Immunology 1996 **88** 636–641

Crystallization of a complex between the Fab fragment of a human immunoglobulin M (IgM) rheumatoid factor (RF–AN) and the Fc fragment of human IgG4

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SUMMARY

Rheumatoid factors (RF) are the characteristic autoantibodies found in patients with rheumatoid arthritis. They recognize epitopes in the Fc region of immunoglobulin G (IgG) and are often of the IgM isotype. In order to analyse the nature of RF–Fc interactions, we have crystallized a complex between the Fab fragment of a human monoclonal IgM rheumatoid factor (RF–AN) and the Fc fragment of human IgG4. The stoichiometry of the complex within the crystals was found to be 2 : 1 Fab : Fc. The crystals diffracted X-rays to 0·3 nm resolution, and the space group was C2, with cell dimensions $a = 16{\cdot}03$ nm, $b = 8{\cdot}19$ nm, $c = 6{\cdot}42$ nm, $\beta = 98{\cdot}3°$. We have also determined the sequence of the variable region of the RF–AN light chain, not hitherto reported. This belongs to the $V_\lambda III$-a subgroup and is closely related to the germline gene *Humlv318*, from which it differs in three amino acid residues. This is the first reported crystallized complex between a human autoantibody and its autoantigen.

INTRODUCTION

Rheumatoid factors (RF) are the characteristic autoantibodies associated with rheumatoid arthritis (RA) which recognize epitopes in the Fc region of immunoglobulin G (IgG). While RFs have been studied in considerable detail with respect to specificity, idiotypy, sequence and *V* gene usage,[1–4] the details of their interaction with IgG Fc have not been elucidated at the level of three-dimensional structure, except by indirect means such as peptide inhibition.[5] The availability of human monoclonal antibodies derived from lymphocytes of patients with RA,[4,6–9] now makes it possible to analyse RF–IgG binding by X-ray crystallography in order to reveal the role of individual V region residues and the precise nature and location of the epitope recognized. This has not yet been achieved for any human autoantibody. Previously we described the crystallization of the Fab fragment of a monoclonal human RF, 2A2, in its uncomplexed form.[10] We now report the crystallization and initial X-ray analysis of a complex between the Fab fragment of another human RF (RF–AN) and the Fc fragment of human IgG4.

RF–AN is a monoclonal IgM,λ produced by an Epstein–Barr virus (EBV)-transformed B-cell line prepared from peripheral blood lymphocytes of a patient with classical RA.[6,11] Like 2A2, RF–AN recognizes the Fc region of the human IgG1, 2 and 4 subclasses, and IgG3 of the G3m(st) allotype, but not of the G3m(b) or G3m(g) allotype, i.e. it has the 'new Ga-related' or Ga2 specificity.[12,13] This specificity correlates with the presence of His435 in IgG reactive with RF–AN, and Arg435 in the non-reactive IgG. This specificity profile parallels that of *Staphylococcus aureus* Protein A (SpA) which has been shown to compete with RF–AN for binding to IgG (Mageed R. & Jefferis R., unpublished data). RF–AN also binds to rabbit IgG, but not that of mouse, rat, swine, goat or sheep.[11] Some mouse and rat IgG subclasses possess His435 and bind SpA, indicating that the presence of His435 is not sufficient for recognition by RF–AN and that other species-specific residues contribute to the epitope.

The sequence of the RF–AN V_H domain has been reported and is derived from a gene in the V_H3 family.[14] The V_L domain, for which only the N-terminal sequence has been previously determined, is a member of the $V_\lambda III$ subgroup.[15] We report here the complete V_L sequence.

The Fc fragment used in this study is derived from a human IgG4 paraprotein (Rea). Compositional analysis of the carbohydrate chains N-linked at Asn297 in each C_H2 domain revealed relative homogeneity with ~62% of sugar chains identical, and >70% lacking terminal galactose (G0-IgG).[16] Polyclonal IgG from rheumatoid sera has higher levels of G0-IgG than non-rheumatoid age-matched controls. Further,

Received 17 January 1996; revised 16 April 1996; accepted 22 April 1996.

