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# Rheumatoid factors in primary Sjögren's syndrome (pSS) use diverse $V_H$ region genes, the majority of which show no evidence of somatic hypermutation

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## SUMMARY

Rheumatoid factor (RF) is the most common autoantibody found in patients with Sjögren's syndrome (SS). To study the genetic origin and the mechanisms acting behind its generation we have characterized and sequenced the immunoglobulin $V_H$ genes used by 10 IgM RF MoAbs derived from peripheral blood of six female patients with pSS. We compared the structure of the RF immunoglobulin $V_H$ genes with those obtained previously from rheumatoid arthritis (RA) patients and healthy immunized donors (HID). $V_H1$ and $V_H4$ were each used by four RF clones, one clone was encoded by $V_H3$ family gene and one by $V_H2$ family gene. This distribution frequency was different from that observed in RA, where $V_H3$ was the dominant family, followed by $V_H1$. Eight different germ-line (GL) genes encoded the clones and all of these genes were seen previously in RA and/or HID RF. Five clones rearranged to $J_H6$, four rearranged to $J_H4$ and one to $J_H5$, in contrast to RF from RA and HID, where $J_H4$ was most frequently used. D segment use and CDR3 structure were diverse. Interestingly, three out of four $V_H4$ clones used the GL gene DP-79 that was seen frequently in RA RF. The degree of somatic mutation in the pSS RF was very much lower than seen in RA and HID RF. All the pSS RF clones except three were in or very close to GL configuration. This indicates that there is little role for somatic hypermutation and a germinal centre reaction in the generation of RF from peripheral blood in pSS.

**Keywords** Sjögren's syndrome rheumatoid factor heavy chain variable region gene

## INTRODUCTION

Sjögren's syndrome (SS) is an autoimmune disorder characterized by chronic inflammation of the exocrine glands, mainly the salivary and lachrymal glands leading to progressive destruction of these glands resulting in dryness of the mouth and conjunctiva (sicca syndrome) [1–3]. The disease can occur alone as pSS or it can be associated with other connective tissue diseases such as systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA), in which case it is referred to as secondary Sjögren's syndrome.

The main immunological feature of SS is mononuclear cell infiltration of the exocrine glands. Most of these cells are T cells (75–85%) and B cells (5–10%) [1,3]. A B cell activation results in hypergammaglobulinemia with elevated levels of circulating immune complexes and production of autoantibodies. Among the autoantibodies seen are rheumatoid factors (RF), anti-Ro/SSA and anti-La/SSB [1–4]. RF is found in the serum and saliva of 60–80% of pSS patients [5,6]. RF is also found in other autoimmune conditions such as RA in about 70% of patients [7–9], SLE as well as healthy donors following certain infections and immunization [10–12], and finally as a monoclonal component (MC) in lymphoproliferative diseases.

The structure and the genetic origins of RF are well characterized in RA and healthy immunized donors (HID) [12–26]. IgM RF from RA are polyclonal and highly mutated with relatively high affinity. This suggests that they have been through a germinal centre reaction with affinity maturation. However, evidence from HID suggests that high-affinity RF B cells are eliminated by the mechanism of peripheral tolerance [18]. The aims of this study were to determine the genetic origin and the structure of RF from pSS patients. This might help to understand the mechanism behind generation of RF in pSS and to determine whether they show

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evidence of being antigen-driven. For this purpose we derived 10 RF MoAbs from the peripheral blood of pSS patients using Epstein–Barr virus (EBV)/hybridoma technology. These MoAbs have diverse V gene usage. Most of the germ-line (GL) genes used were already identified in RF from RA but were used in different frequencies. Interestingly, seven out of 10 clones have very few somatic mutations, indicating that they have not been activated through a germinal centre reaction. Three other clones have moderate to high number of mutations, indicating a germinal centre process.

