal peroneus longus tendon and left distal peroneus longus tendon, and synovialis of the right cubita (Table 1). The durations of local disease were 5 months, 2 months and 2 weeks, respectively.

Histopathology and immunohistochemistry of synovial tissue

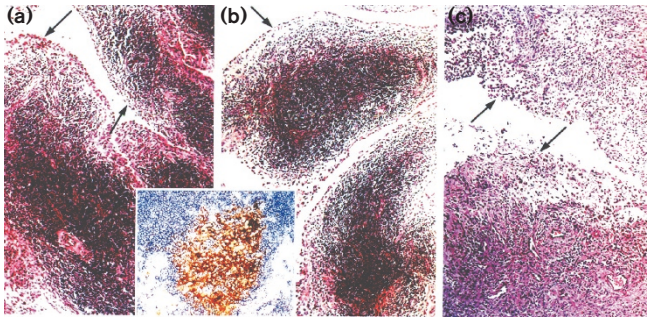
A heterogeneous inflammatory infiltrate could be observed in the different locations. In both locations with longer disease duration (right peroneal tendon [5 months] and left peroneal tendon [2 months]) a very intense inflammatory infiltrate, with Ki-M4-positive FDC-containing germinal centres (Fig. 3a, 3b and insert), could be observed (inflammatory score 5). However, in the right cubita (disease duration 2 weeks) a low, diffuse and nonfollicular infiltration with marked oedema (inflammatory score 2) was detected (Fig. 3c). The latter synovialitis histopathologically showed a more acute inflammatory reaction, whereas in the right and left peroneal tendons the morphological pattern of a typical chronic tendosynovitis was present. Immunohistochemically, the right and left peroneal tendons exhibited a dense follicular-like infiltration, with Ki-M4-positive FDC and peripherally located CD20-positive B lympocytes representing germinal centres (insert in Fig. 3a and 3b). In the right cubita only a very low and diffuse nonfollicular distribution of lymphocytes without Ki-M4-positive FDC could be recognized.

Comparison of the mutated VH segments with the germline genes

Presence of pseudogenes

From the 55 analyzed clones (Table 2), seven expressed nonfunctional rearrangements (pseudogenes) with stop codons, and 48 were found to express functional genes. The existence of pseudogenes has been largely described in IgV genes amplified from genomic DNA of healthy [1,23] and diseased [4,24] individuals. Furthermore, the presence of pseudogenes in diseased individuals is largely related to specific mutations on the RYGW motifs [4]. Based on these findings, it is not surprising that we found pseudogenes in the genomic DNA amplificates (*k194/81*, *k194/120* and *k194/126*). However, we also identified pseudogenes in the cDNA amplificates (*k194/30*, *k194/33*, *k194/130* and *k194/135*), which have not yet been described in the literature. These pseudogenes could be the product of a PCR artifact (maybe due to an elevated number of cycles) that introduced STOP-codons in the IgV-gene sequence. However, because the sequences were read in both directions (5′–3′ and 3′–5′), and both readings yielded the same confirmatory results, we do not consider the pseudogenes in the cDNA amplificates to be PCR artifacts. The existence of such pseudogenes could be explained by the findings of Drapkin *et al* [25] that DNA repair enzymes are

Figure 3



Histopathology (haematoxylin and eosin) and immunohistochemistry (double staining: indirect immunoperoxidase and alcaline phosphatase in insert of parts a and b) of rheumatoid synovial tissue from three different anatomical locations of the RA patient. These are as follows: **(a)** right peroneal tendon sheath; **(b)** left peroneal tendon sheath with inserted figure showing Ki-M4 positive FDC network (brown) surrounded by $CD20^+$ B lymphocytes (blue), representing a germinal centre; and **(c)** synovial membrane from the right cubita. Arrows indicate enlarged synovial intima (original magnification 350×).

part of the RNA polymerase II transcription initiation process. Hence there could be a defective DNA repair mechanism that, in the case presented here, could lead to the introduction of STOP codons in the RNA molecule.

