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The binding constants of IgM rheumatoid factors and their univalent fragments for native and aggregated human IgG

S. DISSANAYAKE*, F. C. HAY & I. M. ROITT *Department of Immunology, The Middlesex Hospital Medical School, London*

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Summary. IgM rheumatoid factors (RF) were isolated from the sera of patients with rheumatoid arthritis and a serologically active Fabμ RF fragment prepared by papain digestion. A radioimmunoassay was developed for the determination of interaction of 19S IgM RF and Fabμ RF with human 7S IgG, heat-aggregated IgG, rabbit 7S IgG, and human pFc'. RF isolated under neutral conditions had a very low binding constant for human 7S IgG (of the order of 10^2 to 10^3 1 mole^{-1}) and a considerably higher value (ca. 10^5) for the aggregated protein and monomeric rabbit IgG. RF obtained under acid conditions which dissociate the complexes with endogenous Ig, had a higher avidity for human IgG monomer as expected and also a comparable reactivity with rabbit IgG. Monovalent Fabμ fragments of 'acid' RF had closely similar affinities for 7S and aggregated IgG suggesting that the enhanced binding with the aggregated protein is essentially dependent on its multivalency rather than the exposure of a new determinant lacking in the native molecule.

INTRODUCTION

19S IgM rheumatoid factor (RF) reacts strongly with immune complexes or aggregated IgG but binds only weakly to native 7S IgG. Whether this preferential reactivity with immune complexes is due to combination with a new antigenic determinant expressed on the complexes, perhaps through a conformational change, or whether the increased binding is the result of the 'bonus effect' of multivalency brought about by the presentation of a polyvalent aggregated complex of Fc regions to the multivalent RF, has been a subject of controversy. Although it has been reported that IgM RF reacts both with native 7S IgG (Schur & Kunkel, 1965; Normansell & Stanworth, 1968; Normansell, 1970; Gaarder & Natvig, 1970) and with heat-aggregated or denatured IgG (Hirose & Osler 1965, 1967; Normansell, 1971), Henney (1969) failed to detect any binding of radiolabelled monomeric IgG to IgM RF. However, the demonstration of 22S complexes (formed from 19S RF and 7S IgG: Kunkel, Müller-Eberhard, Fudenberg & Tomasi, 1961; Normansell & Stanworth, 1968; Normansell, 1970) and of hidden rheumatoid factors with specificity for native IgG (Gaarder & Natvig, 1970) show that at least some of the rheumatoid factors with specificity for native IgG occur as complexes in the serum. These 22S complexes can be dissociated to yield the constituents 19S IgM RF and 7S IgG on treatment with weak acids (Normansell & Stanworth, 1966) and hence isolation of rheumatoid factors by gel filtration in acid buffers should release the bound RF with greater specificity for native IgG.

* Present address: Department of Biochemistry, Faculty of Medicine, Peradeniya Campus, Peradeniya, Sri Lanka.

Correspondence: Dr F. C. Hay, Department of Immunology, Middlesex Hospital Medical School, Arthur Stanley House, 40–50 Tottenham Street, London, W1P 9PG.

The intrinsic association constants have been used to compare the reactivity of IgM RF with native and aggregated IgG. The association constant of IgM RF with native IgG, as determined by ultracentrifugation was $3\text{–}5 \times 10^5$ 1 mole^{-1} (Normansell, 1970); significantly, the association constant for aggregated IgG, estimated from the binding of the monomer of IgM RF to sheep red cells coated with aggregated IgG was of the same magnitude (Normansell, 1971). On this basis, the increased binding of RF to aggregated IgG has been attributed to the multivalent nature of the aggregate.

In view of the importance of this point, we decided to re-examine these interactions using the univalent Fabμ fragment prepared from IgM RF so that contributions to the binding energy caused by multivalency could be unequivocally excluded. In addition, using IgM RF isolated under dissociating and non-dissociating conditions with respect to the 22S complex, we have determined the binding constants with human 7S IgG, rabbit 7S IgG, aggregated IgG and human pFc'. Our results support the view that the enhanced reactivity with aggregated IgG is due essentially to the multivalent nature of the aggregate.

MATERIALS AND METHODS

Rheumatoid sera

Sera giving a high titre in the sheep cell agglutination test (SCAT) were obtained from three patients (E.W., M.S. and J.H.) with classical RA and stored at $-20°$.