## PATIENTS AND METHODS

*Lymphocyte isolation and EBV transformation*

Peripheral blood samples were obtained from six female patients with pSS aged between 36 and 77 years, fulfilling at least four of the preliminary European Classification Criteria for pSS [27]. They were either $RF^+$ by Waaler agglutination test and/or latex test measured by standard nephelometry assay [28]. Mononuclear cells were isolated by high-density gradient centrifugation using Lymphoprep (Nycomed, Oslo, Norway). The cells were incubated with 5 ml of supernatant from the B958 cell line containing EBV for 1 h at 37°C with 5% $CO_2$. After 1 h, Dulbecco's modified Eagle's medium (DMEM; Sigma, St Louis, MO) + Hams F-12 + 10% fetal calf serum (FCS) was added to keep the number of cells at a concentration of $10^6$ cells/ml, and the cells were incubated at 37°C and 5% $CO_2$. Phytohaemagglutinin (PHA; Sigma) was added after 24 h at a concentration of 10 $\mu$g/ml.

*Generation of antibody-secreting hybridomas*

After 2 weeks the EBV-transformed B lymphocytes were fused with a mouse plasmacytoma cell line according to the method described before [29]. In brief, the fusions were performed with transformed B lymphocytes and the mouse plasmacytoma OURI in the ratio 1:1 in the presence of polyethylene glycol 45% (Sigma) in PBS. The fusion products were suspended in a DMEM culture medium + 10% FCS supplemented with hypoxanthine/aminopterin/thymidine (HAT; Sigma) at dilution of 1:50 and Ouabain at 1 $\mu$M.

*ELISA and cloning*

The supernatants from cell lines obtained from the fusions were screened after 2 weeks by ELISA for IgM and IgA RF activities. Briefly, 96-well ELISA plates (Maxisorp; Nunc-immuno plate, Roskilde, Denmark) were coated with human IgG ($\gamma$-kabi; Pharmacia, Uppsala, Sweden) 10 $\mu$g/ml in PBS. Plates were blocked with 1% bovine serum albumin (BSA). Following washing, supernatants were added and the plates were incubated at 37°C for 1–2 h. The plates were washed again and goat anti-human IgM ($\mu$-chain specific) or goat anti-human IgA ($\alpha$-chain specific) alkaline-phosphatase conjugate (Sigma) were added and incubated at 37°C for 2 h. Finally, the plates were washed as before, and Sigma 104 phosphatase substrate (1 mg/ml in diethanolamine buffer pH 9·8) was added and the absorbance was measured after 20 min at 405 nm. Uncoated trays blocked with BSA were used to eliminate antibodies giving unspecific binding. RF-secreting cell lines were cloned repeatedly by limiting dilution as described before [17].

Positive RF clones were tested in ELISA against Fc fragments of human IgG, rabbit IgG (10 $\mu$g/ml each; Sigma), Fab and $F(ab')_2$ of human IgG (5 $\mu$g/ml each; Calbiochem-Novabiochem, La Jolla, CA), tetanus toxoid (5 $\mu$g/ml; NBCI, Cambridge, UK), single-stranded DNA (ssDNA) (50 $\mu$g/ml; gift from Professor O. Mellbye, University of Oslo, Norway), and human IgG subclasses (10 $\mu$g/ml; a gift from Professor T. Michalsen, University of Oslo).

*Preparation of mRNA, cDNA and sequencing*

Total mRNA was extracted as described previously [12,30] and reverse transcribed to cDNA as described before [12,18]. $V_H$ region genes were amplified by polymerase chain reaction (PCR) using specific $V_H$ leader primers and c$\mu$ constant region primer as before [19,20], except for clones RF SN2, RF SN4 and RF VR1 (HY4) leader primer was used (5′ CTGGTGGCAGCTCCCA-GATG 3′) and for RF EF2 (HY1) leader primer was used (5′ GCTCCAGGTGCCCACTCCCA 3′) and for RF SN1 (HM3R) (5′ TCGGACCGTATCCGACGGG GAATTCTCACA 3′) constant region primer was used. The PCR products were gel purified and directly sequenced in both directions [14] using the same primers as for PCR. The closest GL genes were identified by search in the V BASE and DNAPLOT sequence directory (I. Tomlinson, H. H. Althaus *et al*., MRC Centre for Protein Engineering, Cambridge, UK, and University of Cologne, Germany).