Deletions and mixed molecules

Among the 48 clones that express functional genes, there were two presenting amino acid deletions on their CDR2: clones *K194/1* and *K194/111*. These detected deletion events should be regarded as the result of somatic hypermutation and not as a PCR artifact, because they were found in the intrinsic somatic hypermutation hotspots [26–29], and also involved triplets from CDR2, which leaves the transcripts functionally in frame without profoundly altering the backbone structure of the molecule, as defined by Wilson *et al* [2].

Two types of mixed molecules were found. Mixed molecules of the first type (*k194/57*, *k194/67* and *k194/109*) are composed of rearrangements of two different IgV genes. These two mixed molecules could be considered PCR artifacts, like the ones described by Bridges *et al* [30] for amplified Vκ gene segments in RA synovium. This could be due to the fact that RNA is very unstable and could have fragmented while the samples had not been snap frozen (for reasons of hygiene it is not allowed to take snap-freezing apparatus into the operating theatre). However, two facts counter the PCR artifact hypothesis. First, the B cells of the RA synovial samples are used in our laboratory not only for IgV analysis, but also for hybridoma production, so their RNA must be intact to allow successful cell fusion [5,6]. Second, the sequences were read in both directions (5′–3′ and 3′–5′), and both readings yielded the same confirmatory results of functional mixed molecules.

Mixed molecules of the second type (*k194/126*, *k194/119*, *k194/30* and *k194/99*) are composed of a IgV gene rearrangement fragmented by insertions of small random sequences. These insertions are different from the ones described by Wilson *et al* [2] since they are not duplicates or parts of IgV genes. On one hand, this could happen because of the insertion of incorrectly amplified fragments, thus resulting in a PCR hybrid artifact. On the other hand, however, the sequences were read in both directions (5′–3′ and 3′–5′), and both readings yielded the same confirmatory results. Also the use of nested PCR to amplify the genomic DNA strongly reduces the possibility of amplification and insertion of incorrect fragments [24,31], thus rendering improbable the hypothesis of PCR artifact.

Although not considering the deletions and the mixed molecules as PCR artifacts, we made the decision not to consider them for further mutational analyses, because a rational comparison with these sequences is not possible.

Table 2

B-cell clones from the different anatomical localisations are shown with closest identified VH germline gene segment and its homology

