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Analysis of V_κ genes in rheumatoid arthritis (RA) synovial B lymphocytes provides evidence for both polyclonal activation and antigen-driven selection

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SUMMARY

To define mechanisms of sustained activation of synovial B lymphocytes in RA, we studied hybridomas established from the local synovial B cell repertoire of two RA patients for V_κ gene expression and for antigen-binding specificity. The analyses revealed that members of the main V_κ families (I, II and III) were utilized at frequencies consistent with random V_κ gene family use. Furthermore, although the hybridomas expressed genes frequently seen in response to other self- and exogenous antigens, only one V_κI- and two of three V_κIII-expressing hybridomas exhibited reactivity with self-antigens. Nucleotide sequence analysis revealed that all hybridomas, with the exception of rheumatoid factor (RF)-producing hybridomas, expressed V_κ genes highly related to known germ-line genes (99·3–100% homology) and that diversity was generated by deletions and random nucleotide insertions at the V_κ–J_κ junction. Examination of the few nucleotide changes seen within the V_κ genes revealed a predominance of silent to replacement changes. Moreover, most of these changes can be attributable either to allotypic variations or to limited random nucleotide replacements independent of antigen selection. In contrast, one IgG-RF (B4D8) exhibited predominantly replacement nucleotide changes in the complementarity-determining regions, suggestive of antigen-driven selection. The random expression of immunoglobulin variable region genes with no, or little, evidence of mutation in the synovial B lymphocyte repertoire, including natural polyreactive antibodies, alongside mutated IgG-RF, suggest that both polyclonal activation and antigen-driven responses occur in RA synovia.

Keywords immunoglobulin genes rheumatoid arthritis

INTRODUCTION

RA is a chronic inflammatory disease that afflicts, primarily, the synovial membrane. It is widely believed that the local production of immunoglobulins, including rheumatoid factor (RF), in the synovium participates in the pathogenesis of tissue injury in RA [1]. The factors that lead to sustained lymphocyte activation in RA synovia, however, remain unresolved. Two alternative mechanisms, antigen-driven and polyclonal activation, have been suggested as possible causes of sustained B cell activation and autoantibody production [2]. In recent years it has been argued that the finding of mutated RF and the production of autoantibodies to local antigens in the synovium, such as collagen type II, implicate antigen-driven immune responses in disease pathogenicity [3–5]. Other studies, however, have suggested that enrichment of B lymphocyte sub-populations characterized by the ability to produce natural autoantibodies, e.g. $CD5^+$ B cells, may contribute to the disease [6,7].

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To understand disease mechanisms in RA it is important to define how lymphocytes are continually recruited to the immune response in the synovium. One approach to achieve this is to study the specificity and the IgV genes expressed by synovial B cells. The use of IgV genes to define mechanisms of lymphocyte selection and clonal dominance is ideal, since IgV genes harbour imprints characteristic of the way B lymphocytes are selected and clonal relationship between polyclonal lymphocyte populations [8].

Functional immunoglobulin genes are assembled during B cell development from a series of discontinuous germ-line gene rearrangements (V, D and J segments) to form a complete VDJ for the heavy and VJ for the light chains in a programmed manner [9]. Specificity and diversity of the primary antibody repertoire are a consequence of the number of germ-line segments and the imprecision of the recombination between these segments. The generation of this repertoire does not require antigen exposure. After exposure to antigen, a secondary response is elicited with evidence of clonal dominance of antibodies with improved affinity and somatic mutations [10]. In humans, variable region genes encoding the κ chain have been the most extensively studied of the immunoglobulin gene loci. The κ locus on chromosome 2 exists

in duplicated form in opposite transcriptional orientations and is thought to contain a total of 75 genes, of which 32 are potentially functional [11,12]. These V_κ genes have been classified into four subgroups (families) based on amino acid sequence similarity in framework region 1. V_κI family is estimated to have 38, V_κII to have 25, and V_κIII to have 10 members [13], while there is only one V_κIV gene located nearest to 5′ to the J_κ region [14].

Although IgV gene family utilization in mature peripheral B cells appears to be essentially random in that it reflects the size of the IgV families [15], a number of studies have described over-utilization of V_H5 and 6 gene families in early B lymphocytes in the fetus and individual conserved V_H genes in adult circulating B cells [16,17]. No clear restriction in the use of V_κ genes has been shown, although there is some evidence for increased use of a gene from the V_κIII family (Humkv325) in B lymphocytes during early fetal life and in chronic lymphocytic leukaemia (CLL) cells in adults [18,19]. A number of recent studies have examined V_κ family gene use in unselected B lymphocytes taken from the synovium of an RA patient by generating cDNA libraries. However, although these studies provide important data on V_κ family use, the specificity of the antibodies encoded by these genes remains unknown [20]. Other studies have examined the V_κ family use in a panel of RF-expressing hybridomas or polyclonal cells [21,22]. Again, the picture is incomplete without information on the V_κ genes used by other B cells in the joint.

We have generated 34 hybridomas from B cells taken from the synovium of two patients with RA [23]. Although hybridoma technology, as well as other methods of B cell immortalization, have limitations in that the process may select for B cell subsets or cells at early phases of the cell cycle, it nevertheless provides a means of relating antibody specificity to the rate of somatic mutations in IgV genes. In this study we report the sequences of seven of the V_κ chains expressed in the synovial hybridomas. The results show that whilst polyclonal activation appears dominant among B cells selectively immortalized by the fusion process, antigen-driven lymphocytes may also contribute to the local immune reaction in RA synovia.

MATERIALS AND METHODS

Generation of B lymphocyte hybridomas

A panel of 34 hybridomas was generated by fusing mononuclear cells from the synovial membranes of two long-standing RA patients with the heteromyeloma cell line SPAZ-4 [23]. Both patients satisfied the American Rheumatology Association (ARA) criteria. Patient A was a 59-year-old woman with classical erosive seropositive arthritis, while patient B was a 38-year-old woman with polyarticular erosive seronegative RA, with disease duration of 6 and 13 years, respectively. The fusion process was carried out using mononuclear cells separated from enzyme-digested synovial tissue using centrifugation over Ficoll–Hypaque (Pharmacia, St Albans, UK). The separated cells were fused with SPAZ-4 cells at a ratio of 5–10:1, using 50% polyethylene glycol 1500 (Boehringer Corporation, Lewes, UK). The resulting hybridomas were screened for immunoglobulin heavy chain isotypes using ELISA, and cloned three times before being selected for further examination.

