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THE BINDING AFFINITIES OF RHEUMATOID FACTORS INTERACTING WITH THE Cγ3 HOMOLOGY REGION OF HUMAN IgG

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SUMMARY

The binding affinities of rheumatoid factors interacting with Gm(a), Gm(x), 'non a' and 'γ4 non a' antigens have been measured by an equilibrium molecular sieving technique using rheumatoid sera of known specificity and pFc' fragments (≡Cγ3 homology region) from IgG molecules of defined subclass and allotype. All the rheumatoid factors studied showed specific binding with the fragments possessing the homologous antigen. The binding affinities of these rheumatoid factors were low, between 10^4 and 10^5 litres/mole. In contrast, no binding occurred between rheumatoid factors and fragments lacking the appropriate antigenic determinant.

INTRODUCTION

Rheumatoid factors (RF) are antibodies directed against antigenic determinants of IgG. RF reacts with both native and aggregated IgG and there has been much discussion of the relative importance of these reactions (Normansell, 1971; Roitt, 1971; Natvig, Munthe & Gaarder, 1971). Recent attempts to measure the binding constants of rheumatoid factors have indicated similar binding with both native and aggregated IgG (Normansell, 1970, 1971). These studies have utilized rheumatoid factors reacting with the Fc region of IgG as also have the studies with relatively homogeneous antiglobulins (Cerottini & Grey, 1969; Abraham, Clark & Vaughan, 1972), but in no case has the precise antigenic specificity been defined.

Gaarder & Natvig (1970) have shown that in any given rheumatoid serum there may be several distinctly different rheumatoid factors with reactivity for native IgG. Other studies have shown that there are two rheumatoid factor sites in the pFc' subfragment of IgG (the Cγ3 homology region). These are, first, a site occupied by the antithetic Gm(a), 'non a' and 'γ4 non a' antigens and, secondly, the Gm(x) site. In the Cγ2 homology region three

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sites are recognized; the antithetic Gm(b^1) and 'non b^1' antigens; the Gm(g) antigen; and the Ga antigen (Natvig, Gaarder & Turner, 1972) (see Fig. 1).

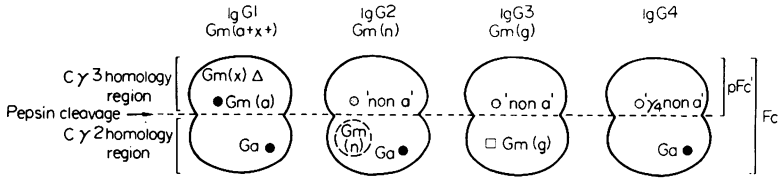

FIG. 1. Schematic diagram of the Fc region of the four IgG-myeloma proteins used in this investigation. The Gm(a) and Gm(x) antigens are present only in the Cγ3 region of IgG1 Gm (a+ x+) proteins. The IgG2 Gm(n) (which is not known to interact with RF) and the IgG3 Gm(g) antigens are located in the Cγ2 region. The Ga antigen, common to all IgG1, IgG2 and IgG4 proteins, is also present in the Cγ2 region. The 'non a' antigen occupies in IgG2 and IgG3 proteins a site corresponding to that of Gm(a) in IgG1 (Gm(a+) proteins. Similarly, the 'γ4 non a' antigen probably occupies this site in IgG4 proteins.

Using rheumatoid factors of defined specificity and pFc' fragments from proteins of known subclass and allotype, we have measured the binding affinities of rheumatoid factors reacting with Cγ3 antigens.

MATERIALS AND METHODS

Isolation of G-myeloma proteins

Human G-myeloma proteins of selected subclass and allotype were isolated by zone-electrophoresis on Pevikon block (Fahey & McLaughlin, 1963) and Sephadex gel filtration (Turner, Bennich & Natvig, 1970a).

