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Reduction of the complement activation capacity of soluble IgG aggregates and immune complexes by IgM-rheumatoid factor

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Summary. The influence of IgM-rheumatoid factor (IgM-RF) on the activation of isolated C1 by soluble IgG aggregates (AIgG) and immune complexes was studied. IgM preparations obtained from the sera of four patients with seropositive rheumatoid arthritis markedly reduced the C1 activation capacity of AIgG, especially when large aggregates were tested. The results of parallel experiments with radiolabelled AIgG indicated that this inhibitory effect of IgM-RF was accompanied by a very large increase of the aggregate size. A comparable IgM preparation isolated from pooled normal human serum influenced neither the size nor the C1 activation capacity of AIgG. The inhibitory effect of IgM-RF on C1 activation was also demonstrated for soluble tetanus–anti-tetanus immune complexes. Thus, in spite of the established C activation ability of IgM-RF and the fact that, in general, larger IgG aggregates and immune complexes activate C1 more efficiently, cross-linking and size enlargement of soluble IgG complexes and aggregates by IgM-RF lead to a decrease of the C1 activation capacity. As a consequence, IgM-RF may reduce plasma complement activation by soluble IgG complexes in the circulation of patients with seropositive rheumatic diseases.

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INTRODUCTION

IgM-rheumatoid factor (IgM-RF) is an autoantibody directed against the Fc part of IgG and is found in the sera of many patients with connective tissue disorders, in particular rheumatoid arthritis (RA) (Carson, 1981). Routinely, IgM-RF is detected by its ability to agglutinate IgG-coated latex particles or erythrocytes. Besides aggregating these IgG-coated particles, IgM-RF can also bind, cross-link, and precipitate soluble immune complexes containing IgG antibodies and soluble non-specifically aggregated IgG (Epstein, Johnson & Regan, 1956; Zvaifler & Schur, 1968; Tesar & Schmid, 1970). Since soluble IgG aggregates and immune complexes can activate the classical pathway of complement (C), it is of interest to know whether and, if so, how their cross-linking by IgM-RF influences C activation. Since the size of soluble IgG aggregates or immune complexes is a major factor determining their efficiency as activators of the classical complement pathway (Doekes, van Es & Daha, 1982, 1984), it might be expected that their reaction with IgM-RF resulting in further aggregation would promote C activation. Furthermore, the binding of IgM-RF to its epitopes might induce C activation. Although early reports indicated an inhibitory effect of IgM-RF on C activation by immune complexes (Zvaifler & Bloch, 1962; Davis & Bollet, 1964), several authors have shown that IgM-RF itself can initiate classical pathway activation when bound to IgG-

coated erythrocytes (Tanimoto *et al.*, 1975) or IgG-coated microwells (Sabharwal *et al.*, 1982). In such studies, the IgG molecules used for coating were reduced and alkylated to render them incapable of C activation without losing their ability to bind IgM-RF.

The influence of IgM-RF on C activation by soluble IgG immune complexes has received less attention. Some reports have indicated an enhancing effect of IgM-RF, in particular with DNA–anti-DNA complexes (Birchmore *et al.*, 1981). However, recent studies revealed that many RA sera were deficient in inhibiting immune precipitation *in vitro* (Naama, Mitchell & Whaley, 1983; Balestrieri *et al.*, 1984), a property of normal fresh sera that depends on classical pathway activation (Schifferli, Woo & Peters, 1982); the lack of this inhibitory effect in RA sera was probably due to inhibition of classical pathway activation by IgM-RF (Mitchell *et al.*, 1984; Balestrieri *et al*, 1984).

We have investigated the influence of IgM-RF on the activation of isolated human C1 by stabilized soluble IgG aggregates. Radiolabelled and non-labelled aggregates of identical size were used to study the effects of IgM-RF on the size of the aggregates and on their C1 activation capacities in parallel. The results indicate that, in spite of an enormous increase in aggregate size, the C1 activation capacities of IgG aggregates are markedly diminished after preincubation with IgM-RF.