Immunoglobulin isotype and cross-reactive idiotype expression

Immunoglobulin heavy and light chain isotypes, IgG subclass and cross-reactive idiotypes (CRI) were characterized using reverse-passive haemagglutination and ELISA [23]. Haemagglutination assays were performed using sheep erythrocytes sensitized with MoAbs specific for heavy or light chain isotypes. ELISA assays were performed using microtitre plates (Linbro Plates; ICN Biomedicals Ltd, Thame, UK) sensitized with polyclonal sheep anti-human immunoglobulin (The Binding Site Ltd, Birmingham, UK). Expression of the V_κIII subgroup and associated CRI was examined using MoAbs C7, 17-109 and 6B6.6 (the last two MoAbs were kindly provided by Professors D. Carson (University of California, San Diego, CA) and W. Koopman (University of Alabama, Birmingham, AL)). Positive and negative controls were used in each assay and bound MoAbs revealed with peroxidase-conjugated sheep anti-mouse immunoglobulin (The Binding Site).

Characterization of antigen-binding specificity

The reactivities of immunoglobulins produced by the hybridomas were studied using ELISA, agglutination or indirect immunofluorescence [23]. Reactivities with human IgG subclass proteins, rabbit IgG, joint-associated antigens, collagen type I and II, heat shock protein p65, single-stranded DNA (ssDNA), histones, cardiolipin, influenza viral proteins and tetanus toxoid were determined in ELISA. Hybridomas were considered reactive with any particular antigen when optical density (OD) values significantly higher (at least 0·1 OD unit higher) than the mean + 2 s.d. of negative controls were obtained. Reactivity with anaerobic intestinal bacteria-associated epitopes was kindly studied by Dr M. Hazenberg (Department of Immunology, Erasmus University, Rotterdam, The Netherlands) using soluble peptidoglycan–polysaccharide complexes, which share antigenic epitopes with 22 out of 40 anaerobic intestinal bacteria, in ELISA [24]. Haemagglutination assays were performed to test for low-affinity interactions with human IgG subclasses (RF activity). Sheep erythrocytes sensitized with human IgG subclass paraproteins using chromic chloride were added to double-folding dilutions of culture supernatants (in HEPES-buffered RPMI containing 2% heat-inactivated fetal calf serum (FCS)) in U-shaped microtitre plates (ICN Biomedicals Ltd) and agglutination titres recorded after 2 h incubation at room temperature. Non-specific interactions with sheep erythrocytes were assessed using erythrocytes treated with chromic chloride but with no sensitizing antigen. Indirect immunofluorescence was carried out using 3T3 fibroblasts or HEp-2 cells cultured as monolayers on 12-well multitest slides (Biodiagnostics, Malvern, UK) in Dulbecco's modified Eagle's medium (DMEM) or RPMI medium, respectively. Slides were fixed in acetone/methanol (1:1) in a dry ice/methanol slurry for 10 min and neat supernatants, or diluted reference sera, applied to individual wells and incubated for 20 min in a humidified atmosphere. Bound antibodies were detected using FITC-conjugated goat anti-human immunoglobulin. Antinuclear and nucleolar specificities were determined by indirect immunofluorescence using HEp-2 cells. Reactivity of the IgM produced by hybridoma A3D5, which has a V_κI light chain 98·3% homologous to that of an anti-thyroglobulin antibody, was tested for reactivity with thyroglobulin by indirect agglutination using gelatin particles sensitized with thyroglobulin (Serodia-ATG, Fujirebio Inc., Tokyo, Japan).

Polyreactivity was confirmed by inhibition experiments using purified antibodies.

cDNA synthesis and amplification

RNA was extracted from cultured cells using guanidinium isothiocyanate [25]. κ-chain cDNA was synthesized from 1 μg of

total RNA using AMV reverse transcriptase (Stratagene Ltd, Cambridge, UK) and a κ-chain constant region primer (KCc1 5′ AAC AGA GGC AGT TCC AGA GTT 3′). Second strand synthesis and amplification were performed using an anchored polymerase chain reaction (a-PCR). First strand cDNA was tailed with poly G using 1 mM dGTP, TdT Mg^{2+} buffer and 48 U of terminal deoxynucleotidyl transferase (TdT) enzyme (International Biotechnologies Inc. Ltd, Cambridge, UK). Second strand synthesis was performed by PCR using a primer for the poly G tail (anch2pc 5′ ACG AAT TCT AGA GTC GAC CCC CCC CCC CCC C 3′) and a κ constant region primer 5′ of the KCc1 primer (KCc2 5′ TGC TTC GGA TCC GAA GAT GAA GAC AGA TGG TGC 3′). Tailed cDNA (4 μl) was mixed with 10 mM Tris–HCl pH 8·3, 50 mM KCl, 1·5 mM Mg^{2+}, 175 μM dNTP in a 50-μl reaction volume, and incubated at 96°C for 5 min, then at 60°C for 5 min. One unit of Taq polymerase was added and the mixture incubated at 70°C for 15 min to allow the anchor primer to extend. KCc2 primer (100 ng) was added and PCR performed for 30 cycles: 94°C 1 min, 60°C 1 min, 72°C 1 min, with a final extension at 72°C for 10 min. The PCR product was cloned into the pCR^{tm}II vector using the TA cloning kit (Invitrogen Corp., R&D Systems Europe Ltd, Abingdon, UK). Four of the resulting colonies were grown in LB medium with 50 μg/ ml ampicillin, and plasmid DNA extracted using the Wizard miniprep kit (Promega Ltd, Southampton, UK) for sequencing.

Nucleotide sequencing

Plasmid DNA was denatured before sequencing by incubation at 37°C for 30 min in 2 mM NaOH, 20 mM EDTA. The DNA was then precipitated with ethanol and resuspended in 7 μl distilled water, 2 μl Sequenase reaction buffer and 1 μl of KCc2 or the anch2pc primer (100 ng). The primer was allowed to anneal to the template for 20–30 min at 37°C before commencing the sequencing reaction using the Sequenase kit (United States Biochemical, Amersham Life Science, Aylesbury, UK) according to the manufacturer's instructions. Plasmid DNA from each colony was sequenced in both directions using the KCc2 or anch2pc primer.

Sequence analysis and database search

Computer compilation of the DNA sequence data was performed by the DNAstar computer software package (DNAstar, London, UK), and the FASTA search program at Daresbury was used to analyse the data.

RESULTS

Synovial hybridomas from RA patients produce monospecific RF, polyreactive antibodies and immunoglobulins with unknown specificity

The fusions yielded 26 IgG- (25 IgG1 and one IgG2) and eight IgM-secreting hybridomas, of which 11 IgG and six IgM expressed κ light chains. All six κ-expressing IgM and one IgG-producing hybridoma (with RF activity) were sequenced. Of the six IgM-producing hybridomas one had RF activity (A2A2) and one was polyreactive (A4A2). Hybridoma A2A2 reacted with human IgG1, 2 and 4 and IgG3 of the G3m(st) allotype, but not IgG3 of the G3m(u) allotype, thus exhibiting a specificity pattern similar to the new Ga specificity [26]. The IgG2 produced by B4D8 reacted with all four human IgG subclass proteins. Hybridoma A4A2 produced an IgM that was polyreactive with cytoskeletal filaments, cardiolipin, vimentin, tetanus toxoid, and IgG Fc (with low affinity for IgG Fc and tetanus toxoid). IgM from the other four hybridomas did not react with any of the tested antigens, and their specificity remains undefined.