*Statistical analysis*

Mann–Whitney *U*-test was used to analyse the differences between the mean numbers of mutations of the three groups of

**Table 1.** Specificities of rheumatoid factor (RF) monoclonal autoantibodies from patients with pSS

| Clone | Human IgG Fc | Rabbit IgG | Fab | $F(ab')_2$ | IgG1 | IgG2 | IgG3 | IgG4 | Specificity |
|---|---|---|---|---|---|---|---|---|---|
| RF MA1 | + | + | − | − | + | + | − | + | Ga |
| RF UL1 | + | + | − | − | + | + | − | + | Ga |
| RF VR1 | + | + | − | − | + | + | − | + | Ga |
| RF LM1 | + | − | − | − | + | + | + | + | Pan |
| RF EF1 | + | − | − | − | + | + | + | + | Pan |
| RF EF2 | + | + | − | − | + | + | + | + | Pan |
| RF SN1 | + | − | − | − | + | + | + | + | Pan |
| RF SN2 | + | − | − | − | + | + | − | + | Ga |
| RF SN4 | + | − | − | − | + | − | − | − | IgG1 |
| RF SN5 | + | + | − | − | + | + | + | + | Pan |

None of the clones showed any reactivity with tetanus toxoid or ssDNA.

RF (pSS, RA, and HID). Fisher's exact test was used to analyse the differences between R:S ratios in the CDRs for the whole panels of RF from pSS, RA and HID. Differences were considered significant at two-sided $P$ values $< 0{\cdot}05$.

## RESULTS

*Specificity of monoclonal RF autoantibodies*

We have derived 10 IgM RF clones monospecific for the Fc region of human IgG from six different patients with pSS (Table 1). Five of the clones (50%) were also found to be reactive with rabbit IgG (RF MA1, RF UL1, RF VR1, RF EF2, RF SN5). Five clones (RF EF1, RF EF2, RF SN1, RF SN5, RF LM1) showed pan specificity (reactive to all human IgG subclasses), while four clones (RF MA1, RF UL1, RF SN2, RF VR1) showed the classical Ga specificity (reactive with IgG1, IgG2 and IgG4 but not IgG3). One clone (RF SN4) was found only reactive with IgG1. None of the 10 clones was reactive with Fab, $F(ab')_2$, tetanus toxoid, or ssDNA.

*V gene sequence analysis*

The sequences of the heavy chains (Table 2 and Fig. 1) revealed that four of the clones (40%) were encoded by $V_H4$ family genes (RF SN2, RF SN4, RF VR1, RF EF1), four (40%) used $V_H1$ family genes (RF MA1, RF UL1, RF EF1, RF SN1), one used a $V_H3$ gene (RF LM1) and one clone used a $V_H2$ gene (RF SN5). All of the $V_H$ genes used have been described before in RF from either RA patients or HID [21–23]. Of the $V_H1$ family clones, one of them (RF MA1) was closest to DP-10 GL gene, RF UL1 was highly homologous to DP-88 GL gene, RF EF2 used DP-14 GL and RF SN2 used DP-7. Interestingly, three of the four $V_H4$ clones were closest to the GL gene DP-79 and all three rearranged to the $J_H4b$ segment. Two of these were from one patient (RF SN2 and RF SN4) and one from a different patient (RF VR1). The fourth $V_H4$ clone (RF EF1) used DP-65 GL gene. The $V_H3$ clone (RF LM1) had closest homology to the DP-46 GL gene and the $V_H2$ clone was closest to the DP-26 GL gene. All of the clones were rearranged to different D-segments (Fig. 2) except two pairs. RF SN4 and RF EF1 used the D3–22 segment in two different reading frames and RF SN2, RF SN5 used the D1–26 segment also in two different reading frames. Analysis of the J segments showed that $J_H6$ was preferentially used, four clones rearranged to the $J_H6b$ segment (RF UL1, RF EF2, RF SN1, RF LM1), while the fifth clone (RF MA1) rearranged to the $J_H6c$ segment (RF MA1). Four clones rearranged to $J_H4b$ (RF SN5, RF SN2, RF SN4, RF VR1), and only one clone used the $J_H5b$ segment (RF EF1).