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the level of G0-IgG has been related to disease activity and progression to erosive changes.[17–19] It has been suggested that G0-IgG may form pathogenic complexes with RF, particularly when the RF is IgG and can form self-associating complexes.[20]

MATERIALS AND METHODS

Preparation of RF–AN IgM and Fab

Procedures for culture of the RF–AN line in a hollow fibre bioreactor, purification of the IgM and preparation of the Fab fragment by trypsin digestion were as described for 2A2 IgM.[10] Unlike 2A2 Fab, the RF–AN Fab fragment gave a single peak on a mono-Q (Pharmacia) column and ran as a single band on sodium docecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) under non-reducing conditions, or as single Fd and light chain bands when reduced (Fig. 1, lane 2).

Deglycosylation of Fab was carried out with *N*-glycanase as described.[10] After removal of the predicted single *N*-glycan from the C_H1 domain, the Fab ran as a single band on SDS–PAGE under reducing conditions, due to superimposition of Fd and light chain (Fig. 1, lane 3).

Preparation of IgG4 Fc

Preparation of the Fc fragment of the IgG4 paraprotein (Rea) by papain digestion was as previously reported.[16]

Cloning and sequencing of V_L cDNA of RF–AN

Total RNA was purified from RF–AN cells by phenol extraction[21] and cDNA made using oligo(dT)$_8$ as primer for first strand synthesis. Polymerase chain reaction (PCR) was carried out using the forward primer 5′-CCC GCT CGA GTG TCA/T GTG GCC CCA GGA CAG-3′, designed on the basis of FR1 of the immunoglobulin $V_\lambda III$ subgroup,[22] and the back primer 5′-GCT CTA GAG GTC AGC TTG GTC CCT/C CCG CCG AA-3′, based on FR4 of the $V_\lambda III$ subgroup.[22] Restriction enzyme sites for XhoI and XbaI were incorporated into the primers for ligation and cloning. The PCR fragment was digested with XhoI and XbaI and ligated into the p-Bluescript plasmid. Clones were identified by enzyme mapping and sequenced. Eight colonies were sequenced.

Crystallization and analysis of crystals

Crystals were grown by hanging drop vapour diffusion on siliconized glass cover slips (Hampton Research, CA) sealed over wells in tissue culture plates each containing 0·5 ml of the reservoir solution. The hanging drops consisted of 2 μl of protein solution containing each protein at 1 mg/ml in 0·1% NaN_3, 20 mM Tris–HCl at pH 7·0, mixed with an equal volume of the reservoir solution to be screened. Crystals were obtained with reservoir solutions containing 17·5 to 22·5% (w/v) polyethylene glycol (mean molecular weight 8000 MW; Sigma, Poole, UK) in 0·1% NaN_3, 100 mM Tris–HCl, pH 7·0, at temperatures between 17·5 and 21·5°.

In order to establish the composition of the crystals, large single crystals were washed free of the protein solution with reservoir solution, and then dissolved in water and analysed by SDS–PAGE (12·5% acrylamide) under non-reducing conditions. The gels were stained with Coomassie Brilliant Blue R, and the tracks scanned with a Joyce–Loebl Chromoscan 3, for comparison with the protein preparations from which the crystals were grown, mixed in various proportions.

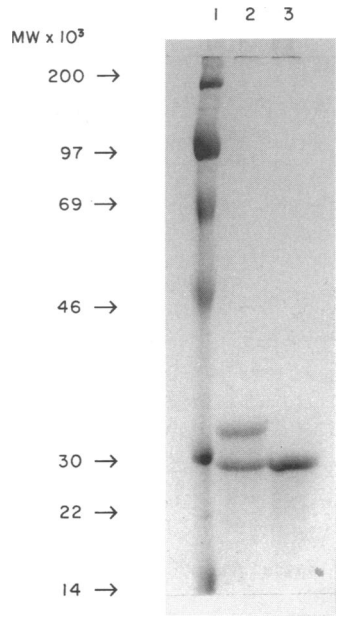

Figure 1. SDS–PAGE analysis (12·5%) under reducing conditions of RF–AN Fab before (lane 2) and after (lane 3) treatment with *N*-glycanase. (Lane 1, markers). After deglycosylation, the Fd (upper band in lane 2) and light chain bands are superimposed.

The crystals were mounted in quartz glass capillary tubes (Glas, W. Müller, Germany) for X-ray analysis. Diffraction patterns were recorded on film using a precession camera, and on an image plate detector (R-AXIS II, Molecular Structure Corporation, Houston, TX), mounted on a rotating anode generator (RU200HB, Rigaku, Japan) emitting CuKα X-rays (λ = 0·5418 nm).