V_κ genes from the three main Vκ families are expressed in synovial B cells

The seven synovial hybridomas expressed genes from the V_κI (A1B4, A2A2 and A3D5), the V_κII (A3B5) and V_κIII gene families (A3B6, A4A2 and B4D8) at frequencies consistent with the level of V_κ family use in normal subjects [27]. The expressed heavy chains were from V_H families 1, 3, 4 or 5 (Table 1).

Six of the seven hybridomas (the exception being B4D8) showed 96·5% or greater homology with their corresponding germ-line genes. A1B4 was 99·6% homologous with the V_κI germ-line gene O2-O12 over the entire V_κ coding region with a single silent nucleotide change at the 3′ end of the gene (V_κ–J_κ junction; T to G; Fig. 1a). A2A2, which produced IgM RF, was 96·5% homologous to O2-O12 with 10 nucleotide differences. Six of these nucleotide differences were silent changes at codons 14, 15, 31, 51, 53 and 94. The remaining four nucleotide changes were replacement changes in the CDRs (one in CDR1 and three in CDR3). Three of four changes in CDR1 and 2 were silent changes, while the fourth resulted in a non-conservative amino acid interchange of alanine to threonine at position 25.

A3D5 was 99·7% homologous with the V_κI germ-line gene O8 with one silent nucleotide difference at the V_κ–J_κ junction (T to C at the third position of codon 95). The sequence of A3D5 also aligned with 97·7% homology to rearranged genes encoding autoantibodies to DNA that express the 3I idiotype in systemic lupus erythematosus (SLE) patients [29], and with 98·3% homology to an autoantibody to thyroglobulin [30]. A3D5, however, did not react with DNA or thyroglobulin, and its specificity remains unknown.

A3B5 V_κ chain gene aligned with 99·7% homology to the V_κII germ-line gene A2 with one nucleotide change in the CDR3 (G to C at position 93), resulting in a non-conservative replacement of the neutral amino acid glutamine to the positively charged histidine. The J_κ gene had two nucleotide changes at positions 96 and 97, resulting in the replacement of tyrosine and threonine with cysteine and serine, respectively. These two changes, however, appear to be due to allotypic variations, since identical changes were seen in six other rearranged J_κ2 genes from normal B cells [31,32]. The A2 gene is frequently expressed in antibodies to a *Haemophilus influenzae* capsular polysaccharide antigen (anti-Hib-PS antibodies). Anti-Hib-PS antibodies use a heavy chain from the V_H3 family in association with different genes from the V_κ2 family. The dominant light chain gene, however, appears to be product of the A2 germ-line gene [33]. Although A3B5 is associated with a V_H3 heavy chain (with 98·9% homology to the DP-31 germ-line gene; [34]) it did not react with any of the antigens tested in this study including soluble peptidoglycan–polysaccharide complexes that share epiotpes with 22 anaerobic intestinal bacteria. Reactivity of the hybridomas with Hib-PS antigen, however, was not examined in this study.

The V_κ genes of hybridomas A3B6, A4A2 and B4D8 were from the V_κIII family. A3B6 aligned with 99·3% homology to the Humkv325 gene. This was in line with the serological data showing A3B6 to be reactive with the MoAb 17.109. The Humkv325 gene is frequently expressed in autoreactive paraproteins and monoclonal and polyclonal RF [35–37]. A3B6, however, had no detectable autoreactivity. The V_κ gene of B4D8 also

