Complement Fixation by Rheumatoid Factor

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ABSTRACT The capacity for fixation and activation of hemolytic complement by polyclonal IgM rheumatoid factors (RF) isolated from sera of patients with rheumatoid arthritis and monoclonal IgM-RF isolated from the cryoprecipitates of patients with IgM-IgG mixed cryoglobulinemia was examined. RF mixed with aggregated, reduced, and alkylated human IgG (Agg-R/A-IgG) in the fluid phase failed to significantly reduce the level of total hemolytic complement, CH_{50}, or of individual complement components, C1, C2, C3, and C5. However, sheep erythrocytes (SRC) coated with Agg-R/A-IgG or with reduced and alkylated rabbit IgG anti-SRC antibody were hemolyzed by complement in the presence of polyclonal IgM-RF. Human and guinea pig complement worked equally well. The degree of hemolysis was in direct proportion to the hemagglutination titer of the RF against the same coated cells. Monoclonal IgM-RF, normal human IgM, and purified Waldenström macroglobulins without antiglobulin activity were all inert.

Hemolysis of coated SRC by RF and complement was inhibited by prior treatment of the complement source with chelating agents, hydrazine, cobra venom factor, specific antisera to C1q, C4, C5, C6, or C8, or by heating at 56°C for 30 min. Purified radiolabeled C4, C3, and C8 included in the complement source were bound to hemolysed SRC in direct proportion to the degree of hemolysis. These data indicate that polyclonal IgM-RF fix and activate complement via the classic pathway.

The system described for assessing complement fixation by isolated RF is readily adaptable to use with whole human serum.

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INTRODUCTION

Complement participates in several biological reactions including immune cytolysis, phagocytosis, immune adherence, and chemotaxis (reviewed in references 1, 2). The role of complement in the pathogenesis of a number of human diseases including rheumatoid arthritis is not clear. The similarity of vascular lesions in rheumatoid arthritis (3) with those of the immune complex disease, experimental serum sickness (4), suggests that complement might play a role in the pathogenesis of rheumatoid vasculitis. The role of rheumatoid factor (RF) [1] in rheumatoid arthritis is also unclear. Patients with highest titers of RF appear to be afflicted with more severe disease (5, 6), and in experimental animals RF appear to augment tissue damage (7, 8). Both RF and complement have been identified in the lesions of rheumatoid arthritis (9, 10). Crucial to the elucidation of the role of RF and complement in the possible pathogenesis of rheumatoid arthritis is the question of whether complement with its potential for producing inflammation and alterations in vascular permeability is activated by RF. Although a number of recent studies (11–15) indicate that RF probably does fix complement under special conditions, other studies (14, 16–21) suggest that RF may, in fact, interfere with complement fixation under other circumstances. Because of uncertainty about the ability of RF to initiate complement fixation, we have employed a different approach based on lysis of RF-sensitized cells by com-

[1] *Abbreviations used in this paper:* Agg-R/A-IgG, aggregated, reduced, and alkylated human IgG; EGTA, ethyleneglycol-bis-(amino ethyl)-tetraacetic acid; GPC, guinea pig complement; HuC, human complement; NHS, normal human serum; PBS, 0.15 M NaCl buffered to pH 7.4 with sodium phosphate; R/A-IgG, reduced and alkylated human IgG; R/A-Hem, reduced and alkylated rabbit IgG-hemolysin; REA, SRC coated with R/A-Hem; RF, rheumatoid factor; SRC, sheep erythrocytes; tHEA, tanned SRC coated with Agg-R/A-IgG; V_o, void volume.

TABLE I

Complement Fixation Test between RF and Agg-R/A-IgG

	Protein concentration	Percent complement fixation with 0.2 mg Agg-R/A-IgG				
		CH_{50}	C1	C2	C3	LFT*
	mg/ml					
Polyclonal IgM-RF						
Ra (Vo)	2.3	2.1	10.1	0	0	64
Ba (Vo)	2.0	15.9	4.3	0	22.6	128
Ca (Vo)	3.5	0	15.1	0	14.9	64
Control						
NHS (Vo)	3.2	4.1	13.9	0	16.1	—
Monoclonal IgM-RF						
IgM DK	2.4	1.5	0	0	24.7	320
IgM Lay	2.9	3.1	0	0	4.1	2,560
IgM Si	2.7	5.3	0	0	12.4	640
Control						
IgM He	2.9	0	0.9	0	13.9	—
Rabbit antihuman IgG (1:10)	6.7	>59.2	76.3	>67.2	18.3	20
Agg-R/A-IgG	0.2	0.7	0	4.2	0	—

* LFT, slide latex fixation test (rheumatoid arthritis test, Hyland Laboratories).

plement in order to demonstrate unequivocally that RF fixes complement. The direct hemolytic assay for RF described in the present communication is readily adaptable to whole serum, and it may be used to assess rapidly the complement-fixing activity of RF present in patients' sera.