IgM rheumatoid factor

(a) *Isolation in acid medium.* RA serum which had been centrifuged at 40,000 *g* for 2 h at 4° to remove lipid, was diluted 1:1 with phosphate-buffered saline (PBS, pH 7·2) and the γ-globulins precipitated at a 45 per cent saturation with ammonium sulphate in PBS. The precipitate was redissolved in PBS, dialysed against citrate-phosphate buffer, pH 4·0, 0·1 M, and chromatographed on Sepharose 6B in the same buffer. The protein concentration in the eluates was continuously monitored at 254 nm. All the fractions were concentrated separately, dialysed against PBS and tested for RF activity by the sheep cell agglutination test (SCAT) and for IgM by immunoelectrophoresis using specific anti-human IgM after adjusting the concentration to 1 mg/ml. The fractions which contained IgM with agglutinating activity were pooled and re-run under acid conditions at least once on Sepharose 6B. This preparation of IgM RF will be called IgM RF (acid).

(b) *Isolation at neutral pH.* To isolate free IgM RF from that bound in the 22S complexes, the ammonium sulphate precipitate was obtained as above, and chromatographed on Sepharose 6B in PBS. The fractions were tested for SCAT positive IgM RF as described. This preparation of IgM rheumatoid factor (19S IgM RF, neutral) was recycled on Sepharose 6B at least twice to remove the contaminating IgG.

Non-rheumatoid IgM

IgM without antiglobulin activity was obtained from the plasma of a patient with Waldenström's macroglobulinaemia by gel filtration on Sepharose 6B at pH 4·0 in citrate-phosphate buffer followed by Pevikon block electrophoresis at pH 8·2 in veronal buffer (*I*, 0·01).

Preparation of Fabμ

Fabμ from IgM RF (acid) was obtained by papain digestion as described by Onoue, Kishimoto & Yamamura (1968). 150 mg of IgM RF (from E.W. serum and pooled E.W., M.S. and J.H. sera) were digested with 10 mg of papain (Worthington Biochemicals, New Jersey) in the presence of 0·01 M cysteine and 0·002 M EDTA at 37° for 24 h. The digestion mixture was chromatographed on Sephadex G-150 at pH 4·5 (acetate buffer, 0·1 M). Two major peaks were obtained and the fractions reactive with anti-κ and λ sera were pooled and used in the binding studies.

Non-antibody Fabμ was prepared from Waldenström IgM by a similar method.

Purification of IgG

(*a*) *7S IgG* human γ-globulins (Cohn Fr. II, Miles Laboratories Research Division, England) were purified by ion exchange chromatography on DEAE-cellulose at pH 8·1 (0·01 M phosphate). 7S IgG was then obtained by gel filtration on a Sephadex G-200 column. Rabbit IgG (Miles Laboratories) was purified on Sephadex G-200 to obtain 7S IgG.

(*b*) *Aggregated immunoglobulins.* IgG was aggregated by heating 7S IgG at 63° for 15 min and polymers of approximate mol. wt 800,000 isolated by gel filtration on a calibrated Sepharose 6B column. Urea denaturation of human 7S IgG was performed by dialysis against 8 M urea, the larger aggregates being removed by gel filtration on Sephadex G-200.

Human pFc'

pFc' of human IgG was prepared by pepsin digestion according to the method of Turner & Bennich (1968).

Radiolabelling of immunoglobulins

Immunoglobulins and fragments were labelled with ^{125}I (Na ^{125}I, IMS4, Radiochemical Centre, Amersham) by the chloramine T method of Hunter & Greenwood (1962). 1 mg of 7S IgG or altered IgG in 1 ml of PBS was treated at room temperature with 1 mCi of ^{125}I and 60 μg of chloramine T in 10 μl of water for 2 min. The reaction was stopped by adding 120 μg of sodium metabisulphite in 10 μl of water. The free iodine was removed by gel filtration on Sephadex G-25 and exhaustive dialysis against PBS.

The Fabμ and pFc' preparations were labelled with ^{125}I (0·5 mCi/mg protein) by the same method.

Specific anti-human IgM serum

IgM with rheumatoid factor activity, obtained from serum E.W., was further purified by Pevikon block electrophoresis. Rabbits were immunized with 300 μg of IgM at 10-day intervals in complete Freund's adjuvant, in incomplete Freund's adjuvant and as an alum precipitate respectively. Rabbits were bled 10 days after the final boosting. Antisera were made specific, as assessed by immunoelectrophoresis and Ouchterlony immunodiffusion against human serum, by absorption with insoluble immunoadsorbents of human IgG and human α-globulins.