RESULTS

V_H and V_L sequences of RF–AN

The V_H sequence has been reported and is derived from a gene in the V_H3 family.[14] The most closely related V_H germline gene appears to be *DP-31*[23] with 93% identity at the amino acid level (7 replacements over 97 amino acids).

N-terminal protein sequencing of the isolated RF–AN light chain had previously indicated that the V_L region corresponded to the $V_\lambda III$ subgroup.[15] This was confirmed by cloning and sequencing the PCR-amplified cDNA (Fig. 2). The translated N-terminal sequence (following the primer) is identical to that determined by protein sequencing of the first 25 residues. On the basis of amino acid sequence homologies, human $V_\lambda III$ light chains have been subdivided into sub-subgroups, *a*, *b* and *c*,[24] indicating the presence of at least three $V_\lambda III$ germline genes. RF–AN is a member of the $V_\lambda III$-a sub-subgroup. One functional $V_\lambda III$-a germline gene, *Humlv318*, has been reported to date,[25] to which RF–AN V_L is highly homologous; in the 240 bp region between the PCR primers, nucleotide sequence identity is 98·8%, while over the whole V_L segment identity at the amino acid level is 97% (Fig. 2). Altogether, five differences between *Humlv318* and the expressed RF–AN V_L segment can be identified: the N-terminal serine of the germline gene

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                 2                               10
                <-------------------------------------------------------------------
RFAN                                                TCA GTG GCC CCA GGA CAG ACG GCC
                 Y   V   L   T   Q   P   P   S   V   S   V   A   P   G   Q   T   A
             S   .   .   .   .   .   .   .   .   .   .   .   .   .   .   K   .   .
Humlv       TCC TAT GTG CTG ACT CAG CCA CCC TCG GTG TCA GTG GCC CCA GGA AAG ACG GCC
318

                    20
                ---------->     ***************** CDR1 ********************
RFAN            AGG ATT ACC TGT GGG GGA AAC AAC ATT GGA AGT AAA AGT GTG CAC TGG TAC
                 R   I   T   C   G   G   N   N   I   G   S   K   S   V   H   W   Y
                 .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .
Humlv318        AGG ATT ACC TGT GGG GGA AAC AAC ATT GGA AGT AAA AGT GTG CAC TGG TAC

                                40                                  ***********CDR2
RFAN            CAG CAG AAG CCA GGC CAG GCC CCT GTG TTG GTC GTC TAT GAT GAT AGC GAC
                 Q   Q   K   P   G   Q   A   P   V   L   V   V   Y   D   D   S   D
                 .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .
Humlv318        CAG CAG AAG CCA GGC CAG GCC CCT GTG CTG GTC GTC TAT GAT GAT AGC GAC

                ***********                 60
RFAN            CGC CCC CCA GGG ATC CCT GAG CGA TTC TCT GGC TCC AAC TCT GGG AAC ACG
                 R   P   P   G   I   P   E   R   F   S   G   S   N   S   G   N   T
                 .   .   S   .   .   .   .   .   .   .   .   .   .   .   .   .   .
Humlv318        CGG CCC TCA GGG ATC CCT GAG CGA TTC TCT GGC TCC AAC TCT GGG AAC ACG

                                                        80
RFAN            GCC ACC CTG ACC ATC AGC AGG GTC GAA GCC GGG GAT GAG GCC GAC TAT TAC
                 A   T   L   T   I   S   R   V   E   A   G   D   E   A   D   Y   Y
                 .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .
Humlv318        GCC ACC CTG ACC ATC AGC AGG GTC GAA GCC GGG GAT GAG GCC GAC TAT TAC

                    *************** CDR3 **********************     100
RFAN            TGT CAG GTG TGG GAT AGT AGT AGT GAT CAT GCT GTG TTC GGC GGA GGG ACC
                 C   Q   V   W   D   S   S   S   D   H   A   V   F   G   G   G   T
                 .   .   .   .   .   .   .   .   .   .
Humlv318        TGT CAG GTG TGG GAT AGT AGT AGT GAT CAT
                                                      --V--^--J--

RFAN            AAG CTG ACC
                 K   L   T

Humlv318
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<-------> determined by protein sequencing