Location	Clone	VH family	Germline	Homology (%)
Left peroneal tendon				
	K194/1#	1	*IgHV1-18*01*	79.4
	K194/3	1	*IgHV1-69*01*	73.6
	K194/4	1	*IgHV1-3*01*	96.6
	K194/5	1	*IgHV1-69*01*	66.9
	K194/6	1	*IgHV1-18*01*	88.2
	K194/8	2	*IgHV2-70*11*	98.1
	K194/9	2	*IgHV2-70*01*	92.0
	K194/23	4	*IgHV4-30-1/4-31*02*	100.0
	K194/24	3	*IgHV3-53*02*	95.1
	K194/25	4	*IgHV4-59*01*	91.0
	K194/26	4	*IgHV4-30-4*06*	89.0
	K194/27	5	*IgHV5-51*01*	96.1
	K194/28	5	*IgHV5-51*01*	85.0
	K194/30##	5	*IgHV5-51*01*	P
	K194/31	5	*IgHV5-51*01*	93.8
	K194/33	5	*IgHV5-51*01*	P
	K194/42	5	*IgHV5-51*01*	98.0
	K194/81	2	*IgHV2-70*01*	P
Cubita				
	K194/57##	1	*IgHV1-18*01//IgHV1-46*01*	ND
	K194/58	1	*IgHV1-46*01*	66.5
	K194/62	1	*IgHV1-18*01*	91.6
	K194/67##	1	*IgHV1-18*01//IgHV1-2*02*	ND
	K194/72	1	*IgHV1-69*01*	95.3
	K194/76	1	*IgHV1-18*01*	97.9
	K194/77	1	*IgHV1-8*01*	94.5
	K194/79	1	*IgHV1-18*01*	75.7
	K194/82	1	*IgHV1-46*01*	80.3
	K194/88	1	*IgHV1-2*02*	91.2
	K194/89	1	*IgHV1-3*01*	86.0
	K194/92	1	*IgHV1-8*01*	88.7
	K194/95	1	*IgHV1-69*01*	76.9
	K194/98	1	*IgHV1-8*01*	96.2
	K194/99##	2	*IgHV2-70*01*	92.0
	K194/100	4	*IgHV4-30-1/4-31*02*	97.0
	K194/101	4	*IgHV4-30-1/4-31*02*	97.0
	K194/102	4	*IgHV4-30-1/4-31*02*	97.0
	K194/103	4	*IgHV4-30-1/4-31*02*	98.0
	K194/104	6	*IgHV6-1*01*	88.0
Right peroneal tendon				
	K194/109##	1	*IgHV1-8*01//IgHV1-18*01*	ND
	K194/111#	1	*IgHV1-18*01*	77.0
	K194/114	1	*IgHV1-18*01*	79.0
	K194/117	1	*IgHV1-18*01*	93.0
	K194/118	1	*IgHV1-18*01*	88.0
	K194/119##	1	*IgHV1-18*01*	96.0
	K194/120	1	*IgHV1-18*01*	P
	K194/121	2	*IgHV2-70*01*	97.0
	K194/122	5	*IgHV5-51*01*	94.0
	K194/123	5	*IgHV5-51*01*	87.0
	K194/124	5	*IgHV5-51*03*	92.0
	K194/125	5	*IgHV5-51*03*	98.0
	K194/126##	5	*IgHV5-51*01*	P
	K194/127	5	*IgHV5-51*01*	94.0
	K194/130	5	*IgHV5-51*01*	P
	K194/135	5	*IgHV5-51*01*	P
	K194/136	5	*IgHV5-51*01*	98.0

#, Clone presenting amino acid deletions; ##, mixed molecule; P, pseudogene; ND, not determined; **Bold** indicates amplification from genomic DNA.

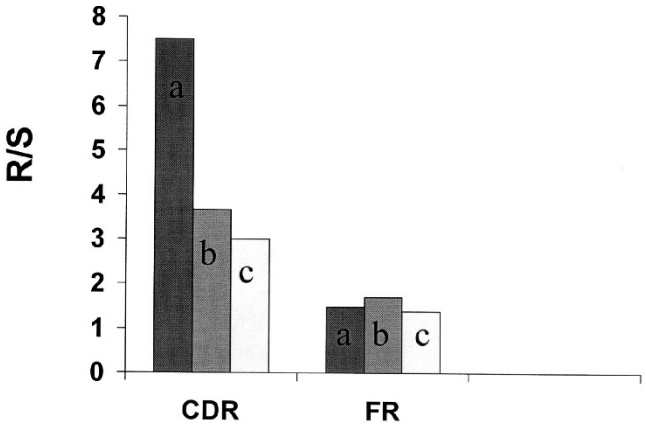
Local overall R/S ratios increase with disease duration

The 41 in-frame functional clones accumulated between 4 and 46 replacements on their amino acid sequence. The R/S ratios in the CDR of all clones from each anatomical region were all higher than 3. There was a direct correlation between the R/S values and the time of local disease duration. Locations with longer disease activity (right and left peroneus longus tendons) also had higher R/S values in the CDR than the location with a later onset (right cubita; Fig. 4).

Heterogenity among the CDR3s

Even though a relatively reduced number of different VH germline gene segments was used, the CDR3s were encoded by D-gene segments that differed in both amino acid sequence and length, and all of the six known human JH gene segments were found. As expected for the normal adult Ig repertoire [32], the JH4 and JH6 segments were the most commonly used.