MATERIALS AND METHODS

Materials

Sephacryl-S300 (Pharmacia Fine Chemicals, Woerden, The Netherlands), peroxidase-conjugated rabbit IgG against human IgM (Dakopatts, Copenhagen, Denmark), *o*-phenylenediamine (Sigma Chemicals, St Louis, MO) and Diaflo XM-50 ultrafiltration membranes (Amicon Corporation, Lexington, MA) were purchased as indicated. Normal human and rabbit IgG and functionally pure human precursor C1 were isolated as described elsewhere (Doekes *et al.*, 1985).

Aggregates and immune complexes

Stabilized soluble aggregates of non-labelled and ^{125}I-labelled human IgG (AIgG) were prepared and fractionated simultaneously in parallel sucrose density gradients (Doekes *et al.*, 1982). They were stored at $-20°$ and used within 2 months.

Tetanus toxoid (TT) was purified from a formalinized filtrate of *Clostridium tetani* cultures (a gift of Dr J. Nagel, Rijksinstituut voor de Volksgezondheid, Bilthoven, The Netherlands) as described earlier (Doekes *et al.*, 1984). The IgG fraction from serum of a healthy individual with a high titre of anti-TT antibodies served as a source of human anti-TT IgG. Soluble TT–anti-TT immune complexes were prepared by mixing 2·8 mg of this anti-TT IgG fraction with 15 μg TT in a total volume of 0·4 ml borate-buffered saline (BBS). After incubation for 30 min at 37° and overnight at 4°, insoluble material was removed by centrifugation (20 min, 1800 ***g***) and the supernatant was used immediately. From preliminary experiments, it was known that the antigen–antibody ratio used resulted in soluble immune complexes with optimal C1 activation capacity.

IgM-rheumatoid factors and normal IgM

Sera were obtained from 20–40 ml of venous blood from four patients with seropositive classical rheumatoid arthritis. Pooled normal human serum (NHS) came from healthy volunteers. IgM-enriched preparations were obtained from the sera by euglobulin precipitation followed by gel filtration at low pH. Serum (10–15 ml) was diluted 1:20 with 2% (w/v) cold boric acid (pH 5·0) containing 0·02% azide, and left to stand overnight at 4°. The precipitate was centrifuged (15 min, 5000 ***g***), washed with 2% boric acid, centrifuged again, redissolved in saline, and dialysed against 0·1 M acetate, 0·25 M NaCl, 2 mM EDTA (pH 4·0). Further purification was accomplished by gel filtration on a 2·5 × 90 cm column of Sephacryl-S300 which was equilibrated and eluted at 20 ml/hr with the same acetate buffer as used for dialysis. The first protein peak eluting from the column was pooled, neutralized with 1 M Tris, dialysed against BBS containing 0·02% azide and 2% (v/v) glycerol, concentrated to 4–5 ml by ultrafiltration, and stored in aliquots at $-20°$ until use.

ELISA for IgM-RF

IgM-RF in sera and in isolated IgM preparations was measured by ELISA. Briefly, samples diluted in phosphate-buffered saline containing 0·1% Tween and 1% fetal calf serum (PBS-Tween-FCS) were incubated for 1 hr at 37° in microwells coated with 10 μg purified normal rabbit IgG. After thorough washing with PBS-Tween, bound IgM was detected by incubation (1 hr, 37°) with peroxidase-labelled rabbit IgG anti-human IgM, diluted 1/500 in PBS-Tween-FCS. After

another wash cycle with PBS-Tween, *o*-phenylenediamine (100 μg/ml) was added in 0·05 M phosphate (pH 5·6) containing 0·02% H_2O_2, and incubated for 20 min at room temperature. The reaction was stopped by adding 10% H_2SO_4 and the optical density at 492 nm was read with a multiscan spectrophotometer.

Determination of aggregate size

In order to assess the effect of IgM-RF or normal IgM on the size of soluble IgG aggregates, 0·1 ml portions of radiolabelled AIgG (160 μg/ml) were mixed with 0·1 ml of BBS, normal IgM, or IgM-RF preparations. The mixtures were incubated for 30 min at 37° and 2·5 hr at 0°, and then diluted with 0·2 ml BBS, after which 0·3 ml samples were centrifuged for 1 hour at 263,000 *g* in 10–30% sucrose gradients as used for the preparation of AIgG. From each gradient, 31–32 fractions were collected and the distribution of radioactivity was determined with a gamma counter.