Specific anti-human IgG serum

Antisera raised in sheep against Fcγ were made specific, as judged by immunoelectrophoresis and Ouchterlony immunodiffusion against human serum, by absorption with insoluble human Fabγ.

Assay of radiolabelled IgM rheumatoid factor by binding to heat-aggregated human IgG

The method involved the incubation of various amounts of ^{125}I-labelled IgM with a constant amount of aggregated IgG, followed by precipitation of the complexes with a specific sheep anti-human IgG and determination of the amount of labelled IgM in the precipitate.

Various quantities of ^{125}I-labelled 19S IgM RF preparations and non-rheumatoid factor IgM in 0·2 ml of PBS were incubated with 200 μg of heat-aggregated human IgG in 0·2 ml of PBS containing a known amount of a standard solution of ^{131}I-labelled human albumin, at 37° for 3 h and overnight at room temperature. After incubation, 0·2 ml of the mixture was withdrawn and added to 0·2 ml of specific sheep anti-human IgG. Precipitation was performed at 37° for 30 min and at 4° for a further 15 min. After mixing on a 'Whirlimixer', 0·2 ml were withdrawn and spun in a Beckman microfuge (15,000 r.p.m./5 min). The supernatant was removed carefully and the unwashed precipitated counted simultaneously for ^{131}I and ^{125}I in a Packard Autogamma Counter, corrections being made for the overlapping of ^{131}I and ^{125}I channels. After correction for the amount of unbound IgM dissolved in the contaminating buffer (calculated from the ^{131}I counts), the results were expressed as μg of ^{125}I-labelled rheumatoid factor and non-rheumatoid factor IgM bound.

Binding of ^{125}I-labelled native, aggregated and urea-denatured human IgG

The method involved the incubation of various amounts of ^{125}I-labelled antigen with a known constant amount of IgM RF, followed by precipitation of the immune complexes with specific rabbit anti-human IgM and determination of the labelled antigen in the precipitate.

100 μg of RF IgM (SCAT titre 256–512 at 1 mg/ml) in 0·2 ml of PBS was added in duplicate to various amounts of ^{125}I-labelled IgG in 0·2 ml PBS (antigen:antibody ratios between 0·5:1–20:1) contained in small glass test tubes (10 × 1·2 cm). To each tube, 0·2 ml of a standard preparation of ^{131}I-labelled human albumin was added as a volume marker. The tubes were incubated at 37° for 3 h and overnight at room temperature with occasional mixing. After incubation the tube contents were mixed and duplicate 0·2-ml aliquots withdrawn; the immune complexes were then precipitated with 0·2 ml of an appropriately diluted rabbit anti-human IgM antiserum, precipitation being performed at 37° for 30 min and 15 min at 4°. After mixing the tube contents on a 'Whirlimixer', 0·2 ml samples were withdrawn, transferred to microfuge tubes and spun at 15,000 r.p.m. for 5 min in a Beckman Type 152 microfuge. The supernatant was removed carefully and the unwashed precipitates counted for ^{125}I and ^{131}I in a Packard Autogamma Counter, corrections being made for the overlapping of ^{125}I and ^{131}I channels. The amount of free antigen present in the contaminating buffer was calculated from the ^{131}I counts. Controls with non-rheumatoid IgM pre-

parations were routinely set up. The results are expressed as μg of native, heat-aggregated or urea-denatured IgG bound to 10^{-4} μmoles of RF IgM corrected for the amount bound to the non-RF IgM control.

Determination of binding affinities of 19S IgM RF isolated at acid and neutral pH

The basic principle of the method was to determine the concentration of free and bound antigen at equilibrium and thereby calculate K_{av} either from a conventional Scatchard (1949) plot or from a 1/b *vs* 1/c plot. The concentration of bound antigen was determined by a method exactly similar to that described above for the binding of labelled IgG to IgM RF with the following modifications. After incubation and before adding the antiserum, 0·1 ml of the mixture was removed and preserved for counting. The amount of total antigen added was calculated from the ^{125}I counts obtained with this fraction. After incubation with the anti-IgM antiserum, 0·2 ml aliquots were spun at 15,000 r.p.m. in a microfuge and 0·1 ml of the supernatant was counted. The amount of antigen remaining free was calculated from these counts. The amount of antigen bound was calculated from the counts in the precipitate corrected for contamination with free antigen and the amount bound by the non-RF control.