Figure 4



Total R/S ratios in the FR 1+2+3 and CDR 1+2 of B-cell clones from each different anatomical region of the RA patient: **(a)** right peroneal tendon sheath; **(b)** left peroneal tendon sheath; **(c)** cubita.

Comparison of the sequences from the same VH family amplified from each location

The comparison was restricted to the VH1 and VH4 families, because they yielded the more relevant results.

VH1 family

The mutational patterns of immunoglobulin VH1 genes was studied by Borretzen *et al* [33] in peripheral blood monoclonal IgM rheumatoid factors of healthy individuals and RA patients. However, this kind of mutational pattern comparison has not been extended to B cells from RA synovial tissue. As is widely known, the primary structure of an antibody, formed by the amino acid sequence, determines all of its chemical and biological properties. Thus, the amino acid sequences that belong to the VH1 family obtained from the three anatomical regions were compared with the amino acid sequences of their closest germline counterparts (Fig. 1a). One result from this comparison is the heterogenity in the CDR3 rearrangements. Moreover, sequences k194/58 and k194/82 are clonally related (confirmed at nucleotide level, data not shown).

Then, the 21 amino acid sequences were compared with the most widely used germline counterpart *IgHV1-18*01* to determine whether a common motif could be discerned (Fig. 1b). As postulated, the conservation of the amino acid sequence of all three FRs is crucial for the interaction with the antigen [34]. In fact, for FR 1+2 we observed highly conserved regions (Fig. 5).

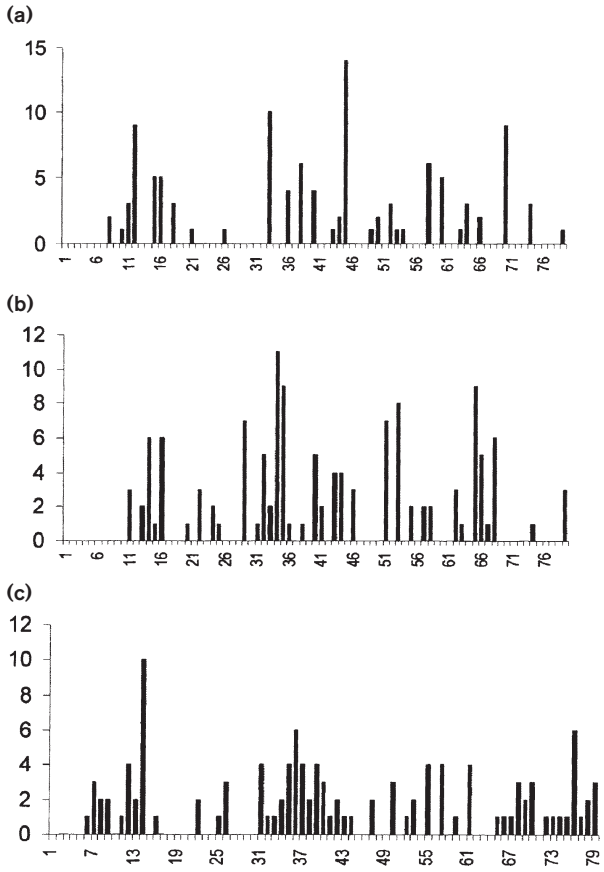
The residues at positions number 6, 10 and 11 from FR1 and the complete FR2 (except positions 22 and 26) showed a high conservation of the amino acid residues. In FR3, we found high sequence diversity (Figs 1b and 5), even though there was still conservation within residues 48–49, 51–52, 54, 56, 58–60, 62–64, 66–67, 69 and 71–75. All the other positions had a total of 30 nonconservative substitutions, and therefore they probably do not play a determining role in the antigen-mediated activation process. As expected, the number of nonconservative substitutions in both CDR1 and CDR2 was highly elevated. Nevertheless, the 3′ end of CDR2, covering residues 41–47, contained a total of 25 substitutions, but only five were nonconservative (Fig. 5). More striking evidence was that residues in position 34 of CDR2 in 11 out of 19 replacements resulted in a proline residue. It could even be speculated that position 34 of CDR2 may be an antigen-selected mutational hotspot, because it does not belong to the defined somatic hypermutation hotspots [28,29]. In position 45 there are 13 substitutions from Leu to Phe, but 12 of them cannot be considered real substitutions because, as shown in Figure 1a, the germline *IgHV1-18*01* is the only one to have a Leu in that position instead of the more frequent Phe. Therefore, in this position we only considered Leu–Ile and one Leu–Phe (for *k194/62*) as real substitutions.