********* CDR (complementarity determining region)

Primer sequences are underlined in the RFAN sequence

Base differences between RFAN and *Humlv318* are bold/underlined in the latter sequence

^ V-J junction

Figure 2. Nucleotide and amino acid sequences of the V_L region of RF–AN, aligned with the $V_\lambda III$-a germline gene *Humlv318.*

is missing from RF–AN V_L; single base changes leading to amino acid replacements occur at codon 16 in FR1 (K → Q) and codon 55 in CDR2 (TCA → CCA, S → P); silent, single base changes occur at codon 45 in FR2 (CTG → TTG) and codon 53 in CDR2 (CGG → CGC) (Fig. 2). The proline residue at position 55 in CDR2 of RF–AN V_L appears to be unique among observed $V_\lambda III$-a proteins.[22,24,25]

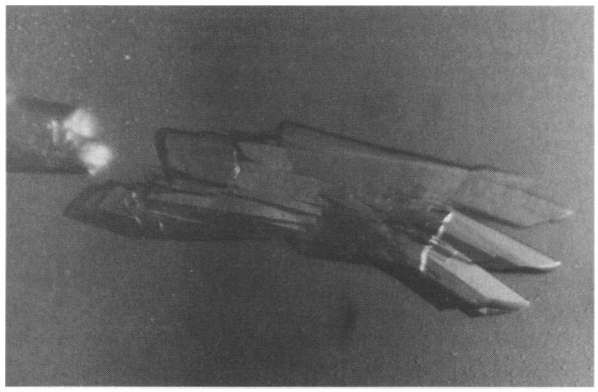

Figure 3. Cluster of crystals of the complex formed between RF–AN Fab and IgG4 Fc. Maximum dimension ≈ 0·6 mm.

Crystallization of the Fab–Fc complex

Crystals appeared in the drops containing a 1 : 1 mixture of the RF–AN Fab and IgG4 Fc, with polyethylene glycol (PEG) as precipitant (see Materials and Methods). They first appeared after a period of 2–3 weeks, and grew to a size typically 0·1 × 0·1 × 0·2 mm over 2–3 months. However, still larger crystals up to 0·1 × 0·2 × 0·6 mm, were obtained by seeding. Smaller crystals, after thorough washing with protein-free reservoir solution, were introduced into 100 μl sitting drops containing the protein solution as before, in sealed perspex wells equilibrated against 3 ml reservoir solution containing PEG at the lower concentration of 15 to 17·5% (w/v). The crystals often grew as irregular clusters, as shown in Fig. 3, and did not display a clear morphology, but could be broken into single fragments for X-ray analysis.

Stoichiometry of the Fab–Fc complex

Large single crystals were washed and analysed by SDS–PAGE under non-reducing conditions, as described above. Figure 4 compares the composition of individual crystals with mixtures

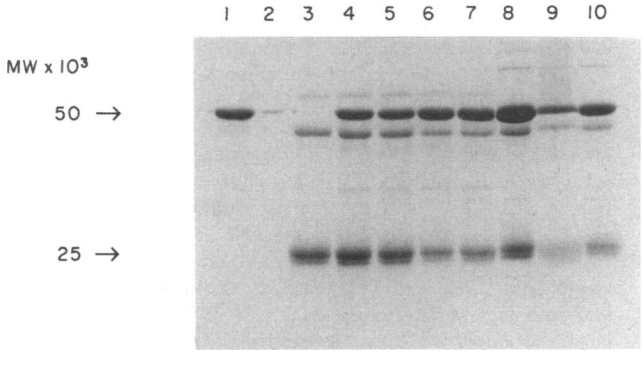

Figure 4. SDS–PAGE analysis (12·5%) under non-reducing conditions of re-dissolved single crystals of the complex of RF–AN Fab and IgG4 Fc, compared with the starting material for crystallization, and mixtures of the two components. Lane 1: RF–AN Fab. Lane 2: blank, showing contamination from lane 1. Lane 3: IgG4 Fc. Lanes 4 & 5: Fab + Fc in ratio 1 : 1. Lanes 6 & 7 : Fab + Fc in ratio 2 : 1. Lanes 8–10: re-dissolved single crystals.