METHODS

Preparation of RF. Sera exhibiting strong RF activity from patients with rheumatoid arthritis were heat inactivated at 56°C for 30 min and incubated with washed packed sheep erythrocytes (SRC) at 37°C for 90 min in order to remove natural antibody to SRC. These sera were then applied to a Sephadex G-200 gel filtration column equilibrated with 0.1 N pH 4.0 acetate buffer. The first peak eluted in the void volume (V_o) contained polyclonal IgM-RF and was dialyzed against 0.15 M NaCl buffered to pH 7.4 with sodium phosphate (PBS) and concentrated by pervaporation. Only electrophoretically heterogeneous IgM was detected in these preparations when examined at a concentration of 4 mg/ml by immunoelectrophoresis employing antisera specific for IgM, IgG, and IgA. Homogeneous monoclonal IgM-RF were isolated from the sera of patients with mixed cryoglobulinemia by previously described methods (22, 23). In brief, IgM Lay and IgM Si (generously provided by Drs. Metzger and Stone, respectively; see references 22, 24, and 25) were purified by DEAE column chromatography at 40°C and IgM DK was isolated by gel filtration on a Sephadex G-200 column in 0.05 M pH 2.5 glycine-HCl buffer. V_o of normal human serum (NHS) obtained from a healthy RF-negative adult was isolated by Sephadex G-200 gel filtration after heat inactivation and absorption of natural antibody in the same manner as with polyclonal RF and used as a control for polyclonal IgM-RF. The serum of a patient with Waldenström's macroglobulinemia which had no RF activity was dialyzed against distilled water and the resulting euglobulin precipitate dissolved in pH 7.4 PBS. The paraprotein, IgM He, was isolated by Sephadex G-200 gel filtration and used as a control for monoclonal IgM-RF. Purity and homogeneity of all four monoclonal IgM (Lay, DK, Si, and He) were verified by immunoelectrophoresis.

Preparation of IgG. Human IgG as Cohn Fraction II obtained from ICN Nutritional Biochemicals Div., International Chemical and Nuclear Corp., Cleveland, Ohio was dissolved in pH 7.4 PBS adjusted to 10 mg/ml, reduced with 0.01 M dithiothreitol at room temperature for 30 min, and alkylated with 0.015 M iodoacetamide at 0°C for 120 min.

Reduced and alkylated human IgG (R/A-IgG) was dialyzed against PBS and concentrated by pervaporation. This preparation was aggregated by incubation at 63°C for 20 min. The aggregated, reduced, and alkylated IgG (Agg-R/A-IgG) was isolated by gel filtration chromatography in PBS (23). Only soluble aggregates eluting in V_o were used in the studies reported. Aggregates were not fractionated further and their average size was not determined.

Source of antisera. Rabbit antihuman IgG was obtained from Terra Marine Bioresearch, La Jolla, Calif. A goat antiserum to rabbit colostrum strongly reactive with rabbit IgG was a gift from Dr. Alexander Lawton of the University of Alabama at Birmingham. These antisera were used after heat inactivation at 56°C for 30 min and absorption of natural antibody to SRC by the same procedure as RF. Monospecific goat antisera to human C1q, C4, C3, C5, C6, and C8 have been previously described (26–30).

Measurement of RF. The RF activity of sera and fractions was measured by the slide latex fixation test (rheumatoid arthritis test, Hyland Laboratories, Los Angeles, Calif.), sensitized sheep cell agglutination test (31), and F II agglutination test (32).

