

Qualitative and quantitative expression of V_{HI} associated cross reactive idiotopes within IgM rheumatoid factor from patients with early synovitis

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Abstract

Monoclonal rheumatoid factors (RFs) of the major Wa cross reactive idiotype group have been shown to express exclusively V_{KIII} subgroup light chains and V_{HI} subgroup heavy chains. A V_{KIII} associated cross reactive idiotope (CRI) (17-109), however, was shown not to be exclusively expressed on IgM paraproteins having rheumatoid factor activity or to be present at increased levels in the sera of patients with rheumatoid arthritis (RA). Three V_{HI} associated CRIs have been defined with monoclonal antibodies and quantitative studies of their representation are reported, together with V_{KIII}, in IgM and IgM RF isolated from the sera of patients with early synovitis, some of whom progressed to classical RA. The results show (a) the probed CRIs were expressed predominantly on IgM RF rather than on non-RF IgM; (b) 5-10% of IgM RFs from patients with classical RA expressed the CRIs, but this represented a lower proportion of IgM RFs than observed for normal individuals or patients with self limiting synovitis; (c) V_{KIII} light chains were highly associated with IgM RFs rather than non-RF IgM (75% and 25% respectively).

It is suggested that the CRIs probed are markers for germline gene encoded antibodies or sequences resulting from minimal mutation of germline genes. The lowered proportion of RFs expressing CRIs in RA may therefore be evidence of polyclonal activation or specific antigenic stimulation, or both, resulting in maturation of the RF response with recruitment of further V_H genes or extensive mutation of germline genes. These studies show that monoclonal RFs are relevant models of RF produced in RA and that the repertoire of RF autoantibodies may be encompassed within a small number of CRI expressing families.

A diagnostic criterion for a number of autoimmune diseases is the specificity of the autoantibodies detected in patients' serum. The restricted specificity of autoantibodies may also be reflected in the expression of cross reactive idiotopes (CRIs) indicative of structural similarities within the antigen binding sites of antibodies. First evidence of these relations was provided by studies of monoclonal rheumatoid factors (RFs) isolated from the serum of patients with lymphoproliferative disorders. Two major idiotypic families of monoclonal RF were identified, designated as Wa and Po, which accounted for 60% and 20% of monoclonal RFs respectively.¹ Protein sequence analysis con-

firmed the structural homology between monoclonal RF proteins expressing CRIs.²

Polyclonal antisera recognising CRIs expressed on monoclonal RF were used to show their expression on polyclonal RF in the serum of patients with rheumatoid arthritis (RA).³⁻⁵ Quantitative studies were not performed, however, as it was recognised that polyclonal anti-idiotypic antisera are complex reagents detecting multiple idiotopes expressed on similar but not identical molecules. Also it is impossible to standardise or reproduce such reagents. More recently, monoclonal anti-idiotypic antibodies have been produced that have allowed structural definition of the light chain idiotope recognised⁶ and led to the isolation of genes encoding for the idiotope expressing proteins.^{7,8} These studies have shown that monoclonal RFs expressing light chain associated CRIs are the product of a small family of V_K germline genes and that quantitative expression within polyclonal RFs may vary between different autoimmune diseases—for example, RA, systemic lupus erythematosus, and primary Sjögren's syndrome.

Our laboratory has produced and characterised monoclonal antibodies recognising CRIs expressed on the heavy chains of monoclonal RFs representative of the Wa CRI group.⁹ From a panel of 163 IgM paraproteins 35% of monoclonal RFs (9/26) expressed the G6 idiotope while only 4% (5/137) of non-RF paraproteins expressed this idiotope.¹⁰ Monoclonal anti-idiotypic antibody H1 recognises an idiotope with more restricted expression within the Wa CRI group. We now report studies to measure the expression of these idiotopes within polyclonal RF present in the serum of patients with RA. In addition to providing information about the genes contributing to the production of this autoantibody, regulation of antibody production by anti-idiotypic reagents may be possible. If polyclonal RF can be resolved into a few CRI expressing antibody families it may be possible by anti-idiotypic treatment to reduce RF production substantially and consequently one aspect of the self perpetuating inflammatory response.

Materials and methods

PROTEIN PURIFICATION

Rheumatoid factors

IgM RF and IgM with no RF activity were purified from the serum samples of four patients with rheumatoid arthritis by a three step procedure; briefly, immunoglobulins were precipitated at 40% saturation with ammonium sulphate and the washed precipitate was redissolved in

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saline. After dialysis against 0.2 M sodium acetate pH 4.4 this preparation was passed over a Sephacryl S-200 column (2×100 cm), equilibrated, and eluted with the same buffer. IgM containing fractions were pooled and dialysed against phosphate buffered saline (PBS; 0.28 M) before passage over a Sepharose-4B IgG column; the unbound fraction was collected as non-RF IgM and the bound fraction, eluted with 3 M KCNS and dialysed against PBS, as IgM RF.