Isolation of pFc' fragments

pFc' fragment was prepared from pooled normal IgG as previously described (Turner & Bennich, 1968) and from four G-myeloma proteins using optimum conditions of pepsin digestion defined in a previous investigation (Turner, Bennich & Natvig, 1970b).

The pFc' fragments were isolated from the peptic digests by gel filtration on Sephadex G-150 (Natvig & Turner, 1971). Isolated fragments were shown to be pure by standard haemagglutination-inhibition tests for a range of antigens known to be present in pFc' fragments of other allotypes and subclasses. Contamination with Cγ2 and Fab antigens was also excluded.

Labelling of pFc' fragments

Fragments (2–5 mg) were labelled with ^{125}I using the iodine monochloride method of McFarlane (1958).

Sera with anti-pFc' activity

Various rheumatoid sera with specificities for Cγ3 antigens were studied. The antibody activities in these sera were shown by haemagglutination inhibition tests to be inhibited by pooled pFc' and Fc fragments but not by $F(ab')_2$ fragment (Natvig *et al.*, 1972). Sera interacting with Gm(a) and Gm(x) antigens were studied directly but sera interacting with

'non a' and 'γ4 non a' antigens were first fractionated on Sephadex G-200 in 0·1 M acetate buffer pH 4·0 (Allen & Kunkel, 1966). Fractions containing IgM (free of potentially inhibiting autologous IgG) were concentrated by ultrafiltration, dialysed against 0·01 M phosphate buffered saline, pH 7·3, and used in the subsequent binding studies.

The baboon serum No. 6 was obtained following immunization with an IgG1 Gm(a+) myeloma protein and was made specific for Gm(a) by absorption with Gm(a−) normal serum.

Rabbit serum IM 112 was obtained from a rabbit immunized with pFc′ fragment prepared from pooled human IgG. The immunization course consisted on one intramuscular injection of antigen in Freund's complete adjuvant followed by four booster injections of alum precipitated antigen.

Binding studies

The binding of ^{125}I-labelled pFc′ fragments by various antisera was studied over a range of ten antigen concentrations (2–20 μg/tube). Preparations of [^{125}I]pFc′ fragments in 0·01 M phosphate buffered saline, pH 7·3 (80 μl) were incubated in triplicate with 20 μl of the antiserum or normal serum at room temperature in 1·0 ml siliconized glass screw capped bottles. The proportion of antigen which was antibody-bound was determined by a modification of the equilibrium molecular sieving technique (Souleil & Nisonoff, 1968; Stone & Metzger, 1968a). After incubation for 1 hr, 0·5 ml of a thick slurry of pre-swollen Sephadex G-200 was added to each bottle. Equilibration was allowed to take place over a period of 4 hr at room temperature during which time the bottles were inverted 15 times/hr. The bottles were then centrifuged, 50 μl of the supernatant removed and the radioactivity determined. The amount of [^{125}I]pFc′ which was antibody-bound was determined using the difference between the radioactivity in the supernatant of bottles containing antiserum and those containing non-rheumatoid human serum. In order to calculate these values, the volume available to the antibody-bound pFc′ (the external distribution volume) was determined by observing the decrease in $OD_{620\ nm}$ of 100 μl of a solution of blue dextran when it was equilibrated under the same conditions with the Sephadex.

The amounts of [^{125}I]pFc′ antigen which were antibody-bound and which were free at equilibrium were determined at each antigen concentration. The total amount of antibody in 20 μl of serum was determined (as binding sites, Ab_t) by extrapolation to $\frac{1}{c}=0$ of the plot of $\frac{1}{b}$ *vs* $\frac{1}{c}$ from the equation:

$$\frac{1}{b}=\frac{1}{K}\times\frac{1}{c}\times\frac{1}{Ab_t}+\frac{1}{Ab_t}$$

where b = antibody-bound antigen
c = free antigen
K = equilibrium constant
Ab_t = total antibody-binding sites

and binding constants (K, litres/mole) determined from $\frac{1}{c}$ when 50% of the sites were occupied by antigen (Steward & Petty, 1972a, b).