C1 activation

For complement experiments, non-labelled AIgG (160 μg/ml) were mixed 1:1 with BBS, normal IgM, or IgM-RF, and incubated for 30 min at 37° and 2·5 hr at 0°. Dilutions of these mixtures were incubated for 30 min at 37° with 0·18 μg C1, in a total volume of 0·1 ml veronal-buffered saline containing 0·1% gelatin, 0·15 mM $CaCl_2$ and 0·5 mM $MgCl_2$, and finally the residual precursor C1's haemolytic activity was assessed as a measure of C1 activation (Doekes, van Es & Daha, 1983).

RESULTS

Isolation of normal IgM and IgM-RF

In preliminary studies, IgM-RF was isolated by affinity chromatography on Sepharose-coupled IgG, followed by elution with glycine-HCl at pH 2·8. Yields of IgM-RF obtained in this way were, however, very low, which might imply a loss of either low-affinity RF not bound to the Sepharose-IgG or of high-affinity RF not eluted at low pH. We therefore used total IgM fractions obtained by euglobulin precipitation and gel filtration on Sephacryl-S300. Gel filtration was performed at a relatively high salt concentration and a pH of 4·0 in order to dissociate immune complexes which might be present in the euglobulin preparation. Figure 1 shows a representative S300 profile in which the activity of IgM-RF, as measured by ELISA, clearly coincided with the high molecular weight protein peak.

Because IgM-RF preparations obtained in this way presumably contain considerable amounts of non-specific IgM, a control preparation of normal IgM (nIgM) isolated in the same way from pooled NHS was included in our experiments. The IgM-RF preparations, designated IgM-RF1, IgM-RF2, IgM-RF3 and IgM-RF4, and the preparation of nIgM, all contained IgM as their major constituent at concentrations of 0·12, 1·45, 0·73, 0·36 and 1·14 mg/ml, respectively. Contaminating IgG and IgA were present in similar amounts (up to 15–20%) in nIgM and the IgM-RF preparations. No IgM-RF was detectable

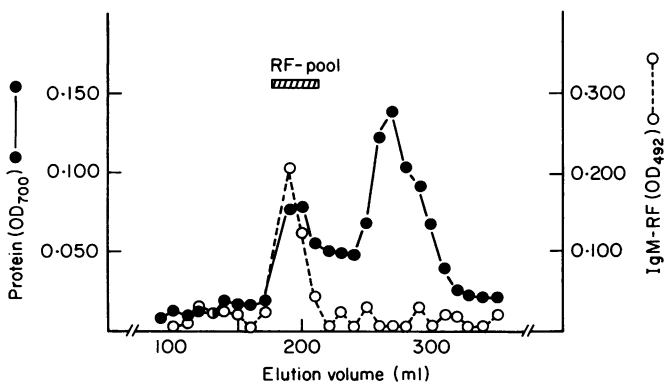

Figure 1. Gel filtration on Sephacryl-S300 of an euglobulin preparation of IgM-RF containing serum. The presence of IgM-RF in column fractions was detected by testing 1/50 dilutions in the IgM-RF ELISA.

in nIgM, whereas IgM-RF1 and IgM-RF4 showed moderate, and IgM-RF2, and IgM-RF3 strong, reactivity in the ELISA for IgM-RF (Fig. 2).

Effect of IgM-RF on the size of AIgG

Re-centrifugation for 1 hour of AIgG of various sizes that had been stored at −20° and were preincubated in BBS for 30 min at 37° and 2·5 hr at 0° before the run confirmed their stability (Figs 3 and 4). In contrast, preincubation of aggregates containing, on average, 40–45 or 80–90 IgG molecules with IgM-RF1 led to such a size increase that most of the material applied to the gradients reached the pellet (Fig. 3a and b). A similar but less pronounced enlargement was found after preincubation of smaller aggregates (18–22 IgG molecules) with IgM-RF1 (Fig. 3c), whereas monomeric IgG and IgG oligomers were apparently not aggregated (Fig. 3d). The other IgM-RF preparations behaved similarly: no effect on the size of monomeric or oligomeric IgG was noted, whereas larger aggregates underwent a very strong increase in size in the presence of IgM-RF. In Table 1, these results are summarized as the relative recoveries of radiolabelled material from the bottom, the lower half, and the upper half of the gradients. It is noteworthy that no gradual changes in aggregate size were found: when enlargement occurred, it always resulted in a shift of recovered radiolabel from the upper half to the bottom of the gradients. Because the control preparation of normal IgM did not influence the size of AIgG (Fig. 4), cross-linking of AIgG by the specific binding of IgM-RF was very probably the cause of the observed phenomenon.