*Analysis of the mutational patterns*

In contrast to what has been found in RF from RA and HID, most of the present clones were found to be highly homologous to their closest GL gene. Surprisingly, four of the clones were found to be unmutated copies of their closest GL genes at the nucleotide level. Three of them used $V_H4$ family genes (RF VR1, RF SN2 and RF EF1), while the fourth used a $V_H1$ family gene (RF SG1) (Table 3). Two other clones had only one silent mutation (RF EF2 and RF SN5). Thus six clones were 100% homologous to the GL product at the amino acid level. One clone had three mutations (RF UL1); two replacement mutations in the $CDR_H1$ and one silent mutation in framework two. Only three clones were moderately to highly mutated comparable to that seen in RF of RA and HID (RF MA1, RF SN4, RF LM1 had six, 11, and 14 mutations per $V_H$ gene, respectively). All three had from two to five replacement mutations in the CDR regions. The mean number of mutations was $3{\cdot}6$ per $V_H$ gene. This is statistically significantly lower than the mean numbers for RA RF (mean 11 and $P = 0{\cdot}0028$, Mann–Whitney $U$-test) and HID RF (mean 10 and $P = 0{\cdot}0051$) [11,22]. The R:S ratio for $CDR_H1 + 2$ for RF in pSS was $2{\cdot}5$ (Table 3), which is not statistically different from the ratios found in RA or HID RF.

**Table 2.** Monoclonal IgM rheumatoid factor (RF) from pSS patients are shown with family gene, the closest identified $V_H$, D and $J_H$ germ-line (GL) gene segment, and the length (L) of $CDR_H3$ amino acid sequence

| Clone | $V_H$ | $V_H$GL | D | $J_H$ | L $CDR3_H$ |
|---|---|---|---|---|---|
| RF MA1 | 1 | 1–69 (DP-10) | D21–9 | $J_H6c$ | 11 |
| RF UL1 | 1 | 1–e (DP-88) | DN4 | $J_H6b$ | 13 |
| RF EF2 | 1 | 1–18 (DP-14) | DN1 | $J_H6b$ | 19 |
| RF SN1 | 1 | 1–46 (DP-7) | D4–17 | $J_H6b$ | 13 |
| RF SN5 | 2 | 2–26 (DP-26) | D1–26 | $J_H4b$ | 13 |
| RF LM1 | 3 | 3–30·3 (DP-46) | D4-b | $J_H6b$ | 21 |
| RF SN2 | 4 | 4–39 (DP-79) | D1–26 | $J_H4b$ | 16 |
| RF SN4 | 4 | 4–39 (DP-79) | D3–22 | $J_H4b$ | 13 |
| RF VR1 | 4 | 4–39 (DP-79) | D6–19 | $J_H4b$ | 11 |
| RF EF1 | 4 | 4–31 (DP-65) | D3–22 | $J_H5b$ | 10 |

## DISCUSSION

RF are autoantibodies against antigenic determinants on the Fc region of human IgG, but many RF are also reactive to IgG of other species, e.g. rabbit IgG [4,17]. Our pSS RF satisfy these criteria. In addition, the majority showed the pan and Ga classical specificity similar to RA RF [17,31].