The equilibrium molecular sieving method of assaying antibody affinity was compared to an ammonium sulphate precipitation method (Steward & Petty, 1972a, using a hapten–antihapten system (NIP-rabbit anti-NIP) (Brownstone, Mitchison & Pitt-Rivers,

1966). The results obtained ($K = 1{\cdot}1 \times 10^6$ litres/mole for molecular sieving and $K = 1{\cdot}3 \times 10^6$ litres/mole for the ammonium sulphate procedure) indicated good agreement.

RESULTS

Reproducible determinations of the binding constants of rheumatoid factors interacting with Cγ3 antigens were readily obtained by the equilibrium molecular sieving procedure.

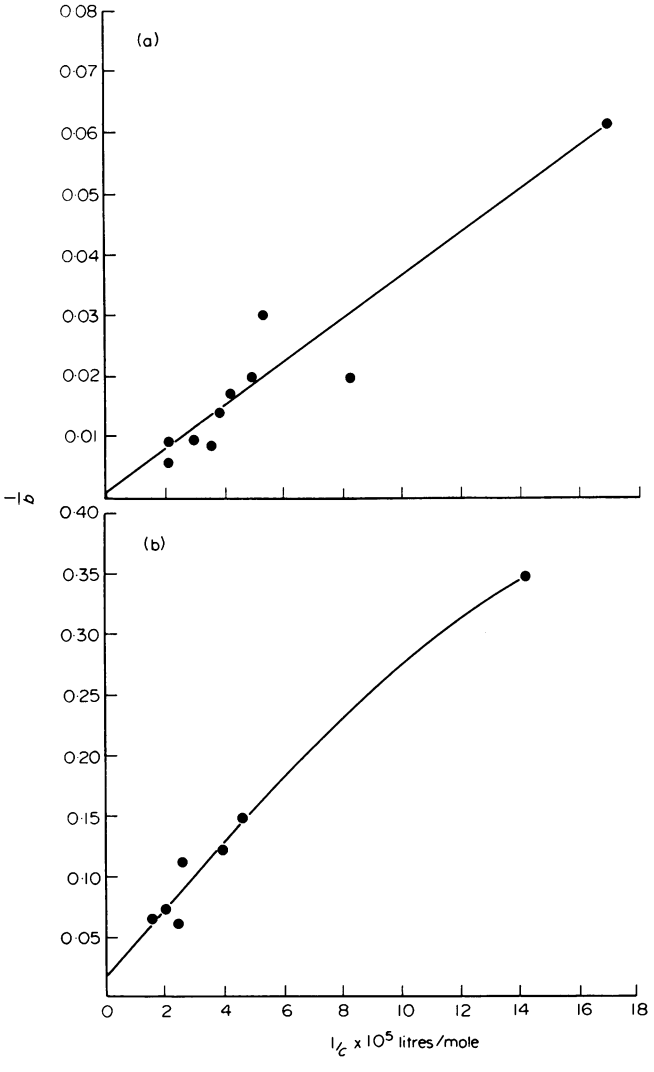

FIG. 2. Representative plots of the binding data obtained with the equilibrium molecular sieving technique. (a) Binding of ^{125}I-labelled IgG1 Gm(a+ x+) pFc′ fragment by a rheumatoid serum (3163) with anti-Gm(x) specificity. Each point represents the mean of triplicate estimations. (b) Binding of ^{125}I-labelled IgG4 pFc′ fragment by the IgM fraction of a rheumatoid serum (2872a) with anti-'γ4 non a' specificity. Each point represents the mean of triplicate estimations.

Representative binding plots are shown in Fig. 2 and the results obtained with various rheumatoid and heteroantisera are given in Table 1.