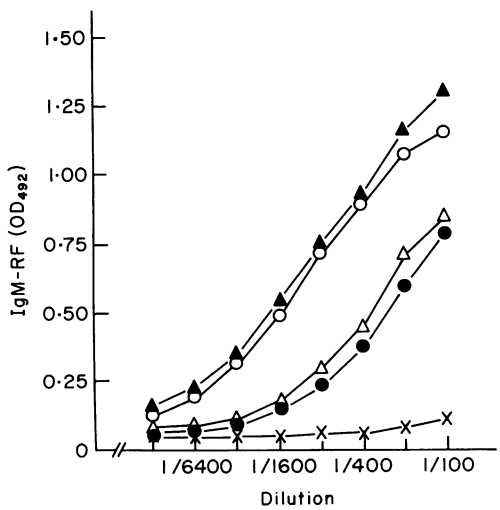

Figure 2. Dose-response curves of the preparations IgM-RF1 (●——●), IgM-RF2 (○——○), IgM-RF3 (▲——▲), IgM-RF4 (△——△) and of nIgM (×—×) in the ELISA for IgM-rheumatoid factor activity.

Effect of IgM-RF on C1 activation

The C1 activation capacity of AIgG preincubated with BBS, nIgM or IgM-RF was assessed by testing at least six dilutions in the C1 activation assay. A representative experiment in which aggregates of 90–100 and 45–50 IgG molecules were used is shown in Fig. 5. Aggregates preincubated in BBS activated C1 in a dose-dependent way with high efficiency: 50% activation was obtained with 25 ng of $(IgG)_{90-100}$ (Fig. 5a) or 34 ng of $(IgG)_{45-50}$ (Fig. 5b). Preincubation of the aggregates with nIgM resulted in a slight shift of the curves to the left, which might be due to an additive effect of some C1-activating material in the nIgM preparation. At high concentrations, both the nIgM and the IgM-RF preparations were found to induce C1 activation on their own (data not shown), presumably indicating denaturation or aggregation of immunoglobulin during euglobulin precipitation. In contrast, preincubation of IgG aggregates with IgM-RF3 diminished their C1 activation capacities, such that 170 ng of $(IgG)_{90-100}$ and 200 ng of $(IgG)_{45-50}$ were required to induce 50% C1 activation (Fig. 5).

Identical experiments were performed with AIgG containing on average 85, 42, 20, and 1–5 IgG molecules. All types of aggregates were preincubated with buffer or with each of the IgM-RF preparations, after which the dilution of each mixture that induced 50% C1 activation was determined. In order to show the effect of IgM-RF more clearly, we calculated the numbers of C1 molecules activated per 1000 IgG molecules under these conditions, i.e. at 50% C1 activation (Table 2). As can be seen, a four- to ten-fold decrease in C1 activation efficiency was found when large aggregates ($\geqq$40 IgG molecules) were preincubated with IgM-RF. Three of the IgM-RF preparations also diminished the C1 activation capacity of $(IgG)_{18-22}$, whereas C1 activation by oligomeric IgG was affected only slightly or not at all.