The results are expressed both as a conventional Scatchard plot of r/c *vs* r, where r = μmoles of antigen bound per μmole of antibody and c = μmoles of free antigen also according to the method of Steward & Petty (1972) where 1/b is plotted against 1/c (b = μmoles of antigen bound, and c = μmoles of antigen remaining free).

The average binding constants were calculated from the Scatchard plots as the reciprocal of the free antigen concentration at r = n/2, where n is the number of binding sites on the RF molecule. From the 1/b *vs* 1/c plots, the total antibody combining sites were determined by extrapolation to 1/c = 0, and K_{av} when 50 per cent of the sites were occupied by the antigen (Steward & Petty, 1972).

Binding affinities of RF Fabμ

Fabμ obtained from RF IgM and the Waldenström IgM were labelled with ^{125}I by the chloramine T method. The biological activity of the labelled Fabμ RF was checked by SCAT inhibition, and only those preparations which inhibited the agglutination of an RF IgM preparation diluted to give a SCAT of $\leq$16 were used in the binding studies. Various amounts of [^{125}I]Fabμ RF were incubated with 100 μg of native or aggregated human IgG in 0·2 ml PBS (antibody: antigen ratios between 1:1 to 20:1) ^{131}I-labelled human albumin was used as a volume marker. In the control experiments, the same amounts of Waldenström Fabμ were used. The immune complexes were precipitated with specific sheep anti-human IgG. The association constants were calculated from 1/b *vs* 1/c plots, where b = μmoles of Fabμ RF bound and c = μmoles of Fabμ RF remaining free.

RESULTS

Isolation of rheumatoid factors

19S IgM RF not linked to IgG to form 22S complexes was isolated by gel filtration in neutral buffer on Sepharose 6B (exclusion limit, 4×10^6) and total IgM RF (bound and unbound) by gel filtration at pH 4·0 (citrate phosphate buffer, 0·1 M). A representative Sepharose 6B fractionation of E.W. serum at neutral and acid pH is shown in Fig. 1. 19S IgM

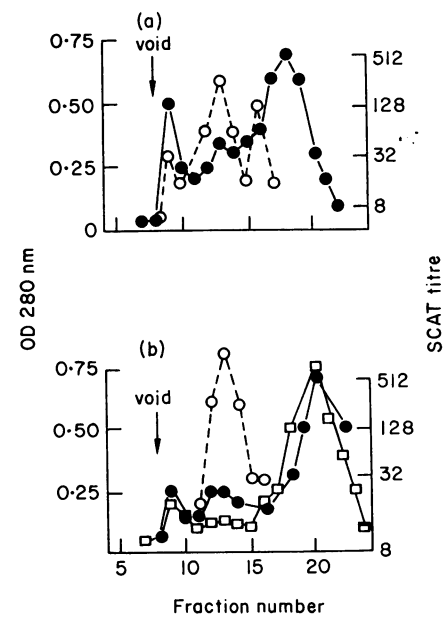

Figure 1. Sepharose 6B fractionation of the ammonium sulphate precipitate of serum E.W. (a) neutral pH (PBS), (b) acid pH (citrate-phosphate buffer 0·1 M, pH 4·0). (●——●) OD serum E.W.; (□——□) OD normal serum; (○ . . . . ○) SCAT activity. Column size 1·6 × 90 cm, sample size 5 ml; 5-ml fractions were collected.

RF isolated at acid pH had a slightly higher SCAT activity than 19S IgM RF isolated at neutral pH, on average one dilution more. On immunoelectrophoresis no IgG was detected, but there was a slight contamination by non-IgM macroglobulins. No attempt was made to remove these contaminants in order to avoid any further fractionation and subsequent deterioration of RF preparations. The two minor SCAT-positive peaks obtained when the serum was separated at neutral pH disappeared on treatment with acid (Fig. 1) suggesting the possibility of immune complexes or aggregates.