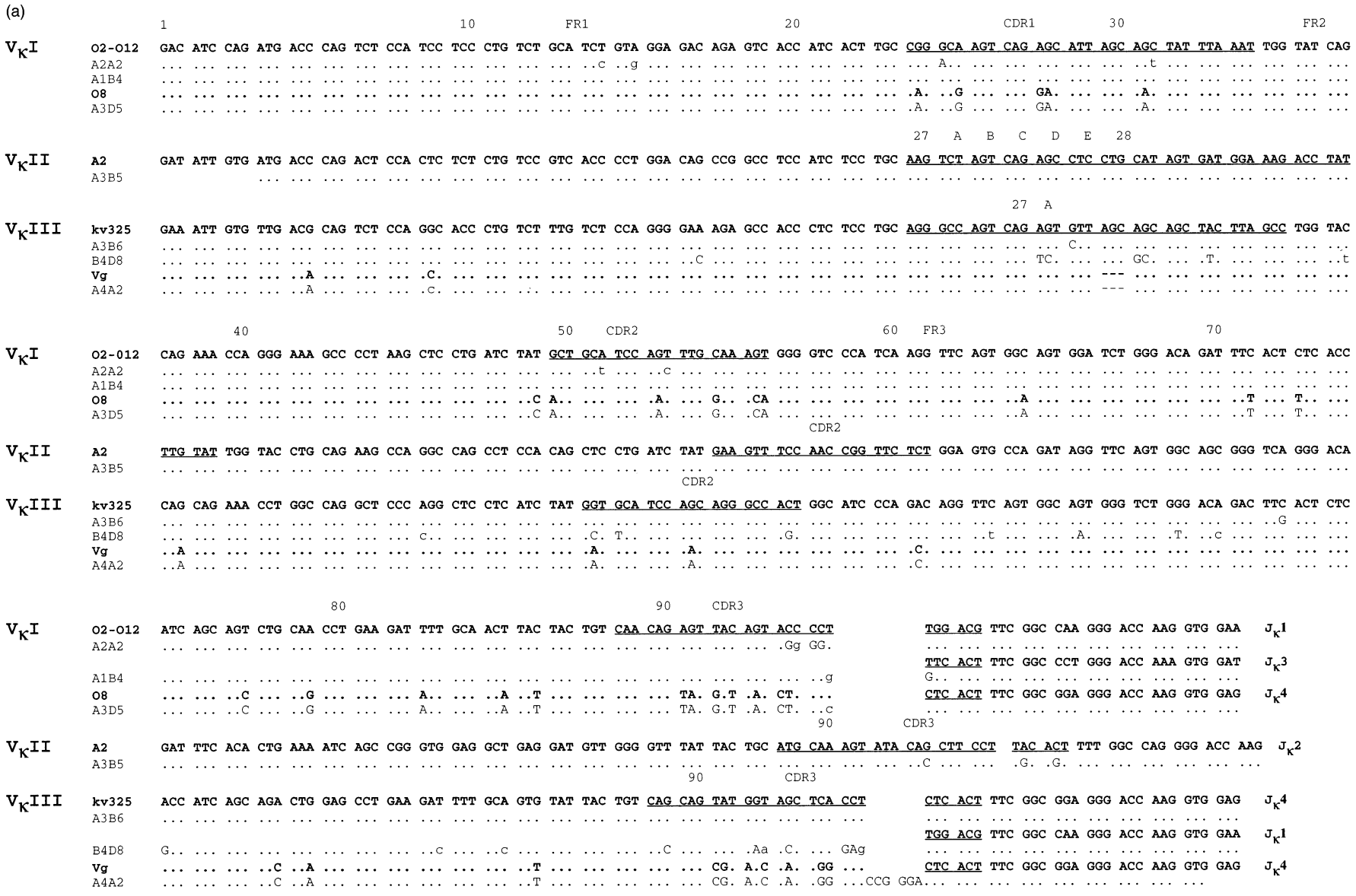

Fig. 1. (a) Nucleotide sequences of the V_κ gene segments expressed in the synovial hybridomas studied. The V_κ sequences are compared with the most homologous germ-line genes (bold), O2-O12 or O8 from the V_κI, A2 from the V_κII and Humkv325 (kv325) and Vg from the V_κIII families. Dots indicate identity with the corresponding germ-line genes. Upper case nucleotide changes indicate replacement changes, while lower case nucleotide changes indicate silent changes. The CDR (underlined) and FR regions are defined and numbered according to Kabat *et al.* [28]. Dashes indicate where gaps have been introduced in the Vg gene to maximize homology with the Humkv325 gene. The J_κ genes associated with each V_κ gene are identified at the right end of the Figure, and the germ-line genes given for comparison. (b) The predicted amino acid sequences of the V_κ genes expressed in the synovial hybridomas studied. The V_κ sequences are aligned with the most homologous germ-line genes from the V_κI, II and III families. Dots represent identity with the corresponding germ-line genes. Numbering is according to Kabat *et al.* [28].

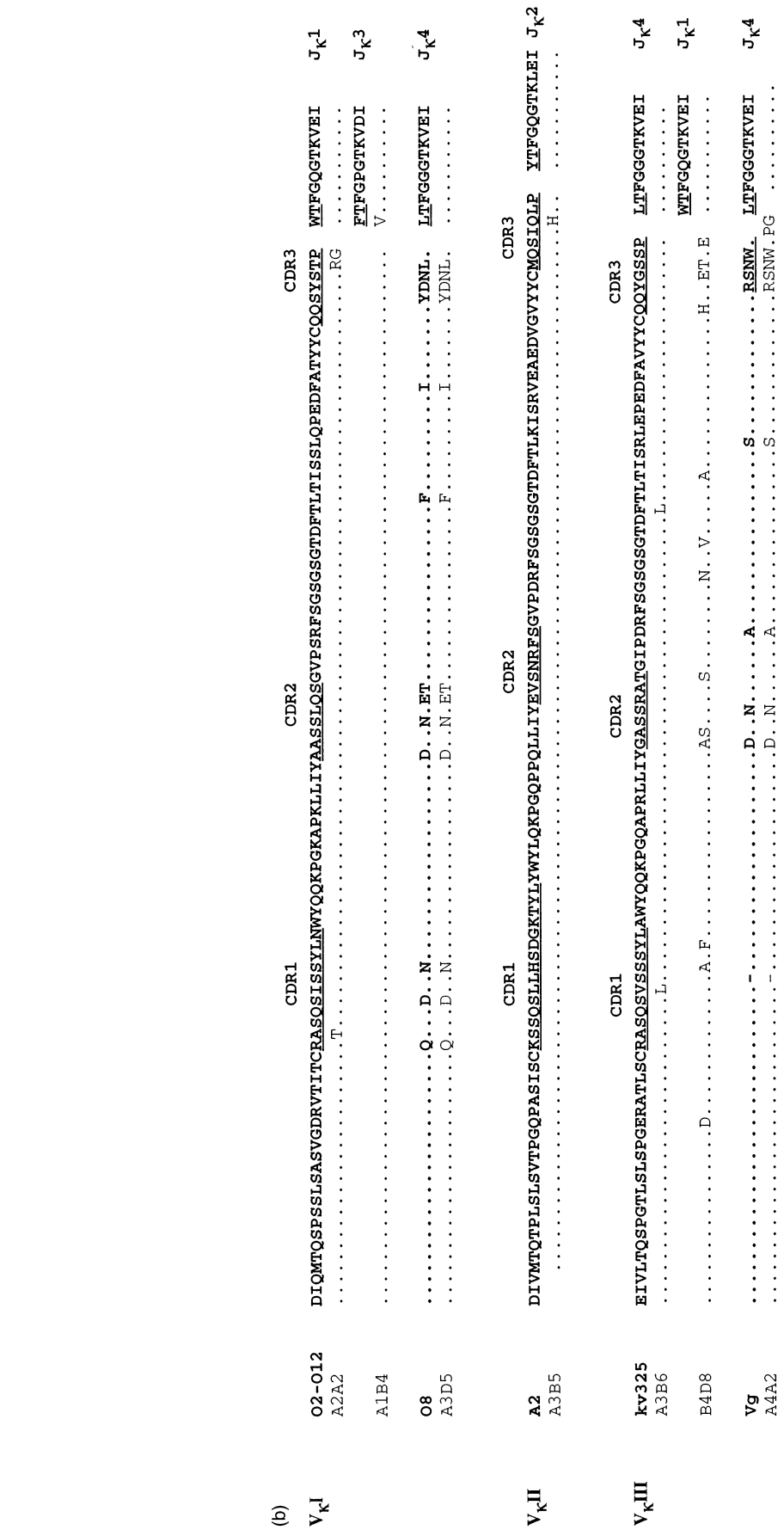

Figure 1.Continued

Table 1. Structural characteristics and specificity of κ light chain expressing synovial hybridomas from RA patients

Hybridoma	Isotype of antibody produced	Specificity of antibody produced	V_H family	Closest V_H germ-line gene (% homology)	V_κ family	Closest V_κ germ-line gene (% homology)
A1B4	IgMκ	NI	3	Vh26 (100)	1	012/02 (99·6)
A2A2	IgMκ	mRF	3	1-9III (97·4)	1	012/02 (96·5)
A3D5	IgMκ	NI	2	ND	1	08 (99·7)
A3B5	IgMκ	NI	3	DP-31 (98·9)	2	A2 (99·7)
A4A2	IgMκ	Polyreactive	4	4.21 (100)	3	Vg (100)
A3B6	IgMκ	NI	1	V_{I-3b} (100)	3	kv325 (99·3)
B4D8	IgG2κ	mRF	1	ND	3	kv325 (91·6)

mRF, Monospecific rheumatoid factor; NI, not identified; ND, not determined.

aligned (91·6% homology) with the Humkv325 germ-line gene and reacted with the 17.109 MoAb. In contrast to the A3B6 hybridoma, which had only two nucleotide changes, B4D8 had 25 nucleotide changes. Ten of the 25 nucleotide changes were in the framework regions, of which six were silent, while four resulted in amino acid replacements (positions 17, aspartic acid for glutamic acid; 65, asparagine for serine; 68, valine for glycine; and 74, alanine for threonine). Two of these amino acid replacements were conservative changes (positions 17 and 68). Thirteen of the 15 nucleotide changes in the CDRs (five in CDR1, three in CDR2 and five in CDR3; Fig. 1 and Table 2) were replacement changes. The alanine at the 5′ end of the CDR2 appeared to be relevant to the binding of the RF-producing hybridomas. Hybridoma A2A2 had a germ-line gene encoded alanine at position 50, while in B4D8 there was a mutation resulting in a change of the glycine to alanine at the same position.