In order to exclude the possibility that the inhibitory effect of IgM-RF was restricted to C1 activation by heat-aggregated IgG, we performed a similar experiment with freshly prepared soluble tetanus–anti-

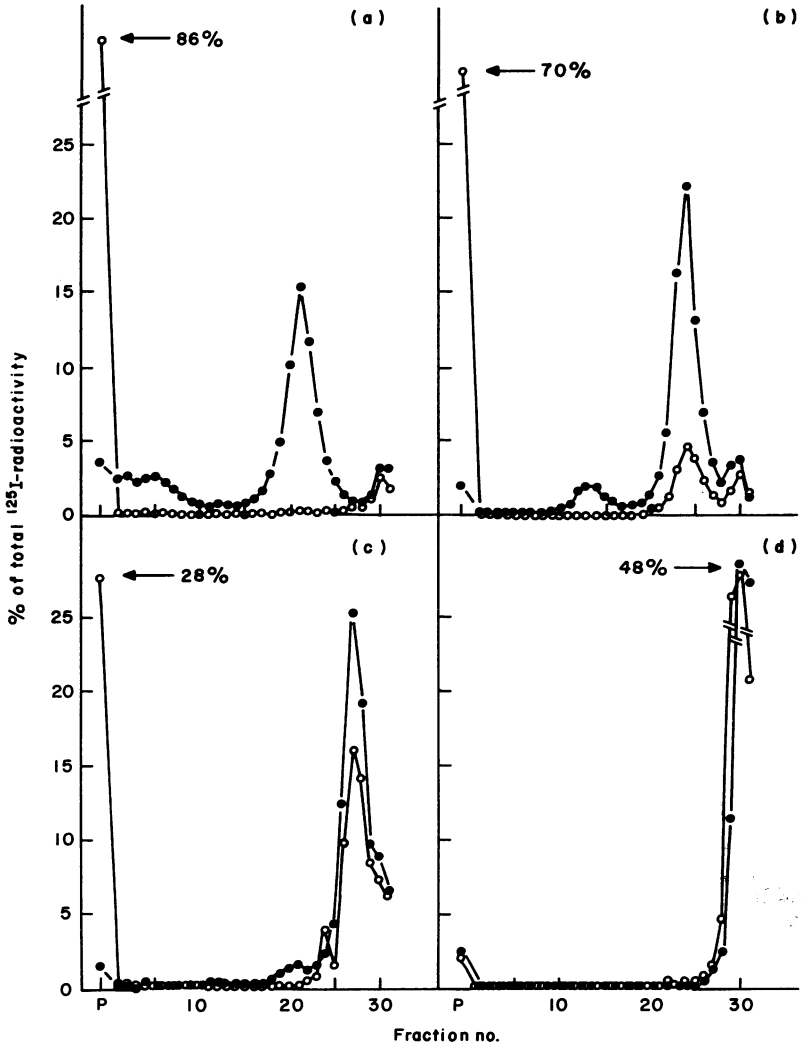

Figure 3. Effect of IgM-RF1 on the size of AIgG. Stabilized ^{125}I-labelled aggregates of (a) 80–90, (b) 40–45, (c) 18–22 and (d) 1–4 IgG molecules were preincubated with BBS (●——●) or IgM-RF1 (○——○) for 30 min at 37° and 2·5 hr at 0° and then centrifuged for 1 hr at 263,000 *g* in 10–30% sucrose gradients. The distribution of radioactivity over the gradients after the run is depicted. Top of the gradients is to the right. P = pellet.

tetanus immune complexes. When prepared at the right antibody–antigen ratio, these complexes induced a definite dose-dependent C1 activation after preincubation in buffer (Fig. 6). The curve was shifted slightly to the left after preincubation with nIgM, whereas preincubation with IgM-RF3 diminished the C1 activation capacity almost four-fold: 50% C1 activation required dilutions of 1:58 and 1:15 after preincubation with buffer and with IgM-RF3, respectively.

DISCUSSION

In this study, we found a marked decrease of the C1 activation capacity of soluble IgG aggregates when the latter were preincubated with IgM-enriched preparations from four sera containing IgM-RF. From parallel experiments with radiolabelled aggregates of identical size, we concluded that a large increase in aggregate size accompanied the loss of C1 activation