In humans, $V_H3$ family genes are the most frequent family used by antibodies (50–60%) [32–34]. This is also true for RF from RA, where $V_H3$ has been found to be the most frequent family (60–80%) followed by $V_H1$ and $V_H4$ [35–37]. However, if RF only derived from blood of RA are considered, $V_H1$ is the predominant family (50%), followed by $V_H4$ (25%) and $V_H3$ (25%) [38–40]. This is similar to RF derived from the peripheral blood of HID ($53{\cdot}4\%$ used $V_H1$, $37{\cdot}9\%$ used $V_H3$, and $5{\cdot}2\%$ used $V_H4$) [12,14]. In pSS RF only one clone used $V_H3$, whereas $V_H1$ and $V_H4$ were equally represented (40% each). This looks closer to RF derived from the blood of both RA patients and normals, but clearly different from the distribution found in the synovial tissues of RA patients. This finding is supported by idiotype studies done previously, where elevated levels of $V_H1$-associated cross-reactive idiotype in the sera from patients with pSS were found [36,41,42]. In addition, our panel also showed a high degree of heterogeneity with eight different GL genes encoding 10 RF clones. This is comparable to RF from RA and HID, which also showed a large degree of heterogeneity. All GL genes utilized by pSS RF were seen previously in RA and/or HID RF. Two GL genes each, used once by our clones ($V_H1$ DP-10 and $V_H2$ DP-26) were seen before in both RA and HID RF; DP-10 was used by several clones from HID, RA, and MC RF [33,43], while DP-26 was used before by two clones from HID and one RF clone from RA synovium [12].

The most frequent GL used by our clones was DP-79 (30%) and this was found to be used by several RF clones from both synovium and peripheral blood of RA but not of HID [22,23].