The V_κ gene of A4A2 aligned with 100% homology to the V_κIII germ-line gene Vg.

CDR3 in synovial V_κ genes exhibit extensive variability

The highest degree of variability in the synovial V_κ genes compared with the corresponding germ-line genes was within CDR3. Five of the V_κ sequences had differences from the corresponding V_κ–J_κ germ-line genes, mostly within the CDR3. Furthermore, most of the nucleotide changes were replacement rather than silent changes. In contrast to earlier studies of RA synovial transcripts, however, the changes within the CDR3 regions did not lead predominantly to charged amino acids, and none had arginine at the V_κ–J_κ junction [20,22]. Only five of 10 amino acid replacements observed in the CDR3 of the examined hybridomas were charged amino acids (position 94, arginine for threonine in A2A2; position 93, histidine for glutamine in A3B5; and 89, histidine and 95, glutamic acid for glutamine and proline, respectively, in B4D8). In both RF-producing hybridomas the changes appeared to be the result of the error-prone repair mechanism that follows the specific endonucleolytic cleavage during juxtaposition, somatic mutations or N-addition in the case of glutamic acid at position 95 in B4D8. The hypermutation machinery favours substitutions rather than deletion/insertion of nucleotides. However, the changes at the V_κ–J_κ junction of B4D8 do not appear to be the result of this predicted pattern of preference, since all three nucleotides have been replaced. The constraint on the length of V_κ CDR3 (nine amino acids) means that 21 different possible nucleotide sequence products of Humkv325-J_κ gene rearrangements, encoding eight different amino acids at position 95a, the splice site, could be encountered if the changes were due to the effect of imprecision in the rearrangement process. However, glutamic acid is not one of these amino acids, and of more than 100 reported Humkv325 sequences in the literature, none had glutamic acid at this position, while two had N-additions resulting in an extra glutamic acid at the junction. This may suggest that the resulting glutamic acid at this position in B4D8 is due to trimming of the terminal 3′ codon of the V_κ germ-line gene followed by N-additions.

Six additional nucleotides were seen at the V_κ–J_κ junction of the expressed gene in A4A2. N-additions at the V_κ–J_κ junction of RF-producing synovial and peripheral blood B cells from RA patients, and more recently normals, have been observed [32,37]. In the vast majority of these reported sequences the N-additions start with CC and have an additional one, two or three G nucleotides, resulting in an extra proline and, occasionally, a glycine. In A4A2 the observed additional nucleotides could be due to N-additions or to the imprecision in the recombination process. In the latter situation, the first CC nucleotides would be from the non-coding germ-line sequence 3′ of the Vg gene, followed by two GG P-nucleotide additions from the second DNA strand [38], while the last two nucleotides would be from the non-coding germ-line sequences immediately 5′ of the J_κ genes, with one mutation resulting in G/A replacement.

Nature and frequency of nucleotide changes in the synovial V_κ sequences

Hypermutation in IgV genes independent of the skewing effect of antigen selection has a preference for purines over pyrimidines (DNA strand polarity), a bias in favour of transition over transversion (purine to purine or pyrimidine to pyrimidine rather than purines to pyrimidines or *vice versa*) and identifiable hotspots resulting from preferential targeting by the mutational machinery [39]. To assess if the nucleotide changes observed in the synovial V_κ genes were due to antigen-induced mutations, random mutations or allotypic variations, the nucleotide changes A2A2, A3B6 and B4D8 were examined for their transition/transversion nature and occurrence in other germ-line or rearranged genes (Table 3). The analyses revealed no preference for changes of purines (A and G) compared with pyrimidines (C and T) in the V_κ genes from A3B6 and A2A2, and no preference for transversion in A2A2. Three of the nucleotide changes in A2A2 (codons 15, 31 and 94) were also seen in one, two and two other rearranged O2-O12 genes, respectively. The 94 codon was also different from the germ-line

Table 2. The distribution of replacement (R) and silent (S) nucleotide changes in the CDR and FR of the κ-expressing synovial hybridomas where the expressed V_κ genes differed from the germ-line genes

Hybridoma	Region	No. of nucleotide changes	No. of amino acid changes	Observed R:S ratios (no. of R:S)	Expected R:S ratios
A2A2	CDR1 + 2	4	1	0·25 (1:4)	3·977
	FR1 + 2 + 3	2	0	0	3·054
A3B6	CDR1 + 2	1	1	∞ (1:0)	3·373
	FR1 + 2 + 3	1	1	∞ (1:0)	3·007
B4D8	CDR1 + 2	8	6	∞ (8:0)	3·373
	FR1 + 2 + 3	10	3	0·67 (4:6)	3·007

All three hybrodmas given in the Table had nucleotide changes that were assessed to be different from the germ-line gene and were assumed, for the purpose of this analysis, to be mutations. Hybridomas with nucleotide changes in the CDR3 only were excluded from this comparison to avoid the possibility of misconstrued inclusion of changes due to the recombination processes for mutations. Nucleotide changes were considered individually within each codon independent of other changes within the same codon. Observed R:S changes were estimated by taking all nucleotide changes regardless of being part of the same codon. The theoretical expected (inherent) R:S mutation ratios were determined according to the formulae given in [39]. These were calculated as the total number of possible single base substitutions resulting in amino acid replacements and excluding those resulting in stop codons.

gene in 5/12 other rearranged O2-O12 genes (in three the T is changed to A and in two to C). The nucleotide changes of C to T and T to C at codons 31 and 53 involved the serine codon AGY (Y = C or T), which has been shown to be a preferred target for the hypermutation machinery [39]. These observations suggest that three to four of the nucleotide changes observed in A2A2 are due to random mutations or allotypic variations rather than antigen selection. In contrast, most of the nucleotide changes in B4D8 involved purines, and showed a preference for transversion. There were identifiable hotspots involving the serine codon AGY (mainly in CDR1, but also FR3 and CDR3), but most of the changes observed in the AGY serine codon involved the second and/or first nucleotides in B4D8. Most of the changes in the B4D8 V_κ gene were unique, with the exception of two changes in the FR regions (codons 46 and 85), which were also seen in other rearranged genes. Changes within the serine residue at position 31 (CDR1) were also seen in other genes, a change to G was seen in three rearranged genes, while the second position (G) was changed to T in two other rearranged Humkv325 genes. The only other nucleotides frequently seen in other rearranged genes were the proline codon at position 95a, where the change in the last T to G was seen in seven other rearranged genes, and to A and C in three and eight genes, respectively.