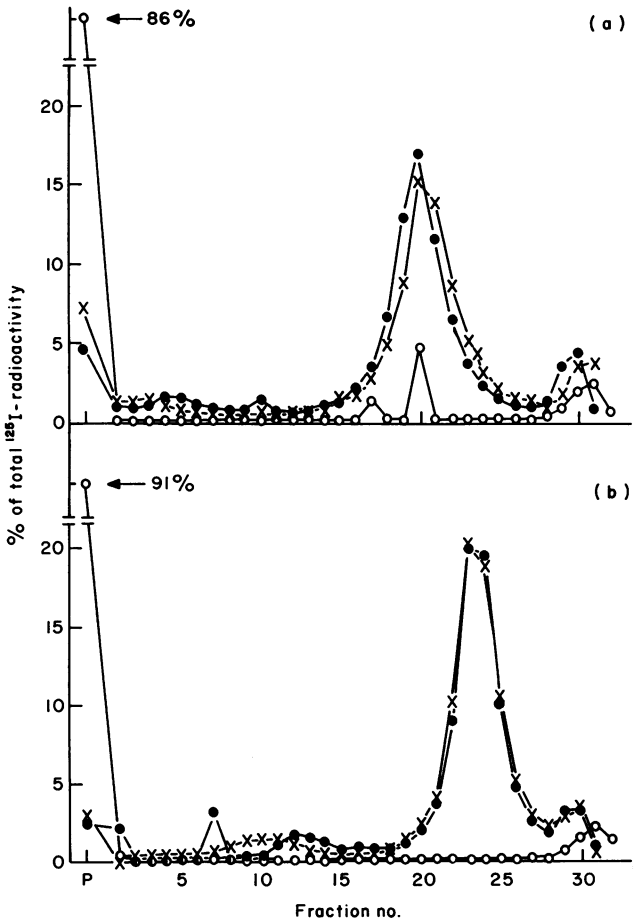

Figure 4. Effect of IgM-RF3 and nIgM on the size of AIgG. ^{125}I-AIgG containing (a) 90–100 or (b) 45–50 IgG molecules were preincubated with BBS (●——●), IgM-RF3 (○——○) or nIgM (×——×), and analysed by ultracentrifugation as described for Fig. 3.

capacity. Both effects were very probably due to the reaction of AIgG with IgM-RF, because preincubation with a comparable IgM preparation made from pooled NHS caused neither size enlargement nor a decrease of C1 activation capacity (Figs 4 and 5). In addition, IgM-RF also inhibited C1 activation by true soluble antigen–antibody complexes (Fig. 6).

The reported enlargement of AIgG agrees with the known capacity of IgM-RF to precipitate soluble IgG aggregates and immune complexes (Zvaifler & Schur, 1968; Tesar & Schmid, 1970). Interestingly, size enlargement seemed to be an 'all or nothing' phenomenon: AIgG apparently cross-linked by IgM-RF reached the pellet of 10–30% sucrose gradients after only 1 hr of centrifugation at 263,000 *g*, which implies an estimated aggregate size of at least 500–600 IgG molecules in the pelleted material. Furthermore, size enlargement was most pronounced with the larger aggregates and did not occur with the 7S fraction from the initial preparative gradient which presumably also contained IgG oligomers. This supports the hypothesis that preferential binding of IgM-RF to IgG aggregates and immune complexes is merely due to the multivalency of polymerized IgG (Normansell, 1971).

Investigations on the role of IgM-RF in C activation have led to apparently conflicting results. Although early studies reported inhibition of C acti-

Table 1. Effect of IgM-RF on the size of AIgG. Recovery (%) of ^{125}I-labelled soluble IgG aggregates from the pellet, lower half (Fr. 1–15) and upper half (Fr. 16–31) of 10–30% sucrose gradients after preincubation with IgM-RF and subsequent re-ultracentrifugation*

		Preincubated with:				
Aggregates	Recovered in:	Buffer	IgM-RF1	IgM-RF2	IgM-RF3	IgM-RF4
$(IgG)_{80-90}$	Pellet	4	86	90	91	91
	Fr. 1–15	24	3	2	1	1
	Fr. 16–31	73	11	8	8	8
$(IgG)_{40-45}$	Pellet	2	70	85	80	76
	Fr. 1–15	10	3	1	7	7
	Fr. 16–31	88	27	14	13	17
$(IgG)_{18-22}$	Pellet	1	28	46	62	47
	Fr. 1–15	2	2	6	3	4
	Fr. 16–31	97	70	48	35	49
$(IgG)_{1-4}$	Pellet	2	2	3	2	6
	Fr. 1–15	1	2	2	2	2
	Fr. 16–31	97	96	95	96	92

* Aggregates (160 μg/ml) were mixed 1:1 with buffer (BBS) or IgM-RF and preincubated for 30 min at 37° and 2·5 hr at 0° immediately before centrifugation.