Measurement of complement utilization. Fresh human serum was obtained from a healthy RF-negative adult. Natural antibody to SRC was removed by repeated absorp-

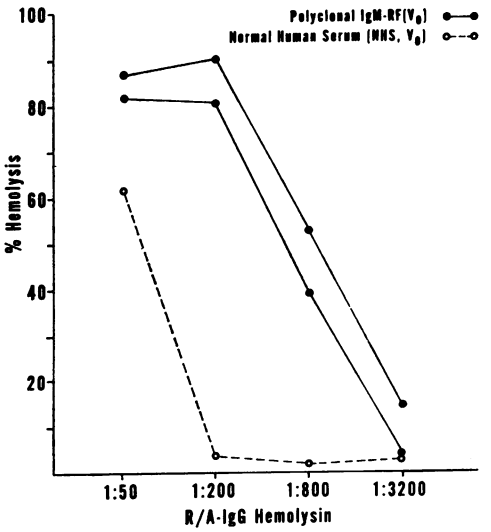

FIGURE 1 SRC coated with R/A-Hem were incubated with constant amounts of two different IgM-RF preparations (●—●) (Ra and Ba, see Table I). Percent hemolysis is plotted versus the dilution of R/A-IgG used to coat the cells. IgM prepared from RF-negative NHS (O---O) is the control. Cell lysis at 1:50 R/A-IgG hemolysin is nonspecific due to residual unreduced hemolysin. At 1:200 R/A-IgG, lysis by IgM-RF is specific.

tion with washed packed SRC at 0°C. Lyophilized guinea pig serum purchased from Hyland Laboratories was reconstituted and absorbed with SRC as above. These absorbed human and guinea pig sera were stored in small aliquots at −70°C until they were used as the human complement (HuC) or guinea pig complement (GPC) source.

Rabbit anti-SRC hemolysin, purchased from BBL, Division of Becton, Dickinson and Company, Cockeysville, Md., was separated into IgM and IgG fractions by Sephadex G-200 gel filtration. SRC were sensitized with IgM-hemolysin at four times the optimal sensitizing dose (IgM-EA) (33).

Testing for complement fixation in the fluid phase by RF (V_0) was done by adding 0.5 ml of each RF preparation and 200 μg Agg-R/A-IgG to HuC or GPC diluted in 0.15 M Veronal-buffered saline containing 0.1% gelatin and optimal Ca^{++} and Mg^{++} (GVB^{++}) and incubating at 37°C for 60 min. The mixture was stored in convenient aliquots at −70°C. Residual CH_{50} was measured by a modification of Mayer's methods using IgM-EA in a total reaction volume of 1.5 ml (34). C1, C2, and C3 hemolytic activities were determined by published methods (34–36).

Hemolysis of sensitized SRC by RF preparations and complement utilized SRC sensitized with reduced and alkylated rabbit IgG-hemolysin (REA), or tanned SRC coated with Agg-R/A-IgG (tHEA). For preparation of the former cells, IgG-hemolysin isolated by gel filtration was reduced and alkylated (R/A-Hem) in order to diminish its complement-fixing activity. The hemagglutination titer of this preparation was 1:40. SRC were sensitized in the same manner as with nonreduced and alkylated hemolysin. For preparation of tanned cells, washed SRC were suspended in pH 7.2 PBS and treated with 1:80,000 diluted tannic acid by a modification of Boyden's method (37). After tannic acid treatment, the cells were suspended to 2% (4×10^8/ml) in pH 6.4 PBS and reacted with an equal volume of 200 μg/ml of Agg-R/A-IgG.

For assessing complement-mediated hemolysis by RF, 0.2 ml of each RF preparation was incubated with REA or tHEA (2×10^8/ml) at 37°C for 60 min and at 0°C for 3 h and the cells were washed three times with GVB^{++}. Serial dilutions of the absorbed HuC and GPC sources were incubated with the sensitized cells at 37°C for 60 min and the degree of hemolysis was assessed by spectrophotometric measurement (412 nm) of hemoglobin released into the supernate.

Radiolabeled complement uptake. Purified HuC components C3, C4, and C8 were prepared by published methods (26–28), trace labeled with ^{125}I by the chloramine-T method (38) without loss of activity, and used for quantitative cell binding studies (39). SRC sensitized with R/A-Hem were reacted with the RF preparations as described in the previous section. To a 1% suspension of these cells was added 0.1 ml of a 1:5 dilution of HuC containing $1–6 \times 10^6$ cpm of ^{125}I-labeled C3 (approximately 20 μg), C4 (approximately 5 μg), or C8 (approximately 5 μg). The suspension was incubated at 37°C for 60 min, centrifuged, and an aliquot of the supernate removed for determination of the degree of hemolysis. SRC stromata were washed three times with PBS by centrifugation at 20,000 rpm in a Spinco 40S rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Stroma-bound radioactivity was determined in a well-type gamma scintillation spectrometer.