Preparation of RF Fabμ

RF Fabμ prepared by papain digestion of 19S IgM RF was isolated by gel filtration on Sephadex G-150 at pH 4·8 (acetate buffer 0·1 M). The fractions that reacted with anti-light chain antisera were pooled and tested by immunoelectrophoresis and inhibition of agglutination of sheep cells coated with rabbit γ-globulin by a very weak IgM RF preparation (SCAT-titre $\leqslant 16$). Some Fabμ RF preparations contained small amounts of IgM Fc subfragments detected by immunoelectrophoresis with specific anti-IgM serum. Only those Fabμ RF preparations that inhibited the agglutination of the diluted IgM RF preparation at a concentration of 1 mg/ml were used in the binding studies.

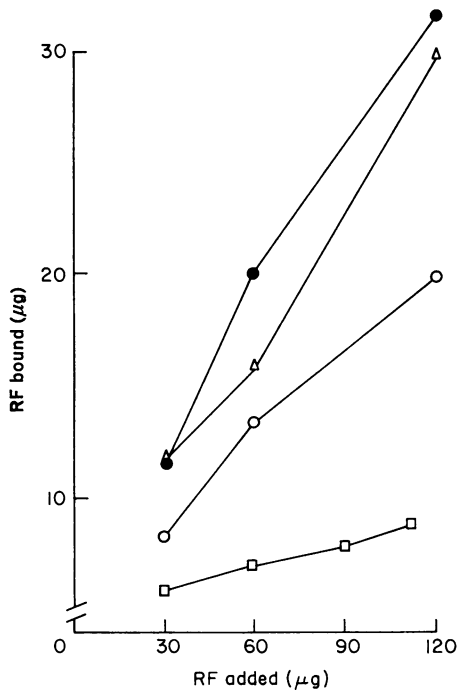

Figure 2. Radioassay of rheumatoid factor: binding of ^{125}I-labelled IgM RF (acid) and non-rheumatoid factor IgM to 200 μg heat-aggregated human IgG. (□) non-rheumatoid factor IgM; (○) void peak from Sephadex G-200 fractionation of serum E.W.; (△) 19S IgM from Sepharose 6B fractionation of serum E.W.; (●) 19S IgM from Sepharose 6B fractionation of serum J.H.

Radioassay of IgM RF

To determine the amount of serologically active RF to use in the avidity studies, the concentration of IgM RF in a given preparation was determined either by absorbtion onto a heat-aggregated human IgG immunoadsorbent or by incubation of ^{125}I-labelled IgM RF with heat-aggregated human IgG and precipitation of the complexes with specific sheep anti-human IgG serum. The amount of RF bound was calculated from the radioactivity in the precipitate (Fig. 2). Of the total protein in the RF (acid, serum E.W.) preparation, 26 ± 7 per cent was shown to bind specifically to aggregated IgG.

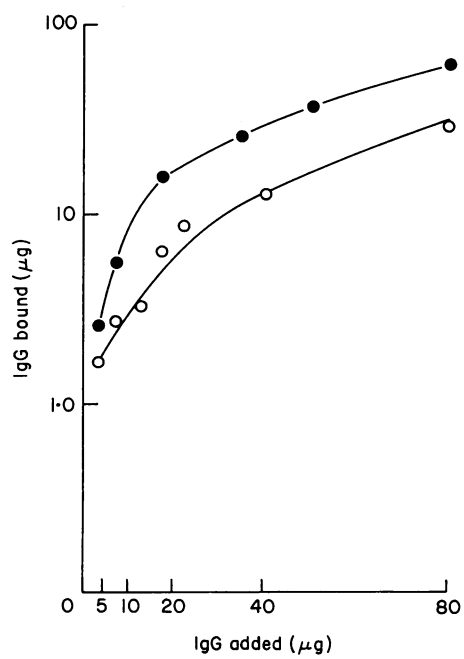

Figure 3. Binding of 19S IgM RF (acid) to ^{125}I-labelled native (○) and aggregated (●) human IgG. Results expressed as binding of antigen to 10^{-4} μmoles of IgM RF.

Binding of 19S IgM RF (acid) to radiolabelled native 7S and aggregated human IgG

The binding of native and heat-aggregated human IgG to 19S IgM RF isolated under acidic conditions was measured as μg of ^{125}I-labelled antigen (native or heat-aggregated human IgG) bound to $10^{-4}\mu$ moles of RF. Aggregated bound approximately twice as well as native IgG on a weight basis (Fig. 3).