Rheumatoid factor and non-RF fractions of RA serum samples R104 and R97 were obtained by affinity chromatography using the Sepharose-4B IgG column as described above. IgM paraproteins having RF activity, Fr and Kok, were similarly isolated from the sera of patients with essential mixed cryoglobulinaemia.

Polyclonal human IgG was prepared from a normal human immunoglobulin preparation (Lister Institute, UK) as the 'breakthrough' fraction from a diethylaminoethyl-cellulose column equilibrated and eluted with 0.01 M phosphate pH 7.0. The heavy chain disease protein Per,¹¹ characterised as an IgG1 Fc fragment, was purified as described previously.¹²

Murine monoclonal antibodies

The production and selection of the monoclonal antibodies C7, G6, G8, and H1 have been described in detail.¹³ The clones were expanded as ascitic tumours and the antibodies isolated from ascitic fluids by elution from diethylaminoethyl-cellulose with 0.03 M phosphate buffer pH 8.0. All monoclonal antibodies were of the IgG1 isotype. $F(ab')_2$ fragments were prepared by pepsin digestion of purified proteins in 0.1 M acetate buffer pH 4.0 using an enzyme/protein ratio of 1:40 (w/w) at 37°C for eight hours.^{14, 15} Undigested IgG was removed by passage of the digest over a Sepharose-4B protein A column equilibrated and eluted with 0.1 M TRIS.HCl pH 8.0 at 4°C. Purity of the $F(ab')_2$ preparations was confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis, using a 10% gel.

QUANTITATIVE ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

IgM rheumatoid factor

Polystyrene microtitre plates (Flow Laboratories, UK) were sensitised with the antigen Fc-Per (20 μ g/ml in PBS pH 7.2), by incubation at 37°C for two hours, then washed three times with PBS containing 0.05% Tween 20 (PBS/Tween). The RF containing sample under test was diluted 1/100, 1/250, and 1/1000 in PBS/Tween and incubated, in duplicate, in antigen sensitised plates for two hours at 37°C. Washed plates ($3 \times$ PBS/Tween) were incubated with a 1/3000 dilution of sheep antihuman IgM peroxidase labelled conjugate (The Binding Site, Birmingham Research Institute, UK) in PBS/Tween for two hours at 37°C. After washing ($3 \times$ PBS/Tween) the plates were developed with *o*-phenylenediamine and the reaction stopped after 30 minutes with 20% H_2SO_4 . Optical density was measured by a Titretrek multiscan ELISA reader (Flow Laboratories, UK). The concentration of RF in the sample was deter-

mined from a computerised four parameter logistic transformation model using the optical density developed for known inputs of the purified IgM rheumatoid factor Fr.

Total IgM and idiotype positive IgM

A capture assay was developed using $F(ab')_2$ fragments of the anti-idiotypic antibodies G6, G8, and H1; total IgM was measured using intact AF6 antibody. ELISA plates were sensitised with antibody at 10 μ g/ml; samples under test were added at a series of dilutions and in duplicate. The assay protocol was as described above for IgM RF and the concentration of IgM or idiotype positive IgM was determined from a computerised four parameter logistic transformation model using the optical density developed for known inputs of the purified IgM rheumatoid factor Kok.

V_{KIII} positive IgM and IgM RF

The assay protocol was as described in the previous section except that ELISA plates were sensitised with $F(ab')_2$ fragments of the V_{KIII} subgroup specific antibody C7.

PATIENTS' SAMPLES

Serum samples were collected from an early synovitis clinic, to which patients had been referred by their general practitioners. On presentation all patients displayed similar clinical manifestations. After 14–20 weeks, however, the disease symptoms were no longer apparent in one group, designated as self limiting synovitis, whereas another group suffered from persistent synovitis and developed the American Rheumatism Association criteria for classical RA.

Results

MEASUREMENT OF TOTAL IgM AND IgM RF

Figure 1 shows the standard curves used for measurement of total IgM and IgM RF.