Taq fidelity

To exclude the possibility of any PCR or Taq-introduced artefacts, V_κ gene DNA from at least four colonies of each PCR product cloning were sequenced in both directions. The V_κ genes were also amplified by the a-PCR protocol on at least two different occasions using different cDNA preparations for cloning and sequencing. All nucleotide changes reported here were confirmed, suggesting that none of the changes was due to PCR errors or Taq infidelity.

DISCUSSION

The main objective of our studies is to define the mechanism(s) responsible for synovial B cell activation in RA. Our approach is based on the consideration that polyclonal activation leads to a stochastic use of IgV genes with no, or random distribution of limited mutations, while antigen-driven selection results in over-expression of a small set of genes and somatic mutation mainly in the CDRs. These mutations usually exhibit a high ratio of nucleotide changes leading to amino acid replacements (R) compared with silent (S) changes [39,40].

The rearranged V_κ genes from the synovial B lymphocyte hybridomas reported here were assigned to the three main V_κ families (I, II and III). Although the number of hybridomas studied is small, and hence insufficient for accurate assessment of frequency, it is nevertheless noteworthy that the frequency of family use was comparable to V_κ gene use in circulating B cells from normal individuals [27,41]. It is also noteworthy that most of the individual genes expressed in the synovial hybridomas were highly homologous to their corresponding germ-line genes and, in the main, had no evidence for somatic point mutations. Furthermore, most genes expressed in the hybridomas have also been found by other investigators to be frequently used in other responses to self and environmental antigens. These observations are consistent with a predominantly polyclonal activation of synovial B cells represented by these hybridomas. The study, however, also provided some evidence for antigen-driven selection of RF-producing synovial B cells.

Sequence analysis revealed that five of the hybridomas, of which four have no identifiable specificity, showed no, or little, evidence for antigen-induced mutations. The five hybridomas had very close homology with known germ-line sequences (99·3–100% homologous). In one of these five hybridomas (A4A2), homology with the related germ-line genes was 100%, while in another two (A1B4 and A3D5) the only nucleotide differences were probably due to deletions and random nucleotide insertions at the V_κ–J_κ junction. Another hybridoma (A3B6) had two nucleotide differences compared with the Humkv325 germ-line gene, but one of these two changes is probably due to antigen-independent random mutation. Since the IgV germ-line gene repertoire in any one individual is the result of a diverse set of haplotypes, it is possible that one or both nucleotide changes observed in A3B6 (and three in A2A2) could be due to polymorphism. Indeed it is

Table 3. Nature of nucleotide changes observed in the V_κ genes from κ-expressing synovial hybridomas

A2A2	Codon No. (location)	F	A3B6	Codon No. (location)	F	B4D8	Codon No. (location)	F
Transition						Transition		
A → G	15(FR1)	2				A → G	30(CDR1)	3
T → C	14(FR1)	0					74(FR3)	0
	53(CDR2)	0				T → C	82(FR3)	0
G → A	25(CDR1)	0				G → A	65(FR3)	0
C → T	31(CDR1)	2					92(CDR3)	1
						C → T	37(FR2)	1
							63(FR3)	1
Transversion			Transversion					
A → T	51(CDR2)	0	C → G	72(FR3)	0	Transversion		
C → G	94(CDR3)	0	G → C	28(CDR1)	0	A → C	17(FR1)	0
	94(CDR3)	0					45(FR2)	1
	95(CDR3)	0					69(FR3)	0
	95(CDR3)	2					84(FR3)	0
						A → T	27A(CDR1)	0
							32(CDR1)	0
						G → C	27A(CDR1)	0
							30(CDR1)	0
							50(CDR2)	2
							89(CDR3)	0
							93(CDR3)	2
						G → T	51(CDR2)	0
							68(FR3)	0
						C → A	95(CDR3)	0
						C → G	56(CDR2)	0
							95(CDR3)	1
						T → A	92(CDR3)	0
						T → G	95(CDR3)	9

Only V_κ genes different from their corresponding germ-line genes are considered in this analysis. Transition refers to nucleotide changes involving purine to purine, or pyrimidine to pyrimidine changes. Transversion refers to nucleotide changes involving purine to pyrimidine and pyrimidine to purine changes. F, Number of other germ-line, or rearranged genes with identical nucleotide changes at the same position.

possible that reciprocal recombination may somatically diversify rearranged genes between maternal and paternal genes and result in changes not present in the original inherited germ-line genes [42]. In contrast, sequence analysis of the IgG RF-producing hybridoma, B4D8 (and to a lesser extent the IgM RF, A2A2) had nucleotide changes probably resulting from antigen-induced somatic point mutations.

The synovial-expressed genes were similar to genes frequently expressed in other responses, but only three of the hybridomas had identifiable specificity. Two of the three V_κI genes (A2A2 and A1B4) were homologous with the O12-O2 gene, which is frequently expressed in responses to both self and exogenous antigens and in B cells from patients with CLL [30,41,43]. However, only A2A2 reacted with IgG. The third V_κI-expressing hybridoma, A3D5, was 99·7% homologous to the O8 germ-line gene, which is frequently expressed in responses to DNA, thyroglobulin and in B cells from patients with CLL [29,30,43], but the antibody did not react with any antigen.

Hybridoma A3B5 expressed a V_κII gene which aligned with 99·7% homology to the V_κII germ-line gene A2. This gene is commonly used in antibodies to a *H. influenzae* capsular polysaccharide antigen (anti-Hib-PS antibodies) in association with V_H3 genes [33]. Although A3B5 is also associated with a V_H3 heavy chain (98·8% homologous with the DP-31 germ-line gene; [34]), it did not react with any of the examined antigens (Hib-PS antigen not tested) and has no defined specificity.

The expressed V_κ genes in three hybridomas (A3B6, A4A2 and B4D8) were from the V_κIII family. A3B6 and B4D8 aligned with 99·3% and 90·9% homology to the Humkv325 gene, respectively. The Humkv325 gene segment is frequently expressed in germ-line, or near germ-line, configuration in IgM_κ paraproteins with various autoantigen specificities [35,36]. The gene is also overexpressed in synovial B lymphocytes and RF-producing B cells in RA patients, but with evidence of mutation and charged amino acids in the CDR3 regions [22,32]. A3B6, however, had no detectable autoreactivity, but the IgG produced by the B4D8 hybridoma was a RF. The expressed V_κ gene in B4D8 had 25 nucleotide changes compared with the Humkv325 gene, of which 13 out of 15 changes in the CDR1 and 2, and 3 were replacement changes consistent with antigen-induced nucleotide mutations [39,40].