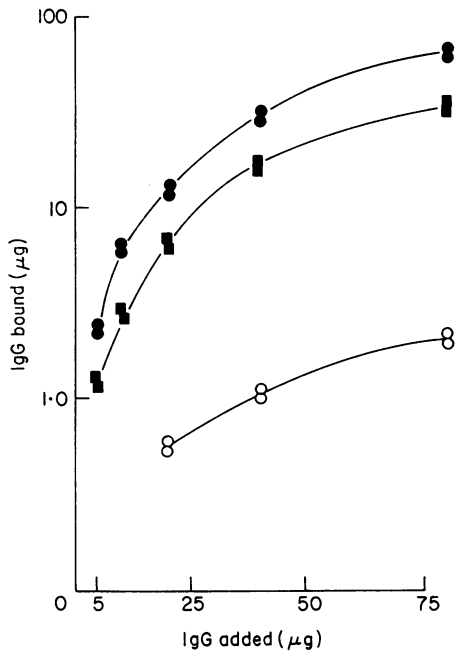

Figure 4. Binding of 19S IgM RF (neutral) to ^{125}I-labelled native (○), urea-denatured (■) and heat-aggregated (●) human IgG. Results expressed as binding of antigen to 10^{-4} μmoles IgM RF.

Binding of 19S IgM RF (neutral) to radiolabelled native and heat-aggregated IgG

19S IgM RF (neutral) bound similar amounts of heat-aggregated IgG as did 19S IgM RF (acid) but only very small quantities of native IgG (Fig. 4). Denaturation with urea increased the reactivity of native IgG with 19S IgM RF (neutral) (Fig. 4).

Binding of Fabμ RF to radiolabelled native and aggregated human IgG

Very little difference was observed in the binding of native or heat-aggregated IgG to Fabμ RF (Fig. 5).

Association constants for 19S IgM RF (acid)

Association constants for the interaction of 19S IgM RF (acid) with native 7S and heat-aggregated human IgG and rabbit 7S IgG are given in Table 1, which presents the results obtained in different experiments.

The K_{av} values are indicative of a relatively weak interaction. They are of the same order of magnitude for native 7S human and rabbit IgG, but were of the order of ten-fold greater for aggregated human IgG.

Association constants for 19S IgM RF (neutral)

The association constants for 19S IgM RF (neutral) with heat-aggregated human IgG and 7S rabbit IgG were of the same order as those for 19S IgM RF (acid) (Table 2). However, with native human IgG, the binding was very low and insufficient for a Scatchard plot. K_{av} for 19S IgM RF (neutral) and 7S native IgG were therefore calculated from 1/b and 1/c plots (Fig. 6) and found to be less than 10^3 1 mole^{-1}.

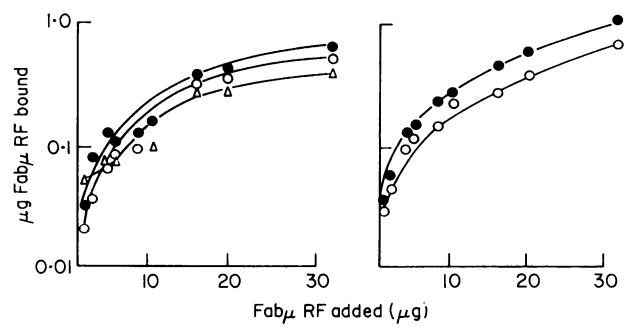

Figure 5. Binding of ^{125}I Fabμ RF to 100 μg native or heat aggregated human IgG in two different experiments. (●) heat aggregated human IgG preparation 1; (△) heat aggregated IgG preparation 2; (○) native 7S IgG.

Table 1. Average binding constants for IgM RF (Acid)

Antigen	K_{av} l. mole^{-1}	
	Individual results	Geom. Mean
7S human IgG	$0{\cdot}8\times10^4$, $1{\cdot}1\times10^4$, $1{\cdot}4\times10^4$	$1{\cdot}1\times10^4$
Agg human IgG	$1{\cdot}8\times10^4$, $1{\cdot}9\times10^5$, $2{\cdot}1\times10^5$	$8{\cdot}9\times10^4$
7S rabbit IgG	$1{\cdot}3\times10^4$, $1{\cdot}2\times10^4$	$1{\cdot}2\times10^4$

Table 2. Average binding constants (K_{av} 1 mole^{-1}) for 19S rheumatoid factors isolated under acid and neutral conditions