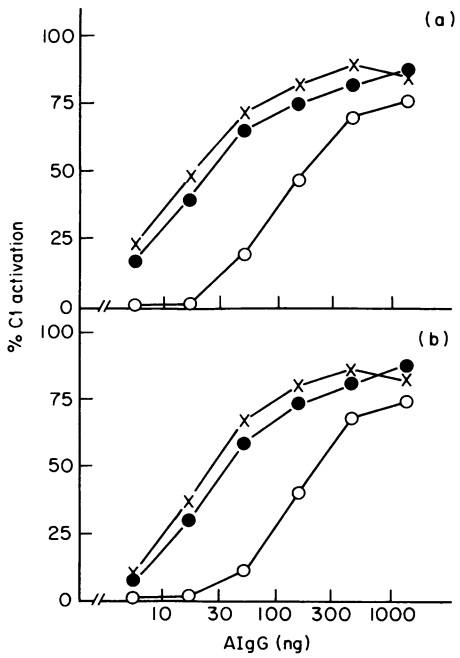

Figure 5. Effect of IgM-RF3 and nIgM on the C1 activation capacity of AIgG. AIgG containing (a) 90–100 or (b) 45–50 IgG molecules were preincubated with BBS (●——●), IgM-RF3 (○——○) or nIgM (×——×). The C1 activation capacity was determined by testing serial dilutions of the preincubated mixtures in a haemolytic C1 activation assay.

vation (Zvaifler & Bloch, 1962; Davis & Bollet, 1964), later ones demonstrated C activation by IgM-RF which was bound to immune precipitates (Zvaifler & Schur, 1968; Tesar & Schmid, 1970; Bianco, Dobkin & Schur, 1974), to IgG-coated erythrocytes (Tanimoto *et al.*, 1975), or to IgG-coated microwells (Sabharwal *et al.*, 1982). However, in most of the latter studies, IgM-RF was bound to chemically modified IgG which could not activate C1 by itself. Schmid, Roitt & Rocha (1970) had shown earlier that the binding of IgM-RF to erythrocytes sensitized optimally with non-modified IgG inhibited C-dependent haemolysis. Truedsson & Sjöholm (1984) recently confirmed this finding and showed that the inhibitory effect was probably due to the same population of IgM-RF molecules which caused haemolysis in the assay described by Tanimoto *et al.* (1975).

Theoretically, the net effect of IgM-RF binding on the C1 activation capacity of IgG complexes is determined by at least four factors, namely: (i) the C1 activation capacity of the complexes before their reaction with IgM-RF, which mainly depends on the density of IgG molecules on a cell surface (Lachmann & Hugh-Jones, 1984) or the degree of polymerization of IgG in soluble complexes (Doekes *et al.*, 1982, 1984); (ii) the extent to which IgM-RF blocks the C1-binding sites on the IgG molecules, directly or via steric hindrance; (iii) the exposure of new C1-binding

Table 2. Effect of IgM-RF on the C1 activation capacity* of AIgG

	C1 activation capacity after preincubation with:				
Aggregate	Buffer	IgM-RF1	IgM-RF2	IgM-RF3	IgM-RF4
$(IgG)_{80-90}$	840	160	80	140	60
$(IgG)_{40-45}$	340	70	60	90	70
$(IgG)_{18-22}$	63	12	45	70	30
$(IgG)_{1-4}$	3·3	1·0	3·9	5·0	2·0

* Activation capacity is expressed as the number of C1 molecules activated per 1000 IgG molecules at the concentration of AIgG that induced 50% C1 activation.