The V_κIII gene expressed in A4A2 was 100% homologous to the Vg germ-line gene. The hybridoma produced a polyreactive IgM that reacted with cytoskeletal filaments, cardiolipin, keratin, myosin, vimentin, tetanus toxoid, and IgG Fc. The Vg gene has also been shown to be expressed in RF-producing B lymphocytes in RA patients, polyreactive natural antibodies and in fetal liver

cDNA [22,31]. The rearranged V_κ gene of A4A2 was associated with the $J_\kappa 4$ gene and had six extra nucleotides at the V_κ–J_κ junction. These extra nucleotides may be the result of *de novo* N-additions or an unusual rearrangement resulting from the use of nucleotides from the non-coding germ-line 3′ end of the V_κ gene, 5′ end of the J_κ and P-additions [38]. Earlier studies of the incidence of N-addition had shown that the process is associated with the activity of the TdT enzyme, which was thought to be active only in pre-B cells before the production of functional heavy chains [44]. In human V_κ genes, N-additions were mainly seen in abortive V_κ–J_κ rearrangements or in translocations involving the κ light locus, suggesting that TdT activity may occur in certain B cells at the stage of development when the rearranging machinery proceeds to the κ locus [45]. However, recent studies of the V_κ CDR3 have revealed non-templated nucleotide additions consistent with TdT activity in normal B cells [31,32].

The lack of antigen binding by the hybridomas may relate to low-affinity interactions or the association of inappropriate heavy and light chains. The question, however, remains, how these cells have been selected for expansion despite the lack of any apparent antigen-driven selection. Currently, there is no definitive answer to this question. It is, however, possible that the expansion of these cells is due to the cytokine milieu of the synovium in RA patients, or the ability of synoviocytes to provide efficient cellular support for the outgrowth of B cells [46]. Alternatively, it is possible that limitations inherent in hybridoma technology may have skewed the selection process in favour of low-affinity surface immunoglobulin-expressing B cells. The lack of specificity in these B lymphocyte hybridomas, however, can not be explained by redundancy within synovial B cells that have undergone 'inappropriate' mutation as a result of antigen-induced expansion, since no, or very little, evidence for mutations was seen in the heavy or light chains of most of the non-antigen-reactive IgM- and IgG-producing hybridomas [34].

In conclusion, the results reported here provide evidence for the lack of mutation in non-RF-producing synovial B cells and the involvement of both polyclonal activation and antigen-driven selection of RA synovial B cells sampled through the generation of hybridomas reported in this study. These observations support our previous studies of V_H gene expression within IgM- and IgG-producing synovial hybridomas and studies of synovial RF by other investigators [3,4,34]. The current data disagree with a previous investigation in which a cDNA library established from the synovium of an RA patient was used [20]. This latter study concluded that synovial V_κ transcripts exhibited extensive evidence for somatic point mutations. The underlying causes for this disagreement are not clear, but may relate to the stage of activation of the selected B lymphocyte for hybridoma production compared with cDNA, which would be dominated by mRNA from plasma cells. Our study also highlights the question of how random B lymphocytes are expanded and maintained without the presence of antigen. It is crucial for the understanding of disease pathogenesis in RA to identify whether this outcome represents abnormal B cell survival, or is due to the unique nature of the synovial milieu.

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REFERENCES

1 Harris ED Jr. Rheumatoid arthritis: pathophysiology and implications for therapy. N Engl J Med 1990; **322**:1277–89.
2 Steinberg AD, Krieg AM, Gourley MF, Klinman DM. Theoretical and experimental approaches to generalised autoimmunity. Immunol Rev 1990; **118**:129–63.
3 Olee T, Lu EW, Huang DF, Soto-Gil RW, Deftos M, Kozin F, Carson DA, Chen PP. Genetic analysis of self-associating immunoglobulin G rheumatoid factors from two rheumatoid synovia implicates an antigen-driven response. J Exp Med 1992; **175**:831–42.
4 Randen I, Pascual V, Victor K, Thompson KM, Førre , Capra JD, Natvig JB. Synovial IgG rheumatoid factors show evidence of an antigen-driven immune response and a shift in the V gene repertoire compared to IgM rheumatoid factors. Eur J Immunol 1993; **23**:1220–5.
5 Tarkowski A, Klareskog L, Carlsten H, Herberts P, Koopman WJ. Secretion of antibodies to type I and II collagen by synovial tissue cells in patients with rheumatoid arthritis. Arthritis Rheum 1989; **32**:1087–92.
6 Plater-Zyberk C, Maini, RN. Phenotypic and functional features of $CD5^+$ B lymphocytes in rheumatoid arthritis. Scand J Rheumatol Suppl 1988; **75**:76–83.
7 Burastero SE, Casali P, Wilder RL, Notkins AL. Monoreactive high affinity and polyreactive low affinity rheumatoid factors are produced by $CD5^+$ B-cells from patients with rheumatoid arthritis. J Exp Med 1988; **168**:1979–92.
8 Berman JE, Alt FW. Human heavy chain variable region gene diversity, organisation and expression. Intern Rev Immunol 1990; **5**:203–14.
9 Tonegawa S. Somatic generation of antibody diversity. Nature 1983; **302**:575–81.
10 Berek C, Ziegner M. The maturation of the immune response. Immunol Today 1993; **14**:400–4.
11 Straubinger B, Huber E, Lorenz W *et al.* The human V_κ locus. Characterisation of a duplicated region encoding 28 different immunoglobulin genes. J Mol Biol 1988; **199**:23–34.
12 Huber C, Schäble KF, Huber E, Klein R, Meindl A, Thiebe R, Lamm R, Zachau HG. The V kappa genes of the L regions and the repertoire of V kappa gene sequences in the human germ line. Eur J Immunol 1993; **23**:2868–75.
13 Meindl A, Klobeck HG, Ohnheiser R, Zachau, HG. The V kappa gene repertoire in the human germ line. Eur J Immunol 1990; **20**:1855–63.
14 Klobeck HG, Bornkamm GW, Combriato G, Mocikat R, Pohlenz HD, Zachau, HG. Subgroup IV of human immunoglobulin κ light chains is encoded by a single germline gene. Nucleic Acids Res 1985; **13**:6515–29.
15 Zouali M, Theze J. Probing V_H gene-family utilisation in human peripheral B-cells by *in situ* hybridisation. J Immunol 1991; **146**: 2855–64.
16 Cuisiner A-M, Guigou V, Boubli L, Fougereau M, Tonelle C, Moinier D. Preferential expression of V_H immunoglobulin genes in early human B-cell ontogeny. Scand J Immunol 1989; **30**:493–8.
17 Stewart AK, Huang C, Stollar BD, Schwartz RS. High-frequency representation of a single V_H gene in the expressed human B cell repertoire. J Exp Med 1993; **177**:409–18.
18 Kipps TJ, Robbins BA, Carson DA. Uniform high frequency expression of autoantibody-associated cross-reactive idiotypes in the primary B cell follicles of human foetal spleen. J Exp Med 1990; **171**:189–96.
19 Kipps TJ, Fong S, Tomhave E, Chen PP, Goldfien RD, Carson DA. High frequency expression of a conserved kappa light chain variable region gene in chronic lymphocytic leukaemia. Proc Natl Acad Sci USA 1987; **84**:2916–20.
20 Lee SK, Bridges L, Koopman WJ, Scroeder HW Jr. The immunoglobulin kappa light chain repertoire expressed in the synovium of patients with rheumatoid arthritis. Arthritis Rheum 1992; **35**:905–13.
21 Thompson KM, Randen I, Natvig JB, Mageed RA, Jefferis R, Carson DA, Tighe H, Førre Ø. Human monoclonal rheumatoid factors derived from the polyclonal repertoire of rheumatoid synovial tissue: incidence