All rheumatoid factors with antibody specificities for the Gm(a), Gm(x), 'non a' and 'γ4 non a' antigens (Sera 7095, 3163, 3070, 4765 and 2872A) had low binding affinities, between 3×10^4 and 9×10^4 litres/mole, when tested with fragments having the appropriate antigen. Every rheumatoid serum was also tested with at least one pFc' preparation lacking the appropriate specific antigen. In such cases the binding was inconsistent and ranged from 0 to 2%.

Of particular interest were the results obtained with fragments bearing the antigens Gm(a), 'non a' and 'γ4 non a' which are thought to occupy similar molecular locations but differ in their amino-acid sequence by one to three residues. Rheumatoid factors interacting with fragments possessing one of these antigens did not show measurable binding with fragments bearing one of the other two antigens. Similarly, another rheumatoid serum

TABLE 1. Total antibody levels and binding affinities of various rheumatoid and heteroantisera for pooled and homogeneous pFc' fragments

Serum			Antigen		Total antibody (Ab_t) nmoles binding sites/ml	Binding affinity (K) litres/mole
Code no.	Origin	Specificity	Origin of pFc' fragment	Antigens present on pFc' fragment		
7095	Rheumatoid	Anti-Gm(x)	Pooled IgG	Gm(a), Gm(x), 'non a'	4·5	$7{\cdot}8 \times 10^4$
			IgG1 Gm(a+x+)	Gm(a), Gm(x)	14·5	$7{\cdot}6 \times 10^4$
			IgG2 Gm(n)	'non a'	—*	—
			IgG3 Gm(g)	'non a'	—	—
3163	Rheumatoid	Anti-Gm(x)	IgG1 Gm(a+x+)	Gm(a), Gm(x)	33·5	$3{\cdot}0 \times 10^4$
			IgG2 Gm(n)	'non a'	—	—
3070	Rheumatoid	Anti-Gm(a)	IgG1 Gm(a+x+)	Gm(a), Gm(x)	11·1	$8{\cdot}0 \times 10^4$
			IgG3 Gm(g)	'non a'	—	—
3071	Rheumatoid	Anti-Gm(a)	Pooled IgG	Gm(a), Gm(x), 'non a'	16·6	$8{\cdot}9 \times 10^4$
			IgG1 Gm(a+x+)	Gm(a), Gm(x)	25·0	$8{\cdot}0 \times 10^4$
			IgG2 Gm(n)	'non a'	—	—
4765	Rheumatoid (IgM fraction)	Anti'-non a'	IgG2 Gm(n)	'non a'	14·3	$6{\cdot}5 \times 10^4$
			IgG3 Gm(g)	'non a'	16·7	$6{\cdot}0 \times 10^4$
			IgG4	'γ4 non a'	—	—
2872A	Rheumatoid (IgM fraction)	Anti-'γ4 non a'	IgG2 Gm(n)	'non a'	—	—
			IgG3 Gm(g)	'non a'	—	—
			IgG4	'γ4 non a'	8·0	$4{\cdot}5 \times 10^4$
3077	Rheumatoid	Anti-Gm(b^1)	IgG1 Gm(a+x+)	Gm(a), Gm(x)	—	—
2130†	Healthy human	Anti-Gm(a)	IgG1 Gm(a+x+)	Gm(a), Gm(x)	6·7	$3{\cdot}6 \times 10^5$
6	Baboon	Anti-Gm(a)	IgG1 (a+x+)	Gm(a), Gm(x)	19·5	$1{\cdot}9 \times 10^5$
IM 112	Rabbit	Anti-pFc'	Pooled IgG	Gm(a), Gm(x), 'non a'	5·2	$1{\cdot}3 \times 10^6$
			IgG1 Gm(a+x+)	Gm(a), Gm(x)	6·6	$6{\cdot}5 \times 10^5$

* Insufficient binding to allow calculation of antibody level or affinity.
† Kindly supplied by the Netherlands Red Cross, Amsterdam.