Miscellaneous. All chemicals were of reagent grade. The sources and use of cobra venom and hydrazine have been previously cited (40–43).

RESULTS

Effect of RF on complement in free solution. Rheumatoid arthritis preparations were incubated with Agg-

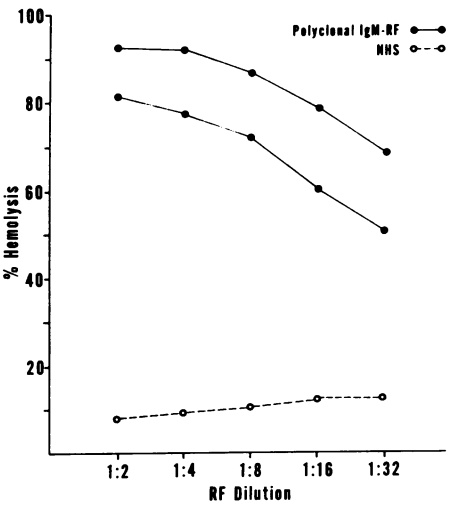

FIGURE 2 The abilities of diminishing amounts of two polyclonal IgM-RF (Ra and Ba) to sensitize REA coated with 1:500 R/A-Hem for lysis by complement is shown. Percent hemolysis is plotted versus the dilution of IgM-RF (●—●). (See Table I for original latex fixation test and protein concentrations.) The negative control is IgM prepared from RF-negative NHS (O---O).

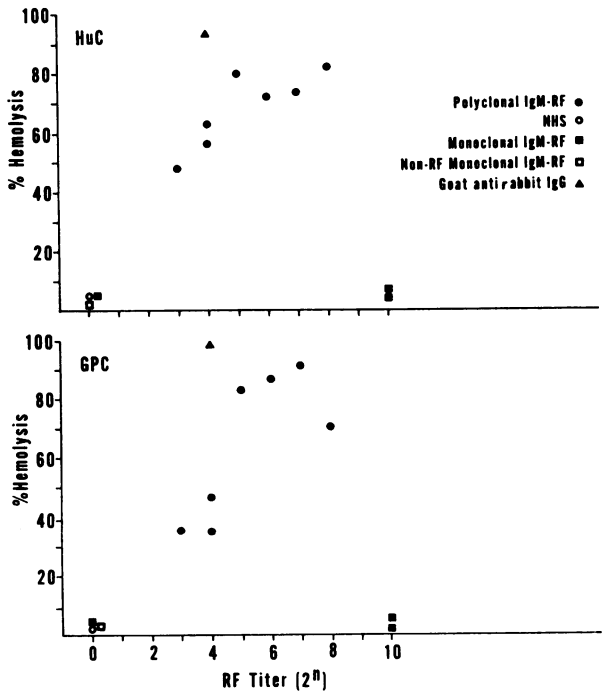

FIGURE 3 Percent hemolysis of REA incubated with various IgM (V_o) preparations and two sources of complement is compared with agglutination titer (2^n) measured by these same cells. Percent hemolysis produced by HuC and GPC correlates directly with agglutination titer for polyclonal IgM-RF (●) ($r_1 = 0.992$, $P < 0.01$ for HuC and $r = 0.882$, $P < 0.01$ for GPC). Non-RF monoclonal IgM (□) and normal human IgM (○) neither agglutinated nor sensitized REA for lysis by either complement source. Monoclonal IgM-RF (■) did not trigger hemolysis. Goat anti-rabbit IgG (▲) was weakly agglutinating but strongly hemolytic with both complement sources.

R/A-IgG (see Methods) following which residual complement (CH_{50}, C1, C2, and C3) was titrated. The studies shown in Table I employed undiluted human serum as a complement source. Identical results were obtained with dilute complement. None of the monoclonal and polyclonal RF significantly reduced CH_{50} or C1, C2, or C3 activities. One polyclonal RF (Ra) and one monoclonal IgM RF (Lay) were also examined at 4°C with results identical to those at 37°C. Fixation by rabbit antihuman IgG, the positive control, was observed.