of cross-reactive idiotopes and expression of V_H and V_κ subgroups. Eur J Immunol 1990; **20**:863–8.
22 Blaison G, Kuntz J-L, Pasquali J-L. Molecular analysis of V_κIII variable regions of polyclonal rheumatoid factors during rheumatoid arthritis. Eur J Immunol 1991; **21**:1221–7.
23 Brown CM, Plater-Zyberk C, Mageed RA, Jefferis R, Maini RN. Analysis of immunoglobulins secreted by hybridomas derived from rheumatoid synovia. Clin Exp Immunol 1990; **80**:366–72.
24 Hazenberg MP, de Visser H, Bras MJ, Prins ME, van de Merwe JP. Serum antibodies to peptidoglycan–polysaccharide complexes from the anaerobic intestinal flora in patients with Crohn's disease. Digestion 1990; **47**:172–80.
25 Chemczynski O, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochem 1987; **162**:156–9.
26 Jefferis R, Nik Jaafar MI, Steinitz M. Immunogenic and antigenic epitopes of immunoglobulins VIII. A human monoclonal rheumatoid factor having specificity for a discontinuous epitope determined by histidine/arginine interchange at residue 435 of immunoglobulin G. Immunol Letters 1984; **7**:191–4.
27 Solomon A. Light chains of immunoglobulins: structural-genetic correlates. Blood 1986; **68**:603–10.
28 Kabat EA, Wu TT, Perry HM, Gottesman KS, Foeller C. Sequences of proteins of immunological interest, 5th edn. Bethesda: US Department of Health and Human Services, 1991.
29 Manheimer-Lory A, Katz JB, Pillinger M, Ghossein C, Smith A, Diamond B. Molecular characteristics of antibodies bearing an anti-DNA associated idiotype. J Exp Med 1991; **174**:1639–53.
30 Hexham JM, Furmaniak J, Pegg C, Burton DR, Rees-Smith B. Cloning of human autoimmune response: preparation and sequencing of human anti-thyroglobulin autoantibody using a combinatorial approach. Autoimmunity 1992; **12**:135–41.
31 Weber J-C, Blaison G, Martin T, Knapp A-M, Pasquali J-L. Evidence that the V_κIII gene usage is nonstochastic in both adult and newborn peripheral B cells and that peripheral $CD5^+$ adult B cells are oligoclonal. J Clin Invest 1994; **93**:2093–105.
32 Bridges SL, Lee SK, Johnson ML, Lavelle J, Fowler P, Koopman WJ, Schroeder HW Jr. Somatic mutation and CDR3 lengths of immunoglobulin κ light chains expressed in patients with rheumatoid arthritis and normal individuals. J Clin Invest 1995; **96**:831–41.
33 Scott MG, Crimmins DL, McCourt DW, Zocher I, Thiebe R, Zachau HG, Nahm MH. Clonal characterisation of the human IgG antibody repertoire to *Haemophilus influenzae* type b polysaccharide. III. A single V_κII gene and one of several J_κ genes are joined by an invariant arginine to form the most common L chain V region. J Immunol 1989; **143**:4110–6.
34 Brown CM, Fitzgerald KJ, Moyes SP, Mageed RA, Williams DG, Maini RN. Sequence analysis of immunoglobulin heavy-chain variable region genes from the synovium of a rheumatoid arthritis patient shows little evidence of mutation but diverse CDR3. Immunol 1995; **84**:367–74.
35 Pons-Estel P, Goni F, Solomon A, Frangione B. Sequence similarities among kappa IIIb chains of monoclonal human IgM kappa autoantibodies. J Exp Med 1984; **160**:893–904.
36 Mageed RA, Walker MR, Jefferis R. Restricted light chain subgroup expression on human rheumatoid factor paraproteins determined by monoclonal antibodies. Immunol 1986; **59**:473–8.
37 Martin T, Blaison G, Levallois H, Pasquali J-L. Molecular analysis of the V_κIII–J_κ junctional diversity of polyclonal rheumatoid factors during rheumatoid arthritis frequently reveals N addition. Eur J Immunol 1992; **22**:1773–9.
38 Lafaille JL, DeCloux A, Bonneville M, Takagaki Y, Tonegawa S. Junctional sequences of T cell receptor $\gamma\delta$ genes: implications for $\gamma\delta$ T-cell lineages and for a novel intermediate of V-(D)-J joining. Cell 1989; **59**:859–70.
39 Betz AG, Neuberger MS, Milstein C. Discriminating intrinsic and antigen-selected mutational hotspots in immunoglobulin V genes. Immunol Today 1993; **14**:405–11.
40 Shlomchik MJ, Marshak-Rothstein A, Wolfowicz CB, Rothstein TL, Weigert MG. The role of clonal selection and somatic mutation in autoimmunity. Nature 1987; **328**:805–11.
41 Klein R, Jaenichen R, Zachau HG. Expressed human immunoglobulin κ genes and their hypermutation. Eur J Immunol 1993; **23**:3248–71.
42 Umar A, Gearhart PJ. Reciprocal homologous recombination in or near antibody VDJ genes. Eur J Immunol 1995; **25**:2392–400.
43 Wagner SD, Luzzatto L. V_κ gene segments rearranged in chronic lymphocytic leukaemia are distributed over a large portion of the V_κ locus and do not show somatic mutation. Eur J Immunol 1993; **23**:391–7.
44 Desiderio SV, Yancopoulos GD, Paskind M, Thomas E, Boss MA, Landau N, Alt FW, Baltimore D. Insertion of N regions into heavy-chain genes is correlated with expression of terminal deoxytransferase in B cells. Nature 1984; **311**:752–5.
45 Klobeck HG, Combriato G, Zachau HG. N segment insertion and region-directed somatic hypermutation in a kappa gene of a t(2;8) chromosomal translocation. Nucleic Acids Res 1987; **15**:4877–88.
46 Dechanet J, Merville P, Durand I, Banchereau J, Miossec P. The ability of synoviocytes to support terminal differentiation of activated B-cells may explain plasma cell accumulation in rheumatoid synovium. J Clin Invest 1995; **95**:456–63.