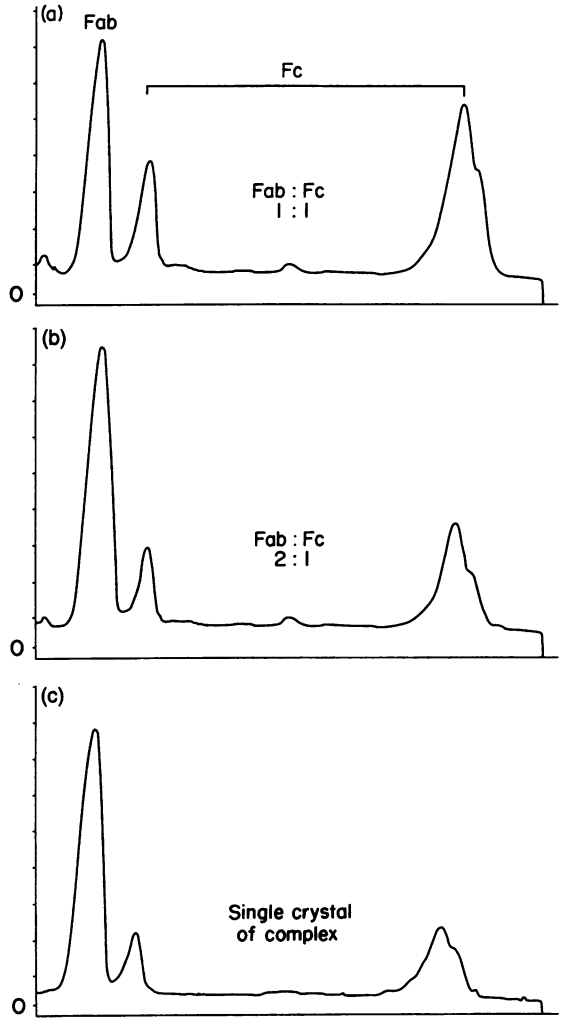

Figure 5. Scans of the SDS–PAGE analysis shown in Fig. 5. RF–AN Fab runs as a single peak, and IgG4 Fc runs as a single higher molecular weight peak, and an unresolved lower molecular weight doublet, as indicated (and see text). (a) RF–AN Fab + IgG4 Fc in a 1 : 1 mixture (Fig. 5, lane 5); (b) RF–AN Fab + IgG4 Fc in a 2 : 1 mixture (Fig. 5, lane 7); (c) Re-dissolved single crystal (Fig. 5, lane 8). Scans (b) and (c) are virtually superimposable, indicating that the stoichiometry of the complex in the crystals is 2 : 1 Fab : Fc.

of Fab and Fc in 1 : 1 and 2 : 1 proportions, as well as each component separately. It can be seen that whereas RF–AN Fab runs as a single band (Fig. 4, lane 1), the IgG4 Fc preparation contains a substantial proportion of molecules in which the inter-heavy chain disulphide bridge is broken (Fig. 4, lane 3), reflecting its production in the presence of reducing agent (1 mM dithiothreitol) and subsequent variable reoxidation with time. The close doublet at 25 000 MW (Fig. 4, lanes 3–10) presumably reflects the heterogeneity in glycosylation at Asn297 in each heavy chain.[16] It is clear from the gel that the crystals contain both Fab and Fc with an apparent ratio of 2 : 1 (Fig. 4, lanes 8–10). This stoichiometry was confirmed from quantitative analysis of gel scans (Fig. 5). The ratio of intact to reduced Fc is the same in the crystals as in the starting material, indicating that no selective crystallization has occurred.

X-ray analysis of crystals

The crystals diffracted X-rays on the rotating anode source to a resolution of 0·3 nm, and although diffraction was weak, they were relatively stable to radiation damage when cooled to 5° during irradiation. The space group symmetry of the crystals was determined by inspection of low resolution precession photographs, and from preliminary data collected on the image plate detector. The space group is C2, with cell dimensions $a = 16{\cdot}03$ nm, $b = 8{\cdot}19$ nm, $c = 6{\cdot}42$ nm, and $\beta = 98{\cdot}3°$. The volume of the asymmetric unit of the crystal is therefore 209 nm, and if this contains one complete $(Fab)_2Fc$ complex with a molecular weight of approximately 150 000 MW, then the V_M ratio (volume of asymmetric unit/molecular weight of contents;[26]) is $1{\cdot}39 \times 10^{-3}$ nm^3/Da. This is well outside the range observed for protein crystals, and would imply an unreasonably high protein density within the crystal. However, assuming that one half of the complex is contained within the asymmetric unit of the crystal, the V_M ratio is $2{\cdot}78 \times 10^{-3}$, which is close to the mean value observed.[26] The complex must therefore be packed within the crystal lattice with a crystallographic two-fold axis coincident with the local two-fold of the Fc fragment, i.e. the two polypeptide chains of the Fc, and the two Fab fragments that are bound to the Fc, are related to each other by an exact two-fold axis of symmetry.