Lysis of SRC coated with R/A-Hem. In contrast to these results which do not show significant complement fixation by RF in free solution, some of the RF preparations readily induced lysis of SRC coated with R/A-IgG hemolysin in the presence of complement. SRC coated with various dilutions of R/A-Hem were incubated with constant amounts of two different polyclonal RF preparations and washed and incubated with complement. Fig. 1 shows that the two polyclonal RF preparations were able to sensitize REA for lysis by complement. The IgM (V_o) prepared from NHS also sensitized the cells for lysis, but only in higher dilution due presumably to residual nonreduced hemolysin. At a 1:200 dilution of R/A-IgG, greater than 80% lysis of the cells sensitized with polyclonal RF was observed, while the control gave less than 5% lysis. In subsequent experiments, REA were prepared with a 1:500 dilution of R/A-Hem.

The effect of diminishing amounts of two polyclonal RF on the ability to sensitize REA for lysis is shown in Fig. 2. Significant hemolysis, 50% and 75%, respectively, was observed even at a 1:32 dilution of these two preparations (original sensitized sheep cell agglutination test titers of Ra and Ba preparations are 1:256 and 1:128). Since the slope of decrement of hemolysis in this and subsequent experiments (see Figs. 3 and 5) indicate the hemolytic end point would have been at 1:256 or 1:512 RF dilution, these results indicate even small amounts of RF are able to sensitize cells coated with R/A-Hem for lysis.

Hemolytic activities of seven polyclonal and three monoclonal IgM-RF preparations were studied in this system. Hemagglutinating activity of the RF on the same sensitized cells was also determined. All seven

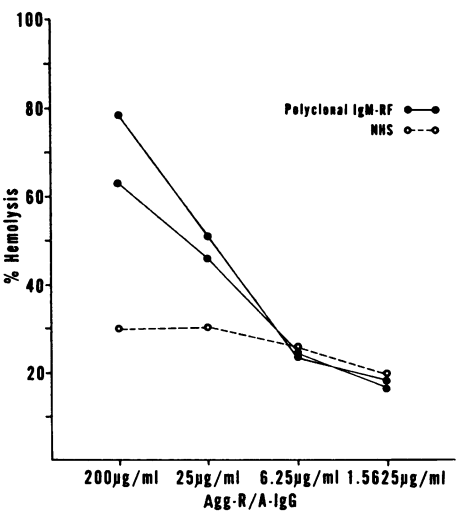

FIGURE 4 tHEA were incubated with constant amounts of the two different IgM-RF (●) and normal IgM (○) shown in Fig. 1. Percent hemolysis with HuC is plotted versus the dilution of Agg-R/A-IgG used to coat the cells. Hemolysis of tHEA incubated with IgM-RF and complement is dependent on the amount of Agg-R/A-IgG used to coat the cells. "Background" hemolysis associated with normal IgM is relatively constant over the Agg-R/A-IgG dose range shown on this figure, and is attributed to nonspecific lability of tanned cells in presence of complement. Doses of Agg-R/A-IgG greater than 200 µg/ml resulted in spontaneously agglutinating tHEA.

polyclonal RF sensitized the cells for hemolysis; all seven also possessed hemagglutinating activity for the sensitized cells (Fig. 3). The correlation between hemolytic activity and hemagglutination titer was significant ($P < 0.01$). Little difference between HuC and GPC was observed (Fig. 3).

Two monoclonal RF did not trigger hemolysis despite avid hemagglutinating activity. A third monoclonal RF (IgM Lay) was not reactive with rabbit IgG. Non-RF monoclonal IgM and normal human IgM (V_o) neither agglutinated REA nor sensitized them for lysis by complement. Goat antirabbit IgG showed high hemolytic activity coupled with a low hemagglutination titer.

Lysis of tHEA. tHEA were also lysed by RF and complement. As shown, lysis was dependent on the amount of Agg-R/A-IgG used to coat the tanned cells (Fig. 4). 200 μg/ml were used routinely in subsequent experiments illustrated by Fig. 5. There appeared to be a general correlation of hemolysis with agglutination titers. Two of the polyclonal RF produced 30–40% lysis. The three monoclonal RFs also induced 30–40% lysis, despite marked hemagglutinating activity. The non-RF monoclonal IgM and the V_o of normal serum had low hemagglutinating activity and also low-grade hemolytic activity presumed to be nonspecific as a consequence of tannic acid treatment. This effect was not seen with the REA. Rabbit antihuman IgG induced marked lysis of tHEA, although its hemagglutination titer was not high.