Antigen	RF (E.W.) acid	RF (E.W.) neutral	RF (pooled) neutral
7S human IgG	$1{\cdot}1\times10^4$	$3{\cdot}2\times10^2$	1×10^3
Agg human IgG	$8{\cdot}9\times10^4$	$3{\cdot}1\times10^4$	$7{\cdot}4\times10^4$
7S rabbit IgG	$1{\cdot}2\times10^4$	$1{\cdot}7\times10^4$	$9{\cdot}2\times10^4$

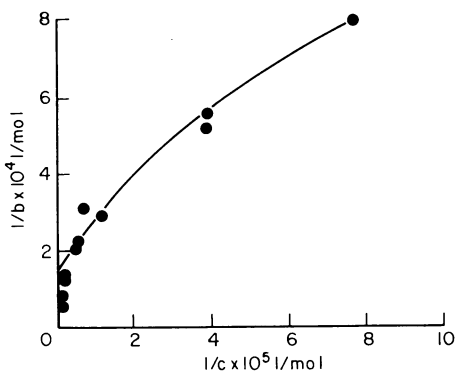

Figure 6. Representative $\frac{1}{b}$ *vs* $\frac{1}{c}$ plot for the interaction of 19S IgM RF (neutral) with heat-aggregated human IgG.

It might be argued that there was sufficient time during the coprecipitation of the complexes with rabbit anti-IgM for the rheumatoid factor to react with the rabbit IgG in the final precipitate and then bind further free radiolabelled IgG. That this is unlikely may be inferred from the considerably lower avidity for monomeric relative to polymeric IgG even though multiple rabbit antibodies would be attached to the human IgM in both cases; the avidity of 'neutral' RF for monomeric human IgG is even lower despite the fact that it binds more strongly than the 'acid' RF to rabbit IgG.

Binding constants for the interaction of 19S IgM RF with human pFc′

The association constant for human pFc′ and 19S IgM RF (acid) was determined by a similar method to that of native and aggregated IgG. The pFc′ was labelled with ^{125}I and the complexes precipitated with specific rabbit anti-human IgM. The values are given in Table 3. These are similar to those found previously by Steward, Turner, Natvig & Gaarder (1973).

Table 3. Average binding constants of rheumatoid factor Fabμ fragments

Antibody*	Antigen†	K_{av} 1 mole^{-1}
19S RF (E.W.)	pFc′	$0{\cdot}6\times10^4$
19S RF (E.W.)	7S IgG	$1{\cdot}1\times10^4$
19S RF (E.W.)	Agg IgG	$8{\cdot}9\times10^4$
Fabμ (pooled) RF	7S IgG	$0{\cdot}9\times10^4$
Fabμ (pooled) RF	Agg IgG	$1{\cdot}5\times10^4$
Fabμ (E.W.) RF	7S IgG	$0{\cdot}2\times10^4$
Fabμ (E.W.) RF	Agg IgG	$0{\cdot}3\times10^4$

* Isolated from serum under acid conditions.
† All IgG of human origin.

Binding constants for the interaction of Fabμ RF with native and aggregated IgG

The method of determination of binding constants was similar to that used for 19S IgM RF, but instead of the conventional method of varying antigen concentrations, a fixed amount of the antigen and varying quantities of ^{125}I-labelled Fabμ RF were used. The complexes were precipitated with specific sheep anti-human IgG serum. The results are shown in Fig. 7 and Table 3. Within the constraints imposed

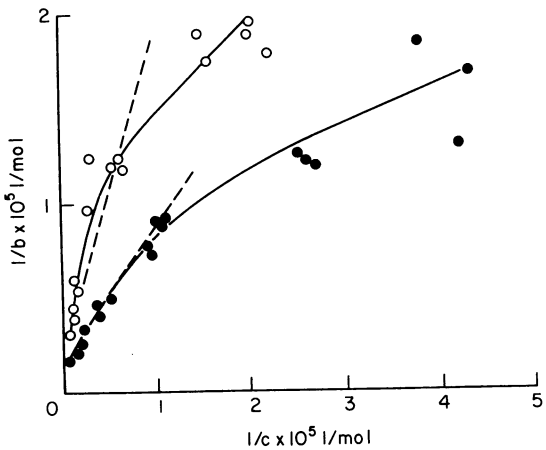

Figure 7. Representative $\frac{1}{b}$ *vs* $\frac{1}{c}$ plot for the interaction of Fabμ RF with native (○) and heat-aggregated (●) human IgG.

by the system, the Fabμ RF preparations showed equal reactivity with the monomer and aggregated IgG. The binding constant of Fabμ (E.W.) for 7S IgG was a little lower that that calculated for the intact IgM RF, but this might easily be attributed to changes occurring in the molecule during the preparation of the Fabμ subunit.