As stated by Chang and Casali [35], the CDR1 is the IgVH gene region with higher susceptibility to amino acid replacement, and this was in fact the case for all the obtained sequences. The CDR1 had the highest number of nonconservative replacement mutations, which makes it very unlikely to be primarily involved in the antigen-mediated activation.

VH4 family

From the seven amplified sequences of the VH4 family (four from the cubita and three from the left peroneal tendon) five had *IgHV4-30-1/4-31*02* as their closest germline counterpart, and the other two had *IgHV4-59*01* and *IgHV4-30-4*06* (Table 2). When comparing the five sequences that

Figure 5



Amino acid replacement plot, calculated across translated amino acid sequences shown in Figure 3b. **(a)** Conservative replacement (Y↔ D, E; ↔ N D, E; L↔ V, A, F, I; S↔ N, T, Q; D↔ E; K↔ R); **(b)** indeterminant replacement (H↔ all amino acids; M↔ all amino acids; G↔ A, V, L, I, F, P; W↔ R, K, S, T, N, Q, D, E; P↔ A, V, I, L,F; K, R↔ N, Q, S, T, Y; C↔ D, E, K, N, Q, R, S, T, Y; Y↔ K, R, N, Q, S, T); and **(c)** nonconservative replacement (R, K↔ E, D; D, Y, E↔ A, I ,L, P, F; S, T, Q, N↔ A, I, L, V, P, F; R, K↔ A, I, L, P,V, F; C↔ A, I, P, V, L, F; W↔ A, I, P, F, V, L; G↔ C, D, E, R, K, N, Q, S, T, Y). The type of replacement was defined on the basis of [45–48].

belong to *IgHV4-30-1/4-31*02* (Fig. 2a) with each other, there was evidence of two different clonal relations. The first clonal relation was between sequences *k194/100* and *k194/101* (Fig. 2b), suggesting that both sequences are derived from a single progenitor cell with the rearrangement *IgHV4-30-1/4-31*02_IgHD4-17*01_IgHJ5*02*. The second clonal relation was between sequences *k194/23*, *k194/102* and *k194/103* (Fig. 2c), suggesting that sequence *k194/102* was derived from *k194/103*, which in turn was derived from sequence *k194/23* that had a progenitor cell with the rearrangement *IgHV4-30-1/4-31*02_IgHD2-2*02inv_IgHJ4*01*. Furthermore, the small number of mutations of all of these five sequences when compared with the germline could be taken as indirect evidence that the germline already encodes a high-affinity antibody, as suggested by Williams and Taylor [8].

Discussion

Various studies have demonstrated that somatically mutated B-cells are present in RA synovial tissue [5,6,8–10] and in human RA hybridomas [7,36]. However, we studied the IgV genes of synovial B cells taken from different anatomical regions, with distinct histopathology and local disease duration, of the same RA patient. The analysis of the 55 IgVH sequences corroborates the findings of other groups that studied a single location, and adds further information on B-cell distribution and activation in RA.

Amino acid deletions and mixed molecules: novel pathways to generate antibody specificities?