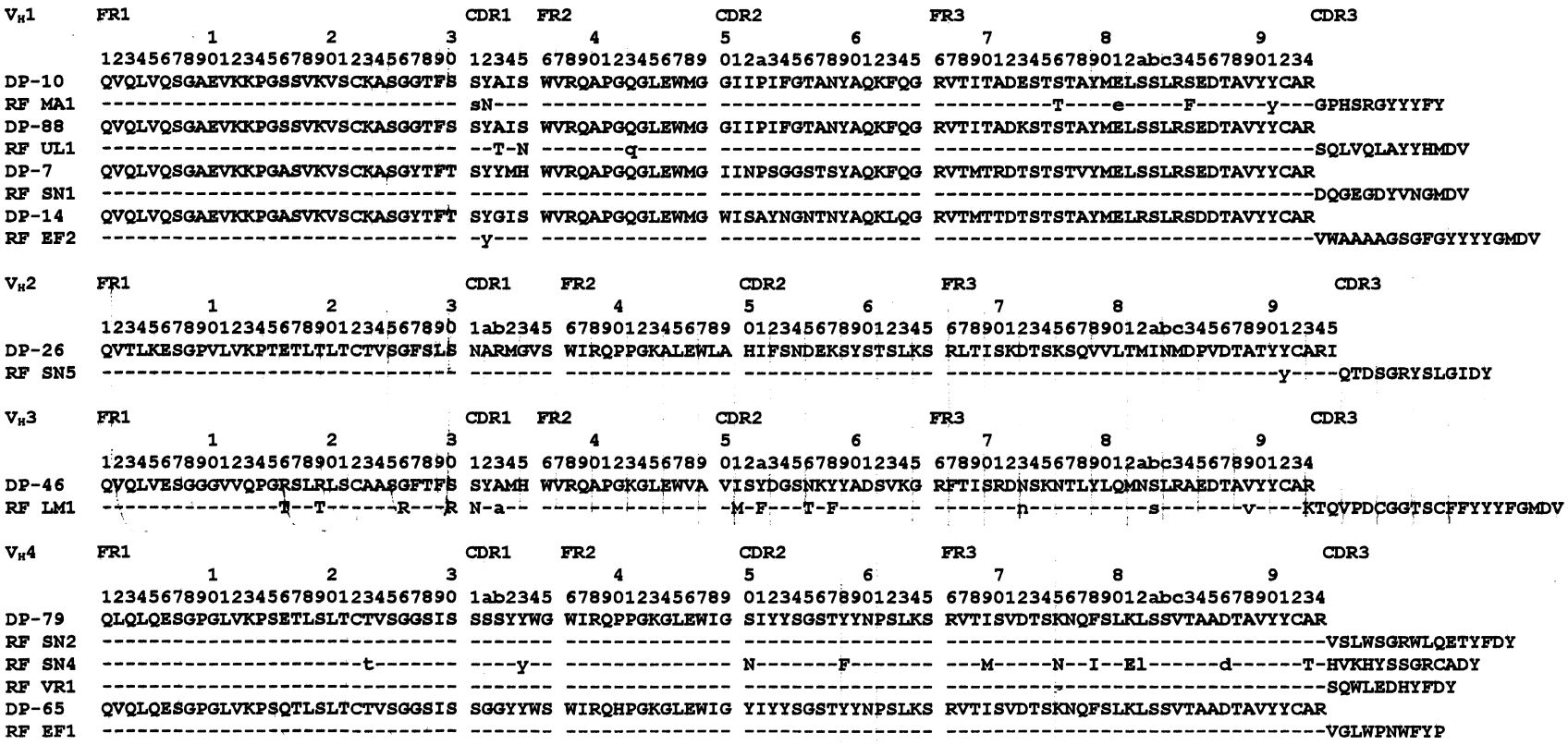


**Fig. 1.** Amino acid sequences of the $V_H$ gene region of IgM rheumatoid factor (RF) from pSS. Closest identified germ-line genes in the V BASE and DNAPLOT are shown. Dashes denote homology, uppercase letters denote replacement mutation, whereas lower case letters denote silent mutation. The codons are numbered according to the definition of Kabat [51].