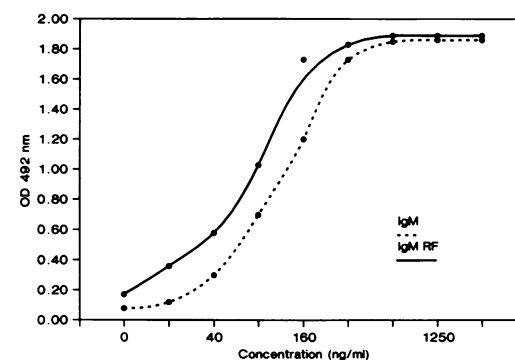


Figure 1: Standard curves for the measurement of total IgM (●—●) and IgM rheumatoid factor (IgM RF) (●---●) using the monoclonal paraproteins Kok and Fr respectively.

Table 1: Concentration of total IgM and IgM RF in the patients' serum samples. Value are given as means (SD)

Subject group	Total IgM (mg/ml)	IgM RF (μ g/ml)
Normals (n=15)	1.032 (0.546)	2.87 (2.47)
Self limiting synovitis (n=20)	1.12 (1.44)	9.71 (13.1)
Persistent synovitis (n=21)	3.46 (2.1)	225 (284)

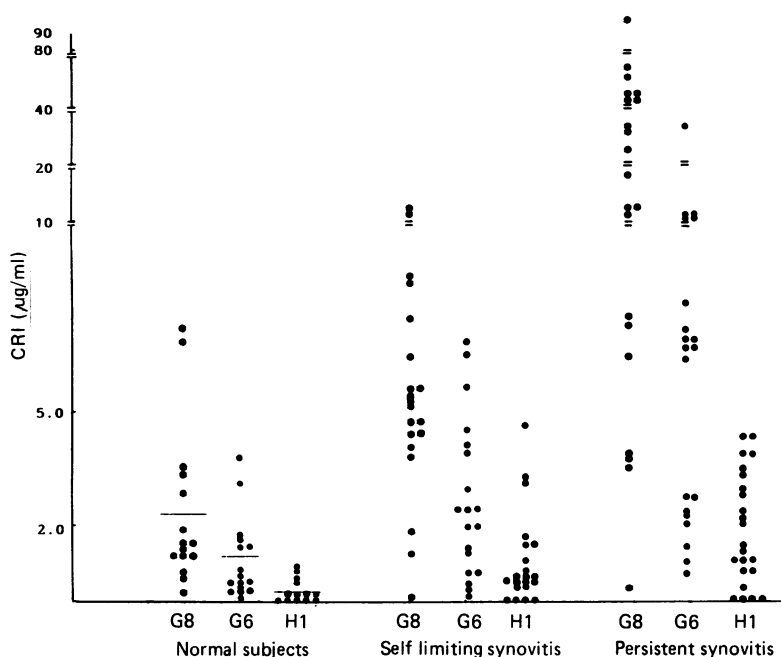


Figure 2: Measurement of G8, G6, and H1 cross reactive idiotopes in serum samples from normal individuals, patients with self limiting synovitis, and those with persistent synovitis. The horizontal lines indicate mean values for G6, G8, and H1 in normal subjects. CRI=cross reactive idiotope.

Dilutions of each serum under test were made and the dilution giving an optical density in the mid-region of the standard curve was used to derive the quantitative data. The results show an increase in total IgM and IgM RF concentrations in the group with persistent synovitis but only in IgM RF in the group with self limiting synovitis (table 1); the increase in total IgM is not accounted for solely by the presence of IgM RF.

MEASUREMENT OF G6, G8, AND H1 CRIS WITHIN POLYCLONAL IgM AND IgM RF

The concentration of G6, G8, and H1 positive IgM was determined by the capture ELISA described and the percentage of total IgM and IgM RF expressing these CRIs was obtained using the values given in table 1. The concentrations of G6 and G8 were twice as high in the group with self limiting synovitis and 12 times

greater in the group with persistent synovitis than in the normal subjects (table 2). A wide spread of G6 and G8 concentrations was observed for normal serum samples, with relatively high values being found for samples from an elderly group of individuals with a mean age of 57 years. The idiotope H1 was detected in a minority of normal serum samples (4/15, 26%), but most samples from the groups with self limiting or persistent synovitis (73%) contained IgM expressing H1 (fig 2). The percentage of total IgM expressing CRI was significantly raised in the groups with persistent synovitis and self limiting synovitis, whereas when calculated as a percentage of the IgM RF a reverse correlation was apparent; thus the percentage of G8, G6, and H1+ IgM RF in normal sera (82.9%, 37%, and 4.35% respectively) was considerably higher than in self limiting synovitis (61.8%, 26.8%, and 11.2%) and persistent synovitis sera (12.7%, 3.2%, and 0.82%) respectively—except for the expression of H1 in the group with self limiting synovitis.