Recently, the introduction of deletions and duplications, in addition to nucleotide exchanges, has been described as a feature of the somatic hypermutation process [2,3]. Amino acid deletions in the IgV genes have only been described in lymphomas and healthy secondary lymphatic tissue, however [2,3]. In the present study we report the existence of such amino acid deletions in the IgV genes from synovial B cells of an autoimmune disease. For the first time, amino acid deletions in the VH genes were found in B cells of an autoimmune disease. The detection of these deletion events in RA synovialitis stresses the functional homology of the synovial membrane to secondary lymphatic tissue.

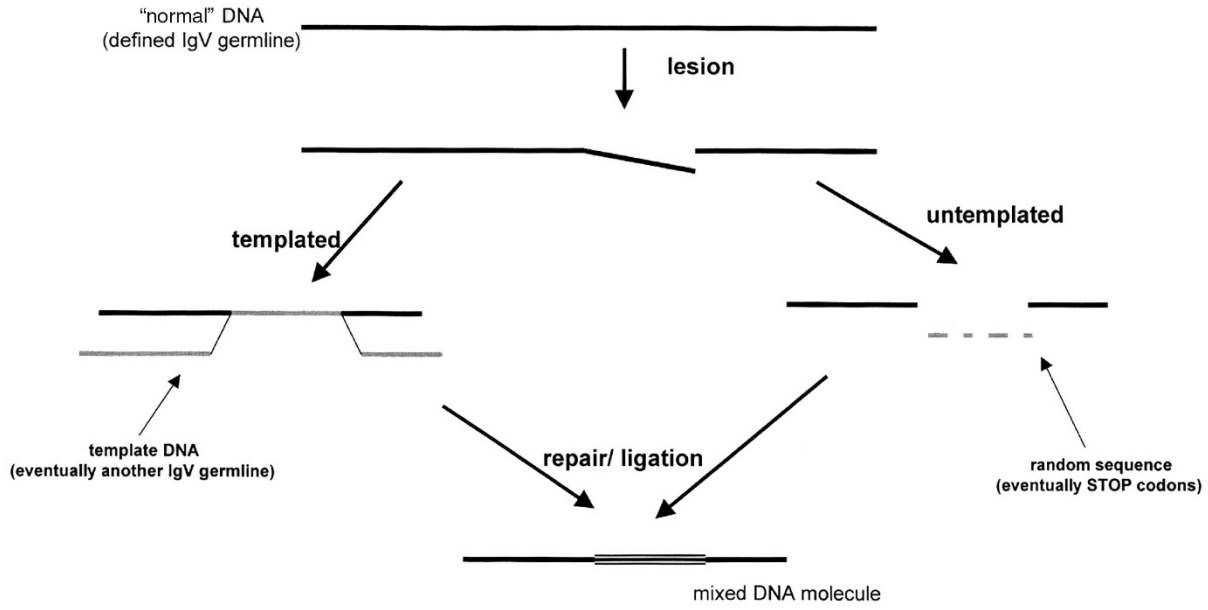
Also for the first time, IgV gene mixed molecules were found that are formed either by two segments of different IgV genes or by a IgV gene rearrangement that is fragmented by random insertions. The mechanisms that underlie the formation of these mixed molecules could be modified pathways to the unified model for somatic hypermutation, as proposed by Maizels [37] (Fig. 6); an initiating lesion could lead to a hypermutation either templated (using another germline gene as template) or untemplated (by inserting small random sequences). In some cases the process of untemplated hypermutation could lead to the insertion of STOP codons, rendering the gene nonfunctional.

Hence, the production of mixed molecules and the introduction of deletions could represent novel pathways for RA synovial B cells to generate new specificities that lead, for instance, to autoreactive antibodies that could contribute to the local and systemic tissue destruction.

Apparent mutational pattern among the 19 amino acid VH1 segments

The comparison of the amino acid sequences of the 19 VH1 segments from the different locations provided some valuable data on the interaction of the RA synovial B cells and their target antigen(s).