 

```
D21-9       GTATTACTATGATAGTAGTGGTTACTACTAC
RF MA1      -AGAGG-CCC------C--TA---T---

DN4         GAGTATAGCAGCTCGTCC
RF UL1      CGAG—CC-----------

D4-17       TGACTACGGTGACTAC
RF SN1      G-GGG-A---------

DN1         GGGTATAGCAGCAGCTGGTAC
RF EF2      CT-GGC--------------G

D4-b        TGGATATTGTAGTAGTACCAGCTGCTATGCC
RF LM1      CCCCG-----G--G------------TCTT-

D1-26       GGTATAGTGGGAGCTACTAC
RF SN2      T-GC--CAA--C------TT
RF SN5      C-G---------------C-

D3-22       GTATTACTATGATAGTAGTGGTTATTACTAC
RF SN4      AC-----AG-AG-G--C--T--GC-G----T
RF EF1      ---GG-T----GCCAA-C-----C-AC-CCT

D6-19       GGGTATAGCAGTGGCTGGTAC
RF VR1      C-AGG-C-----------AGG
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**Fig. 2.** Nucleotide sequences of D segment of pSS rheumatoid factors (RF) compared with the closet germ-line (GL) D segment. Upper case denotes mismatches with the GL.

Interestingly, all our DP-79 clones rearranged to the $J_H4$ segment which was similar to most DP-79 RF clones from RA which also used the $J_H4$ segment. This suggests that a combination of $V_H$ DP-79 and $J_H4$ might be important in RF specificity. The other GL genes used by our clones, and also seen before in RA RF, were DP-46, which were similarly used by two clones from RA synovium [22]. DP-14 and DP-7 were each used by one RF clone from RA synovium [22,23]. DP-88 and DP-65 GL genes were used by RF from HID and pSS but not from RA [12,22]. Although the frequency of $V_H$ family use was different, in general the GL gene usage of pSS RF seemed more similar to RA RF than HID RF.

**Table 3.** Total number of mutations, R:S ratios of $CDR_H1+2$ and $FR_H1+2+3$ of the $V_H$ segment of IgM rheumatoid factor (RF) of pSS in different $V_H$ families and the overall panel of IgM pSS RF

| | | CDR1+2 | | CDRs | FR1+2+3 | | FRs | |
|---|---|---|---|---|---|---|---|---|
| $V_H$ | Clone | R | S | R:S | R | S | R:S | Total |
| $V_H1$ | RF MA1 | 1 | 1 | 1 | 2 | 2 | 1 | 6 |
| | RF UL1 | 2 | 0 | | 0 | 1 | | 3 |
| | RF EF2 | 0 | 1 | | 0 | 0 | | 1 |
| | RF SN1 | 0 | 0 | | 0 | 0 | | 0 |
| | Total $V_H1$ | 3 | 2 | 1·5 | 2 | 3 | 0·67 | 10 |
| $V_H2$ | RF SN5 | 0 | 0 | | 0 | 1 | | 1 |
| $V_H3$ | RF LM1 | 5 | 1 | 5 | 5 | 3 | 1·67 | 14 |
| $V_H4$ | RF VR1 | 0 | 0 | | 0 | 0 | | 0 |
| | RF SN2 | 0 | 0 | | 0 | 0 | | 0 |
| | RF SN4 | 2 | 1 | 2 | 5 | 3 | 1·67 | 11 |
| | RF EF1 | 0 | 0 | | 0 | 0 | | 0 |
| | Total $V_H4$ | 2 | 1 | 2 | 5 | 3 | 1·67 | 11 |
| | Total | 10 | 4 | 2·5 | 12 | 10 | 1·2 | 36 |

A striking finding is the preferential use of the $J_H6$ segment (50%). Generally $J_H4$ is the most frequent J segment used (45–65%), followed by $J_H6$ (about 20–25%) [44,45], and this was also true for RA and HID where $J_H4$ is the most frequently used by RF [22]. This might be due to selective activation of B cells clones. D-segment use was diverse.

It has been postulated that the generation of autoantibodies can occur mainly through either an antigen-driven response [35], as in RA and HID RF, or through other mechanisms such as unspecific polyclonal or monoclonal activation. In the former mechanism the B cell undergoes a germinal centre reaction where the immunoglobulin V regions undergo a process of somatic hypermutation, affinity maturation and selection [35]. Clonal expansion and class switch have been seen in RF from RA. Following polyclonal and monoclonal activation the antibodies generated have low affinity and they are very close or directly encoded by the GL, as in MC RF.

The majority of pSS RF were 100% homologous to a GL gene product (six out of 10); to our knowledge only one RF clone out of 55 from RA synovium and only one out of 34 from HID were encoded directly by the GL [12,22,23,25]. Three of our pSS RF clones had a substantial number of mutations [11,29]. In the first one (RF LM1) there was evidence of targeting the replacement mutation in the CDR5 regions (R:S = 1 + 2); and this indicates that this RF had been selected during the germinal centre reaction. The second clone (RF SN4) had 11 mutations, but most of those mutations were in FR regions (eight mutations), while the third clone (RF MA1) had only one replacement and one silent mutation in $CDR_H1$ and the rest in $FR_H3$. This indicates that there was no pressure in the CDR region of these two clones but they had still undergone a germinal centre process.

Our data suggest that there is little or no role for somatic mutation in the generation of most RF in pSS. This has been suggested indirectly by previous studies with idiotype markers [34,36,37,41–43,46–48]. One explanation for this is that there may be intrinsic B cell defects resulting in failure to diversify the GL by somatic mutations [46]. The possible mechanism of generation of those antibodies is through unspecific polyclonal activation and expansion of B cells without an antigen-driven process and T cell help. This could be part of the immunological and pathological processes acting in SS, as indicated by the considerable number of SS patients who develop hypergammaglobulinaemia and lymphoma and monoclonal gammopathy due to monoclonal B cell activation and production of MC RF.

However, Stott *et al*. and Bahler & Swerdlow studied rearranged variable region genes without defined antibody specificities within the labial salivary glands from patients with SS and showed evidence of antigen-driven germinal centre response [49,50]. These findings suggest that our RF from peripheral blood of pSS might not be involved in the local pathology of the disease.

In conclusion, our data suggest that there is very little role for somatic mutation in the generation of RF from peripheral blood in pSS.

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