MEASUREMENT OF G6 AND G8 IDIOTOPES IN PURIFIED IgM RF AND NON-RF IgM

Affinity chromatography on immobilised IgG was used to prepare IgM RF and non-RF IgM from the serum samples of two patients from the group with persistent synovitis who had high concentrations of IgM RF. It is apparent that G6 and G8 expressing molecules were recovered predominantly as IgM RF (table 3).

MEASUREMENT OF V_{KIII}±IgM AND IgM RF FROM RA SERA

Parallel and essentially overlapping standard curves were established for known inputs of the IgM RF paraproteins Kok and Fr. Non-RF IgM and IgM RF were prepared from RA serum samples Nos 1-4 as described in 'Materials and methods'. Serum R104 was fractionated to give RF and non-RF containing pools.

For RA serum No 1 10% of total IgM expressed the V_{KIII} subgroup, while the percentages for IgM RF and non-RF IgM were 23%

Table 2: Concentration and relative proportions of G6, G8, and H1 cross reactive idiotopes in the serum samples of patients and normal subjects

Subject group	G8 (µg/ml)*	G8/IgM (%)	G8/IgM RF (%)	G6 (µg/ml)*	G6/IgM (%)	G6/IgM RF (%)	H1 (µg/ml)*	H1/IgM (%)	H1/IgM RF (%)
Normal individuals	2.38 (2.1)	0.23	82.92	1.06 (0.924)	0.1	37	0.125 (0.24)	0.012	4.35
Self limiting synovitis	6.0 (3.6)	0.53	61.8	2.61 (2.35)	0.23	26.8	1.09 (1.39)	0.097	11.22
Persistent synovitis	28.5 (27.4)	0.82	12.7	7.27 (7.19)	0.21	3.2	1.84 (1.47)	0.053	0.82

*Values are means (SD).

Table 3: Proportion of G6 and G8 in purified rheumatoid factor (RF) and non-RF IgM from patients with persistent synovitis

Patients with persistent synovitis	G6		G6/IgM RF (%)	G8	G8/IgM RF (%)	
	Total (µg/ml)	% in IgM RF			Total (µg/ml)	% in non-RF IgM
R104	8.16	72	2	35.84	83.5	4.1
R97	60.16	92.3	2	188.2	87	5

Table 4: Measurement of $V_{KIII}+IgM$ in purified IgM and IgM RF isolated from rheumatoid arthritis serum samples

Rheumatoid arthritis serum samples	Total IgM ($\mu\text{g/ml}$)	IgM RF ($\mu\text{g/ml}$)	$V_{KIII}+IgM$ ($\mu\text{g/ml}$)	$V_{KIII}+IgM/\text{total IgM}$ (%)
<i>No 1</i>				
Unpurified	9675	3960	973	10
IgM RF	160.7	129.8	36.8	23
Non-RF IgM	4.0	0.21	0.46	11.5
<i>No 2</i>				
Unpurified	1625	900	NT	—
IgM RF	84	66	11.5	14
Non-RF IgM	54	1.15	10.56	19
<i>No 3</i>				
Unpurified	1069	255	NT	—
IgM RF	56.3	81.1	15.04	27
Non-RF IgM	93.67	4.0	8.0	8.5
<i>R104</i>				
Unpurified	2764	294.4	NT	—
IgM RF	26.9	10.88	6.0	23
Non-RF IgM	62.7	0.515	5.04	8
<i>No 4</i>				
Unpurified	300	65	NT	—
IgM RF	2.87	0.99	2.56	89
Non-RF IgM	8.8	0.17	2.69	30

and 12% respectively (table 4). This bias in favour of the use of V_{KIII} within IgM RF was similarly seen in serum samples Nos 3 and 4; in sample No 4 nearly 90% of the IgM RF expressed the V_{KIII} light chain. Obviously the proportional expression varied widely and RA serum sample No 2 IgM RF gave a value of only 14%. When serum R104 was split into RF and non-RF fractions about 25% of the RF was shown to use the V_{KIII} light chain.