Role of complement in lysis. In order to clarify the mechanism of hemolysis, HuC was treated in several ways known to interfere with complement activity. The altered complement was employed for lysis of REA sensitized with polyclonal RF. These treatments included chelation of divalent cations with EDTA and EGTA, heat inactivation, and treatment with hydrazine and with cobra venom factor. Each of these prevented lysis (Fig. 6). Monospecific antisera to C1q,

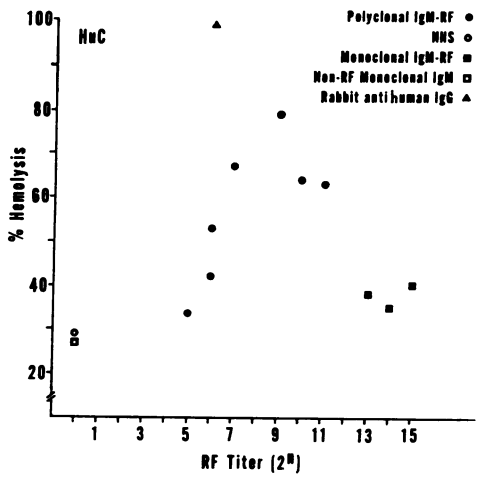

FIGURE 5 Percent hemolysis of tHEA by IgM-RF and HuC is plotted versus RF titer determined with the same cells. Percent hemolysis correlates with RF titer for polyclonal RF (●) ($r = 0.813$, $P < 0.01$). In spite of high agglutination titer monoclonal RF (■) produce only slightly greater hemolysis than that associated nonspecifically with RF negative controls (○, □). The positive control, rabbit antihuman IgG (▲), is strongly hemolytic although its agglutination titer is relatively low.

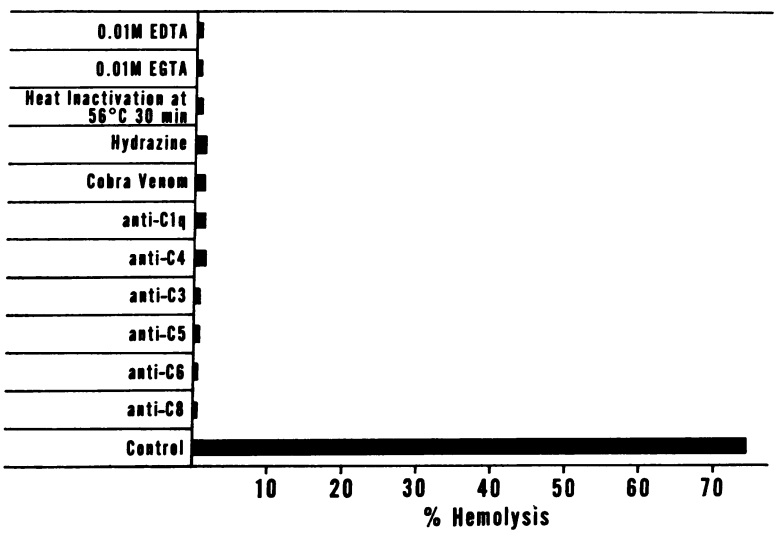

FIGURE 6 The HuC source was treated in various ways known to interfere with complement activity. Percent hemolysis of REA incubated with polyclonal IgM-RF (Ra) and variously treated complement are compared. (74% hemolysis shown at the bottom of the figure, in which untreated HuC was used.) All treatments abolished lysis.

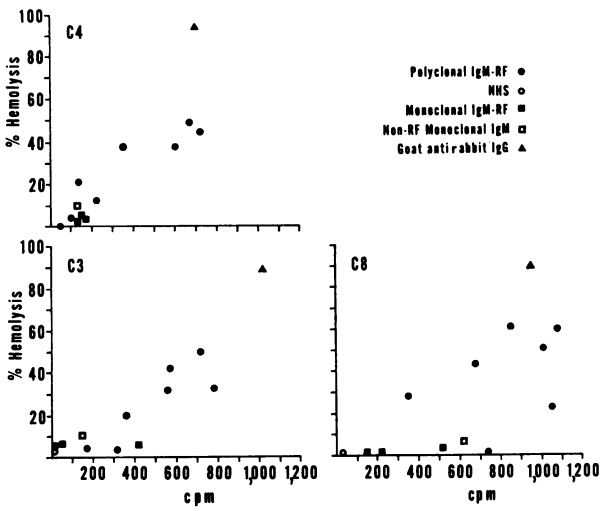

FIGURE 7 Percent hemolysis of REA incubated with IgM-RF and complement is plotted versus erythrocyte membrane uptake of ^{125}I-radiolabeled purified HuC components, C4, C3, and C8, expressed as counts per minute. The uptake of C4 and C3 exhibit a high degree of correlation with percent hemolysis ($r = 0.923$, $P < 0.01$ and $r = 0.863$, $P < 0.01$ for C4, and C3, respectively). Correlation for C8 uptake did not reach the significant level with the number of examinations performed.