(3077) with a specificity for an antigen not present in the pFc′ region (Gm(b^1)) showed no binding with IgG1 Gm(a+x+) pFc′.

A serum from a sensitized human donor and a baboon heteroantiserum, both reactive with the Gm(a) antigen, showed binding affinities of $>10^5$ litres/mole. A rabbit anti-human pFc′ serum also showed a higher binding affinity ($0{\cdot}6$–$1{\cdot}3 \times 10^6$ litres/mole).

Despite the differences in binding affinity, the calculated Ab_t values in rheumatoid sera were comparable to those obtained for the sensitized human serum with anti-Gm(a) specificity and two heteroantisera reacting with pFc′ antigens (Table 1).

DISCUSSION

This investigation has shown that serum rheumatoid factors with specificities for the Cγ3 antigens of IgG have relatively low binding affinities (10^4–10^5 litres/mole). These findings are similar to those of other workers using monoclonal antiglobulins of undefined serological specificity from patients with diseases other than rheumatoid arthritis. For example, Cerottini & Grey (1969) and Stone & Metzger (1968b) reported association constants of the order of 6×10^3 to 6×10^4 litres/mole for monoclonal IgG and IgM cryoglobulins which reacted with the Fc region of human IgG. Abraham *et al.* (1972) recently reported, in a patient with hypergammaglobulinaemic purpura, a homogeneous IgA antiglobulin which reacted with an Fc antigen shared by IgG1, IgG2 and IgG4 proteins—possibly the Ga antigen known to be commonly involved in rheumatoid factor reactions with the Cγ2 homology region (Allen & Kunkel, 1966; Gaarder & Natvig, 1970). Using the equilibrium molecular sieving technique, an association constant of $1{\cdot}5 \times 10^6$ litres/mole was calculated for this antiglobulin.

Normansell (1970, 1971) measured the binding constant of rheumatoid factors obtained from the sera of patients with rheumatoid arthritis. He obtained the low value of 4×10^5 litres/mole, using both native and heat aggregated IgG as antigen. This value probably represents the pooled effect of a number of different systems including antigens from both the Cγ2 and Cγ3 homology regions.

Using pFc′ fragments prepared from selected myeloma proteins we have measured the binding characteristics of what are virtually single systems, though even here it is impossible to exclude the contribution of other, unknown, antigenic sites. The very poor binding for pFc′ fragments lacking antigens against which the rheumatoid sera had agglutinating antibody, supports the specificity of the reaction.

The low binding constants observed in the six rheumatoid sera are similar to the values obtained by Normansell (1970, 1971) and contrast with the rather higher values for the sensitized healthy human, the immunized baboon and the immunized rabbit. Since it is likely that the rheumatoid sera contain (on the patient's own IgG) the antigens reacting with the rheumatoid factor, one explanation for the low affinity antibody is *in vivo* elimination of the higher affinity antibody. This would be less likely in the human and other animals actively sensitized with foreign IgG. However, some such *in vivo* antibody elimination may have occurred in this group also since the binding constants obtained were lower than is usual in animals hyperimmunized with other antigens (Soothill & Steward, 1971). This is possible since the Cγ3 homology region is probably very similar in different species; for example there is 66% sequence homology between the human and rabbit Cγ3 regions. An alternative explanation is that the patients with rheumatoid arthritis (a presumed chronic soluble complex disease (Winchester, Agnello & Kunkel, 1969; Natvig *et al.*, 1971; Munthe

& Nativig, 1972; Zvaifler, 1973) have the characteristic of making low affinity antibody which has been shown in certain in-bred strains of mice (Soothill & Steward, 1971) reported to be prone to chronic soluble complex disease. Such antibody achieves little or no immune elimination of antigen (Alpers, Steward & Soothill, 1972) so there is presumably little elimination of antibody. A possible way of differentiating between the two main hypotheses is to compare the affinity of R.F. in the joint fluid with that of R.F. in the serum of the same patient.

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