Discussion

Cross reactive idiotopes have been shown to define structurally related molecules in numerous antigen specific antibody systems.¹⁶ Furthermore, shared idiotypity often results from the expression of germline genes, closely related families of germline genes, or genes that have undergone minimal somatic mutation.^{17, 18} These relations have been formally demonstrated for rheumatoid factors that occur as monoclonal paraproteins in about 10% of patients with Waldenström's macroglobulinaemia. Thus the monoclonal RF protein Sie was used as immunogen in the production of the 17-109 antibody recognising a light chain CRI; 17-109 allowed the cloning of the gene from a 17-109 expressing lymphoblastoid cell line and subsequently a germline gene (K_V325) that encoded exactly the primary amino acid sequence of the V_K region of protein Sie.⁸ The 17-109 idiotope was shown not to be confined to RF, though it was expressed in polyclonal RF, particularly in the serum of patients with primary Sjögren's syndrome.¹⁹

We have developed murine monoclonal anti-idiotypic antibodies recognising V_H region CRIs, whose expression is essentially restricted to monoclonal IgM paraproteins with RF activity. As these RFs arise in patients having lymphoproliferative disorders, but not necessarily suffering from associated symptoms of RA, it is essential to determine the relevance of studies of these monoclonal RFs to polyclonal RFs in RA. Thus we have developed qualitative and quantitative ELISAs to investigate CRI expression in the serum of selected patient groups.

In this study the CRIs recognised by antibodies G6, G8, and H1 were measured in serum samples of patients attending an early synovitis clinic. Retrospective analysis allowed these patients to be divided into those with self limiting synovitis and those with persistent synovitis progressing to classical RA. It was part of our thesis that germline encoded RF sequences, and hence expression of CRIs, may be characteristic of early disease, whereas in longstanding disease recruitment of new gene families or mutation within those originally expressed may lead to a reduced proportion of RFs expressing a given CRI.

The total IgM concentration was normal for the patients with self limiting synovitis, whereas the IgM RF concentrations were raised threefold (table 1); for the patients with persistent synovitis total IgM concentrations were raised (threefold) as were the IgM RF concentrations (80-fold). Table 2 and fig 2 summarise the concentrations of G6, G8, and H1 CRIs expressed in these sera and give absolute values for the probed CRIs and percentage values for the CRIs within total IgM and IgM RF. As expected for a group of normal individuals RF was detectable, but unexpectedly the G8 and G6 idiotopes formed 83% and 37% of the IgM RF respectively. In the groups with self limiting and persistent synovitis these values were reduced to 62% and 27% and to 13% and 3% respectively. The highly restricted CRI H1 constituted 4%, 11%, and 0.8% of IgM RF present in the normal group and the groups with self limiting and persistent synovitis respectively. These proportions are very similar to those reported by Nelson *et al.*,²⁰ who have recently shown that only a minor proportion (3%) of RF from patients with RA expresses a common idiotope.

Our previous studies have shown that the G8 and G6 CRIs are often coexpressed on the same IgM paraprotein and hence the values for expression of these CRIs as a percentage of IgM RF in normal individuals are similarly suggestive of coexpression. The same could be true for the groups with self limiting and persistent synovitis, but the percentage values do not

require this explanation. In further experiments populations expressing G6 have been purified by affinity chromatography and also shown to express G8. We also showed preferential expression of V_{KIII} light chain within polyclonal RF purified from RA sera when compared with the non-RF component (75% and 25% respectively). These results may appear to be at variance with the study of Williams *et al.*,²¹ who proposed a negative selection for V_{KIIIb} expression within polyclonal RF. Williams *et al.*,²¹ however, used the antibody JG-B1 recognising a V_{KIIIb} epitope expressed within IgM but not IgG molecules, while antibody C7 recognises V_{KIII} IgM and IgG molecules. It is possible that the JG-B1 antibody recognises a V_{KIII} epitope requiring germline gene sequences for expression, while C7 recognises an epitope determined by a conserved stable framework sequence. Alternatively, JG-B1 may recognise the product of a V_{KIII} gene family that is a minor component of polyclonal RF and IgG.

A coherent interpretation of these data would be that in normal individuals germline genes encoding IgM molecules having RF activity are expressed at low levels. The fact that few genes encode this antibody specificity is reflected in the high incidence of CRIs detected. Aetiopathological mechanisms leading to RA result in the recruitment of other genes encoding RF specificities. This may arise through activation of new families of V_H genes by a process of polyclonal activation or antigen induced maturation of a specific antibody response, leading to somatic mutation of the germline genes encoding RF or other genes that encode antibody to an exogenous antigen and whose product also binds the self antigen IgG.

As a proportion (10%) of polyclonal RF expresses the probed CRI this study shows that monoclonal RFs can be considered relevant structural analogues of a proportion of RFs produced in RA. Possibly, therefore, the total polyclonal RF response may be included within a relatively small library of CRIs, and monitoring of the expression of these CRIs might be indicative of disease progression and hence prognosis. Alternatively, anti-CRI antibodies could contribute to the development of selective treatments for modulation of this form of self reactivity.

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