Figure 6



Generation of mixed molecules according to Maizels [37]. In RA synovial tissue mixed molecules could be the result of a templated or an untemplated hypermutation DNA repair process.

All of the VH1 sequences had mainly conservative mutations in the FR and nonconservative in the CDR, thus, agreeing with the results from Wedemayer *et al* [38]. When resolving at 2.1 Å, the crystal structure of a germline antibody Fab fragment and its complex with hapten, they observed an expansion of the binding potential of the primary antibody repertoire. This expansion derived from configurational stability due to antigen binding and somatic mutations, nonconservative mutations in the CDR that raised the affinity for the hapten, and conservative mutations in the FR.

Another important finding was the almost overall conservation of the mutational cold spots and 'structural cold spots' [4] among the 19 VH1 segments. The interesting absence of mutations in positions 17, 23, 28, 30, 48, 56 and 71 suggests the existence of more 'structural cold spots' in the VH1 family than those described.

During the germinal center reaction (for instance in the follicles of the two earlier lesions of this patient), rearranged B cells with low-affinity receptors improve their affinity by somatic hypermutation [39,40]. Nevertheless, these mutations can also decrease the affinity instead of expanding it. Therefore, as reported by Meffre *et al* [41], under appropriate regulation VDJ rearrangements take place in mature B cells of human tonsil. Hence, the heterogenity observed on the CDR3 of the 19 VH1 sequences could be due to a reactivation of the rearrangement process in order to rescue these RA synovial B cells from deleterious somatic mutations, or to further increase their binding affinity.

Based on the above findings, there appears to be a conserved mutational pattern among all 19 VH1 segments, hence suggesting that in all three RA lesions of this patient the synovial B cells were activated by a restricted number of antigens. This is strengthened by the replacement in position 34 of CDR2, which could be interpreted as an antigen-selected mutational hotspot.

Cyclic re-entry of mutated rheumatoid arthritis synovial B-cells in the hypermutation process

The increment of mutations with antigen dose [42] possibly indicates that the maturation of the immune response is a continuous process with the production of an increasing number of hypermutated memory B cells with time. In the special case of RA, the local joint destruction may release antigens that lead to the hypermutation process. Characteristic for B-cell hypermutation are the elevated R/S ratios in the CDR. In the present study there was a direct association of the overall R/S ratios with the duration of local disease. Synovial B cells were shown to undergo a germinal center-like reaction in RA [5,16]. Therefore, we suggest that the activated B cells from this patient have undergone a local maturation in the germinal center-like structures detected in the peroneal tendons (left and right). On the other hand, the fact that one lesion was free from FDCs and exhibited only an acute inflammatory infiltrate could support the model proposed by Oprea and Perelson [43]; the already mutated germinal centre B cells from the peroneal tendons might have migrated into the cubita synovial tissue, as has been shown for closely located finger joints [44], and

re-entered in a cyclic hypermutation process. The apparent existence of a mutational pattern on amino acid level of clones obtained from the different regions could support this hypothesis. However, the existence of clonally related B-cells in the cubita and left peroneal tendon leaves no doubts that, in the patient studied, there is cyclic re-entry of the mutated B cells from the early RA lesions in the hypermutation process [11] that sequentially colonize new germinal centers, as proposed by Kepler and Perelson [12]. These reactivated B cells then invade new anatomical regions, leading to the perpetuation of the chronic inflammation in RA.

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Authors' affiliations: Maria M Souto-Carneiro, Veit Krenn, Ralph Hermann and Hans-Konrad Müller-Hermelink (Institute for Pathology, University of Würzburg, Würzburg, Germany) and Achim König (Orthopedic Hospital, University of Würzburg, Würzburg, Germany)

Correspondence: Maria M Souto-Carneiro, Institute for Pathology, University of Würzburg, Josef-Schneider-Strasse 2, D-97080 Würzburg, Germany. Tel: +49 931 201 3779; fax: +49 931 201 3440; e-mail: path119@mail.uni-wuerzburg.de