C4, C3, C5, C6, and C8 included in the complement source were also highly inhibitory. In order to rule out complement depletion by immune complexes formed between complement components and the antisera as the reason for inhibition of lysis, anti-C1q, anti-C5, and anti-C6, and anti-C8 were reduced and alkylated. After this treatment which greatly impairs complement fixing potential, the antisera were able to inhibit lysis of RF-sensitized REA by complement.

In addition, cells sensitized with REA were incubated with RF and HuC into which were incorporated trace amounts of ^{125}I-labeled C3, C4, or C8, as outlined in Methods. Fig. 7 indicates that a good correlation was obtained between the binding of ^{125}I-labeled C3, C4, or C8 and the percentage of lysis.

DISCUSSION

Complement fixation by IgM-RF remains a controversial subject despite many years of investigation. Early studies indicated that RF inhibited fixation of complement by IgG (16–20). It was concluded that RF was incapable of complement activation and that it blocked the fixation of complement by IgG. A rational basis for this view was provided by the discovery that the antigenic site for RF (44, 45) and the binding site for the first component of complement, C1 (46), were both located on the Fc fragment of IgG. However, methodologic limitations made interpretation of many of these studies uncertain. In these reports aggregated IgC, which is highly anticomplementary, was used as antigen and whole hemolysin was used to sensitize SRC for titration of residual complement. Whole hemolysin contains both IgM and IgG anti-SRC antibody and since RF may react with IgG-sensitized SRC, these cells are not satisfactory indicator cells for titration of residual complement.

Reduction and alkylation of aggregated IgG markedly impairs its fixation of complement (47). Using this antigen, Zvaifler and Schur obtained the first evidence of complement fixation by RF (11). Subsequent studies reaffirmed these observations (12) but failed to provide incontrovertible proof that RF was responsible for the complement activation since whole serum was used as the source of RF and the possibility that some other non-RF anticomplementary substance was associated with the RF-IgG complexes was not excluded. SRC sensitized with whole hemolysin were used in titration of residual complement, further contributing to difficulty of interpreting experimental results. Tesar and Schmid extended and clarified these observations using purified RF and indicator cells sensitized with IgM hemolysin (13). However, in all these reports RF proved an extremely weak complement-fixing antibody. Since IgM antibodies are generally quite effective in complement fixation (48), some uncertainty remained.

In experiments reported here we were not successful in demonstrating any significant reduction of either whole hemolytic complement or individual complement components by RF-IgG complexes in fluid phase. However, it should be pointed out that we used considerably less aggregated IgG and RF than was used in the reports cited above. This was done because preparations of R/A-IgG are not totally incapable of fixing complement and we were concerned that further aggregation by RF of weakly complement-fixing IgG aggregates might account in part for the experimental results of other investigators.

In contrast, SRC sensitized with Agg-R/A-IgG or REA were readily hemolyzed by even small amounts of polyclonal RF in the presence of added complement. Three lines of evidence clearly indicate that hemolysis is mediated by complement. First, uptake of radiolabeled C4, C3, and C8 correlates directly with percent hemolysis. Secondly, hemolysis is abolished by chelation of divalent cations with EDTA or EGTA, treatment of complement with hydrazine or cobra venom, and by incubation of complement at 56°C for 30 min. Thirdly, monospecific antisera reactive with individual complement components are highly inhibitory when added to the HuC source. The demonstration that uptake of the early complement component, C4, correlates directly with percent hemolysis and that hemolysis is inhibited by EGTA (49) and antisera to early com-

plement components, indicates that under the conditions employed, sensitized SRC are lysed by RF and complement through the classical pathway. Contrary to previous reports in the literature (11, 12), GPC is as effective as HuC in lysing the SRC sensitized with RF. Our findings are in agreement with the recent demonstration of complement fixation by RF reported by Tesar and Schmid (15) and contrast with the report of McDuffie and Brumfield (21).