DISCUSSION

We report here the first crystallization and characterization of a complex between the Fab fragment of a rheumatoid factor and its antigen, the Fc fragment of IgG. This is also the first reported crystallization of any autoantibody complexed with its autoantigen. The Fab fragment was prepared from the well-characterized monoclonal IgM RF–AN derived from transformed B cells of an RA patient, while the Fc belongs to the human IgG4 subclass. The stoichiometry of the complex in the crystals is 2:1 Fab:Fc. From the space group and cell dimensions it is clear that the asymmetric unit of the crystal contains one half of the complex, and thus it follows that the two Fab fragments bind symmetrically to either side of the Fc. In this respect, the RF Fab–Fc complex resembles those formed between domain B of SpA and human IgG1 Fc,[27] and the neonatal Fc receptor and rat IgG Fc.[28] In all of these structures, the stoichiometry of the complex is 2 : 1 ligand : Fc, and a crystallographic two-fold axis relates the two halves of the complex. However, in the recently determined structure of the complex formed between domain C2 of streptococcal Protein G (SpG) and human IgG1 Fc[29] the stoichiometry is also 2 : 1, but the two halves of the complex are related by a non-crystallographic two-fold axis of symmetry.

The RF Fab–Fc crystals diffract to a resolution of 0·3 nm, sufficient to enable the structure of the complex to be determined. The relationship between the Fc epitope recognized by RF–AN and the binding sites on Fc for SpA and SpG will be of particular interest. It is expected that these will overlap, on the basis of the subclass and allotypic specificity of RF–AN,[12] competition between SpA and RF–AN for IgG binding, and the overlap between the binding sites for SpA and SpG on Fc.[29]

We have also determined the complete V_L sequence of RF–AN (Fig. 2), and found that it is highly homologous to that of the functional germline gene *Humlv318*,[25] which belongs to the $V_\lambda III$-a subgroup. Although there are only three differences at the nucleotide level over the 240 bp sequenced (98·7% identity) and three at the amino-acid level in the complete V region (97% identity; Fig. 2), it is not certain that *Humlv318* is the precursor gene of RF–AN V_L. The absence of the N-terminal serine and the presence of glutamine at position 16 occur in several other human monoclonal $V_\lambda III$-a light chains,[24] suggesting the possible existence of a closely related $V_\lambda III$-a germline gene.[25] Although the $V_\lambda III$ subgroup comprises about 20% of all λ light chains,[30] its occurrence in RFs sequenced to date is low. The *Humlv318* gene is expressed in one other monoclonal RF, G9, prepared from rheumatoid synovial cells,[31] but G9 has a different specificity and a different V_H segment (V_H4·18) from RF–AN. A distinct $V_\lambda III$ gene is expressed in RF mAb60.[4] It is noteworthy that one of the possible somatic mutations leading to an amino acid substitution occurs in a CDR (Pro55, CDR2), raising the possibility of selection by autoantigen. The V_H sequence of RF–AN is not quite so closely related to its nearest germline precursor, *DP-31*, from which it differs at seven amino acid positions.[14,23] Considerable variability in heavy chain CDR3 in particular has been documented among RFs.[14]

Determination of the structure of this complex will define the nature of the interaction between RF V regions and the Fc autoantigen, and will reveal the precise location of the epitope. It will also provide a basis for understanding the role played by germline gene sequences and somatic mutation in the generation of RF activity.

ACKNOWLEDGMENTS

We thank Carl Spicer and Sally Humphreys for production of cell supernatants. We are grateful to the Arthritis and Rheumatism Council (UK) for their support for this work (Project Grants S0142 & S0512), and BJS thanks the Nuffield Foundation for the award of a Science Research Fellowship.

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