It is possible that the apparent equal reactivity with monomer and aggregated human IgG could be due to binding to the sheep IgG bound in the complex after the addition of the sheep anti-human IgG. This seems unlikely, however, as the binding constant for Fabμ RF to both aggregated and monomer IgG was similar to that for whole IgM RF reacting with monomer IgG.

DISCUSSION

The classical method of isolation of IgM RF is gel filtration of RF serum in acid buffer on Sephadex G-200, followed by the adsorption of the macroglobulin fraction on to insolubilized IgG and elution of the rheumatoid factors. Gel filtration in acid buffer dissociates the 22S complexes and releases the bound IgM RF. Our preliminary studies showed that acid elution from the immunoadsorbent may not release the very high affinity antibodies and that exposure to acid buffers at this stage may result in the denaturation of some IgM RF. We have therefore avoided the use of affinity chromatography but have carried out these studies with the appropriate IgM serum fractions.

The curvature observed in the Scatchard plots and sometimes in the 1/b *vs* 1/c plots are indicative of the heterogeneity of the rheumatoid factors with respect to their binding affinities. Hence any extrapolation is inaccurate and due to the errors involved in the determination of the absolute antibody concentration, the calculated values for K are only approximately correct (Werblin & Siskind, 1972). Irrespective of the method and conditions of isolation, 19S IgM rheumatoid factors from the three sera had binding constants of the order of 10^4–10^5 1 mole^{-1}, for heat-aggregated human IgG. The binding constants reported by Normansell (1970; 1971 and 1972) are of the same order of magnitude, but the constants reported for native and aggregated human IgG are not directly comparable because of the differences in the methods involved. A monoclonal IgA RF was found to have a binding constant of $1{\cdot}5 \times 10^6$ 1 mole^{-1} (Abraham, Clark & Vaughan, 1972), while lower constants (10^3–10^4 1 mole^{-1}) were reported for monoclonal IgG and IgM cryoglobulins which reacted with the IgG Fc (Cerrottini & Grey 1969; Stone & Metzger 1968), although the circumstances are different from those operating in the rheumatoid diseases where the polyclonal rheumatoid factors presumably arise through antigenic stimulation of the immune system. It may be pertinent to recall that IgM antibodies in general tend to be of lower affinity than IgG (Hornick & Karush, 1972) and the values reported here—at least for the more avid RF molecules released by acid treatment—are of the same order as those found after stimulation by other antigens such as DNP linked to phage $\phi \times 174$. There were interesting differences in the reactivity of 19S RF with monomeric IgG depending on the pH used for isolation of the rheumatoid factor. The material obtained under acid conditions was of higher avidity for 7S human IgG than that

isolated at pH 7·2 since at neutrality the high avidity antibodies are complexed to IgG (present as aggregates? cf. Hay, Nineham, Torrigiani & Roitt, 1976). The 'acid' RF showed comparable reactivity with rabbit and human monomeric IgG but the average binding constant for aggregated human IgG was some eight times higher. The low avidity RF obtained at neutral pH bound much more strongly both with the aggregated protein and surprisingly with rabbit monomeric IgG; indeed the binding constant for rabbit monomer was almost the same as that for aggregated human IgG. In other words, unlike high avidity RF, the low avidity antiglobulin recognizes a determinant on rabbit IgG which is not present on the human molecule.

Some increase in binding with the aggregated human protein would be anticipated due to multivalence and the greater 'bonus effect' seen with the lower avidity RF is consistent with such a model. We were still unable to say whether the increased binding to aggregated IgG could be explained entirely on this basis or whether exposure of a new 'rabbit-like' determinant on aggregation was making a contribution. The problem was circumvented by the use of monovalent Fabμ derived from 19S rheumatoid factor, isolated under acid conditions, which excluded any influence due to multivalency. This reagent bound monomeric and polymeric human IgG with closely comparable avidities which strongly indicates that the increased binding of 19S RF to aggregated IgG is largely, if not entirely, a multivalence effect. However, we cannot rule out the possibility that a different mechanism might operate for the binding of 19S rheumatoid factor isolated in neutral buffer.

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