The direct hemolytic system described in this report for demonstration of complement fixation has proven considerably more sensitive than the fluid phase system. This is presumably because few molecules, probably only one, of cell-bound IgM-RF are needed to sensitize cells for lysis while multiple antibody molecules are needed in free solution to produce measurable complement reduction. These results are quite similar to those reported for IgM antibody specific for human blood group substance A (50). A second advantage of the direct hemolytic system is that anticomplementary substances which may be present in RF preparations are removed by washing the cells before incubating them with complement.

While all polyclonal RF fixed complement in the direct hemolytic system, it is of interest that the three monoclonal RF did not. One of these RF was isolated by acid gel filtration which may have altered its ability to fix complement. None of the three was heated to 56°C which has been reported to abolish anticomplementarity of mixed cryoglobulins (51). Of course, our sample is small and other investigators have reported that some monoclonal RF are complement fixing (51). However, it should be pointed out that none of those studies employed R/A-IgG and, therefore, one cannot differentiate complement fixation by RF from that produced by IgG in the mixed cryoprecipitate.

We have previously shown that efficiency of complement fixation by IgG antihapten antibody correlates directly with its binding energy (52). Although there are only a few reported determinations of RF binding energies, they are all of similar magnitude (24, 25, 53–55). A recent study suggests that while strength of RF-IgG interaction may exert some influence on complement fixation it is probably of secondary importance (15). It was shown that RF isolated from high-titer rheumatoid sera were more efficient in complement fixation than RF isolated from low-titer sera. The influence of binding energies was minimized by adjusting all purified RF preparations to the same agglutinating strength.

Schmid, Roitt, and Rocha (14), have shown that RF are, in general, weakly complement fixing, capable of augmenting hemolysis of cells suboptimally sensitized with hemolysin but inhibiting hemolysis of optimally sensitized cells. In the latter case the weak activity of IgM-RF is substituted for the stronger complement fixation by IgG hemolysin.

IgM-RF may be composed of antibodies which are intrinsically weak in complement fixation or may be composed of a mixture of complement fixing and noncomplement fixing antibodies, accounting for these observations. We believe our finding of noncomplement fixing monoclonal RF is pertinent to this point. Failure to fix complement might reflect differences in μ-chain subclasses. Although IgM subclasses have not been clearly demonstrated, one may be encouraged to this belief by analogy with IgG subclasses which do exhibit differences in complement fixation (56). In this regard, rabbits immunized with *p*-arsanilic acid conjugates have been clearly shown to produce IgM responses consisting of complement-fixing and noncomplement-fixing antibodies (57).

Both complement-fixing and noncomplement-fixing monoclonal RF probably exist, although we believe that the latter are more frequently encountered. It is reasonable to assume that polyclonal RF represent the collective specificities and biological activities of many monoclonal RF, most of which do not fix complement. Thus, weakly complement-fixing polyclonal RF are composed of a low percentage of complement-fixing and a greater percentage of noncomplement-fixing IgM antibodies.

Noncomplement-fixing IgM may lack the C1 binding site or may possess configurations that interfere with C1 binding. Plaut, Cohen, and Tomasi have shown that pentameric Fc μ is considerably more active on a molar basis with regard to complement fixation than is the parent IgM molecule from which it is derived (58). This is consistent with the latter view stated above.

It is clear that antibody responses to some immunogens are restricted in immunoglobulin class and subclass (59, 60). In certain experimental situations the mode of immunization is important (61). It seems likely that antigenic sites in the Fc region of IgG, to which the host responds, are of importance in determining the type of RF produced. Thus, some Fc antigens would select for noncomplement-fixing RF while others would select for complement-fixing RF, reflecting differences in specificity for antigen.

The in vivo relevance of complement-fixing IgM-RF is difficult to assess. It is clear that RF may inhibit or augment complement fixation under appropriate experimental conditions. However, there are compelling reasons to suspect that it contributes to the inflammation of rheumatoid arthritis. Patients with highest titers of RF have severest disease and are more likely to have systemic vasculitis (5). It is within this group

that one finds patients who exhibit depressed serum complement titers (6) and hypercatabolism of C3 (62). Human RF have been shown to augment immune damage in experimental animals lending further evidence supporting a pathogenetic role (7, 8).

The direct hemolytic assay of RF described in this report is readily adaptable to whole serum and can be used to assess rapidly the complement-fixing activity of RF from large numbers of patients. This should provide important correlations with respect to the natural course of disease, and response to therapy in patients with rheumatoid arthritis.

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