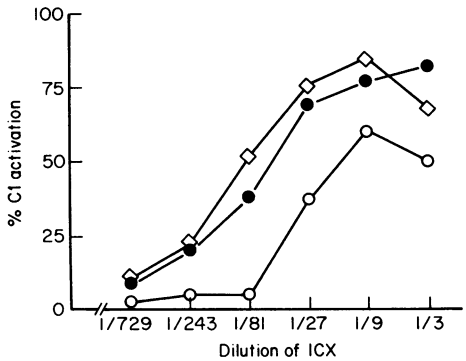

Figure 6. Effect of IgM-RF3 and nIgM on the C1 activation capacity of soluble TT–anti-TT immune complexes. Freshly prepared soluble TT–anti-TT complexes were preincubated with BBS (●——●), IgM-RF3 (○——○) or nIgM (◇——◇) and tested in the C1 activation assay.

sites on IgM-RF itself through its binding to the IgG molecules; (iv) the capacity of IgM-RF to spatially rearrange the IgG molecules to either increase or decrease their effective binding avidity for C1.

The present study shows that IgM-RF can increase the size of soluble IgG aggregates remarkably. Although, in general, larger aggregates activate C1 with higher efficiency, it is possible that in these very large aggregates a major proportion of the IgG molecules can not participate in C1 binding and activation. Alternatively, the observed inhibition of C1 activation was due to a blockage of C1-binding sites by IgM-RF.

Apparently, lost C1-binding sites were not replaced by new ones exposed on bound IgM-RF. This may be explained by recent insights on C1 binding and activation by IgM antibodies. Conformational changes play an important role in this process (Brown & Koshland, 1975; Siegel & Cathou, 1980; Borsos, Chapuis & Langone, 1981), such that only the 'staple' form of antigen-bound IgM can bind and activate C1 (Feinstein *et al.*, 1983). This IgM conformation will predominate among IgM molecules bound to surfaces with a high density of repetitive determinants, a condition that was fulfilled in the studies which showed C1 activation by IgM-RF bound to IgG-coated erythrocytes. On the other hand, the arrangement of IgG molecules in soluble complexes may vary widely and may be changed by the binding of IgM-RF. Therefore, IgM-RF bound to some soluble IgG complexes might assume a C1-activating 'staple' conformation, but after binding to other complexes remain in the non-activating 'star-like' or planar form (Feinstein *et al.*, 1983). This could explain the discrepancy between our present results and those of Tesar & Schmid (1970) and of Birchmore *et al.* (1981), who found an enhancing effect of IgM-RF on C activation by soluble immune complexes, which was ascribed to C activation by IgM-RF itself. On the other hand, our results are in agreement with those of Kline Bolton, Schrock & Davis (1982), who studied C activation by erythrocyte-bound AIgG in the presence and absence of IgM-RF.

Naama *et al.* (1983) studied the ability of patients' sera to inhibit immune precipitation, a phenomenon known to depend on classical pathway activation by the growing lattice of soluble immune complexes (Schifferli *et al.*, 1982). They noted that RA sera could abolish this inhibitory effect, presumably due to the presence of IgM-RF (Mitchell *et al.*, 1984). Balestrieri *et al.* (1984) confirmed these results by demonstrating inhibition by purified IgM-RF of two C-dependent phenomena: the prevention of immune precipitation,

and the C-dependent resolubilization of immune precipitates. Although it cannot be completely excluded that the apparent inhibitory effect of IgM-RF was simply due to precipitation of soluble complexes by IgM-RF itself, Naama *et al.* (1983) did show a parallel decreased C4 consumption, which suggests that IgM-RF indeed inhibited C activation by soluble immune complexes.

The present study may explain why there is little evidence for complement activation in the sera of RA patients. Despite frequently high concentrations of circulating soluble immune complexes or aggregates, depressed serum complement levels are a rare finding. Complexes of $C\bar{1}s$ bound to $C\bar{1}$-inactivator, a marker of C1 activation, could also not be detected (Inman & Harpel, 1983; Hack *et al.*, 1984). In contrast, C activation via the classical pathway is one of the features of local synovial inflammatory reactions (Ruddy & Austen, 1970; Inman & Harpel, 1983). The study of Kaplan *et al.* (1980) which demonstrated that the enhanced C4 catabolism in RA patients occurred mainly extravascularly, also points to the synovium as the main site of C activation. Thus, it may be suggested that local conditions in the synovium influence the conformations and interactions of immune complexes and/or rheumatoid factors in such a way that C activation is promoted. In the circulation, however, IgM-RF may prevent immune complexes from activating complement by binding to C1-binding sites on IgG in a conformation in which its own Fc-parts cannot activate C1.

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