Effect of Rheumatoid Factor on Complement-Mediated Phagocytosis

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ABSTRACT The frequency and amount of IgM rheumatoid factor (RF) in the blood of patients with rheumatoid arthritis (RA) correlate with the severity of the disease and the number of complications. Though previous studies of RF in subacute bacterial endocarditis have shown that RF inhibits phagocytosis of microorganisms by granulocytes, the presence of low levels of complement (C) in blood and synovial fluid of patients with the highest titers of RF suggests that an interaction between RF and C may contribute to the inflammatory process in RA. We thus employed a quantitative methodology to examine the effect of RF on complement-dependent phagocytosis of sheep erythrocytes by rabbit granulocytes. Addition of 2500 molecules of IgM RF to sheep cells heavily coated with IgG antibody (195,000 molecules per cell) resulted in virtually complete inhibition of uptake of C3 (β_{1c}) and prevention of phagocytosis, an effect resulting from inhibition of uptake of C1 by the cells. When erythrocytes coated with only 34,000 molecules of IgG antibody were employed, phagocytosis was similarly inhibited. However the effect of RF on such cells was shown to be primarily mediated through inhibition of C4 rather than C1 uptake. Although the results do not exclude the participation of an IgM RF of higher avidity, present only in the tissues in rheumatoid inflammation, circulating IgM RF probably does not play a potentiating role in rheumatoid inflammation.

INTRODUCTION

Whether rheumatoid factor (RF)[1] (anti-IgG antibodies present in the sera of most patients with rheumatoid ar-

thritis [RA]) contributes to the pathogenesis of the inflammation of synovium or other tissues in this disease is not known. Though the experiments of Hollander and his collaborators (1, 2) (who found that injection of autologous IgG into quiescent joints could produce exacerbations of synovitis) were interpreted on the basis of an immune complex reaction between injected IgG and RF, this work has not been successfully repeated (3). Davis has postulated that RF may actually inhibit inflammation produced by immune complexes (4). In support of this latter concept Messner and his associates (5) found that IgM RF isolated from patients with subacute bacterial endocarditis was able to inhibit polymorphonuclear leukocyte phagocytosis of the infecting organisms coated with 7S IgG antibody. The inhibitory effect observed seemed to be primarily directed at the phagocytosis-promoting effect of fresh normal serum.

In the present study we have pursued the question of the effect of IgM RF on complement-mediated phagocytosis employing a quantitative methodology aimed at defining the effect with respect to quantities of antibody and RF involved as well as seeking to identify the point in the complement sequence at which RF acts. The results show that IgM RF does inhibit complement-mediated phagocytosis by blocking fixation of both C1 and C4. Interference with C1 fixation predominates at high levels of IgG antibody whereas interference with C4 fixation plays an important role at lower levels of antibody.

METHODS

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[1] *Abbreviations used in this paper:* C, complement; EM, effective molecule; RA, rheumatoid arthritis; RF, rheumatoid factor.
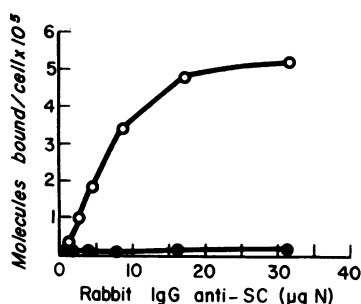
FIGURE 1 Binding of rabbit ^{125}I-labeled IgG to sheep erythrocytes (E); IgG added to 5×10^7 E. (○), anti-sheep cell IgG; (●), normal IgG.

cipitate the euglobulin. The precipitate was dissolved in pH 8.0 Tris (0.1 M), NaCl (0.2 M) buffer, and the IgM fraction isolated by chromatography on Sephadex G-200. In order to eliminate small amounts of IgG bound to RF the rheumatoid IgM was further purified by sucrose density gradient ultracentrifugation in a 5–20% sucrose gradient in pH 3.5, 0.1 M glycine buffer. Final yield of IgM from the original pool was about 10%. Normal IgM was similarly prepared from a pool of two sera.

The IgG fraction from rabbit serum was prepared by chromatography of a 50% saturated ammonium sulfate precipitate of normal or immune rabbit serum on DEAE-cellulose (DE-32, Whatman) at pH 8.5, 0.01 Tris buffer plus additional NaCl to achieve a relative salt concentration of 0.04 M (conductivity, 4500 reciprocal Ω at 25°C). The antiserum was a pool from five rabbits immunized intravenously with whole sheep cells.

Complement components. Guinea pig C1 and C2 were purified according to the method of Nelson, Jensen, Gigli, and Tamura (6). Human C3 (β_{1c}) was isolated by chromatography on TEAE- or DEAE-cellulose according to the method of Müller-Eberhard and his associates (7, 8). Chromatography on hydroxyapatite as used by these workers for final purification of β_{1c} did not prove to be a reproducible method in our hands for eliminating the major contaminant, C5, while still preserving full C3 activity. However our final preparation from DEAE-cellulose contained less than 5% contamination by C5 as determined by radial immunodiffusion analysis. Human C4 was prepared by the method of Müller-Eberhard and Biro (9). The final preparations contained small amounts of C1 inhibitor (less than 1 effective molecule per 100 effective molecules of C4).

Complement titrations. All titrations were carried out in tubes by modifications of the basic methodology of Mayer (10) except that one-half of all the recommended volumes were used and all tubes contained 7.5×10^7 sheep erythrocytes. C1 was titered with EAC₄, prepared by removing C1 from EAC₁,₄ by incubation in pH 7.5 barbital buffer, 0.075 M relative NaCl and 0.01 M EDTA, at 37°C for 10 min followed by an additional incubation after washing at 0°C for 12 hr. C2 was measured with EAC₁,₄ prepared by incubating EA with 1000 effective molecules (EM) C1 per cell followed by addition of 50 EM C4 per cell. For C4 determination EAC₁, made by addition to EA of 1000 EM C1 per cell (200–350 EM C1 actually bound per cell as determined by C1 transfer test [11]), was employed. C3 (β_{1c}) was measured by immune adherence of EAC₁,₄,₂ (12) in microtiter plates. Serial dilutions of sample in 0.025 ml glucose-veronal buffer (6) were first made. Then 0.025 ml of EAC₁,₄,₂, 4×10^7/

ml, were added, these having been prepared by addition of 75 EM of C2 per cell to EAC₁,₄. After 10 min at 37°C, 0.025 ml of a suspension of human erythrocytes, 4×10^7/ml, in barbital buffer containing 0.01 M EDTA was added, together with 0.025 ml of 0.4 M NaCl. The cells were mixed and allowed to settle for 2 hr, at which time the agglutination patterns were read.

Radiolabeling. Rabbit IgG, human IgM, and human C3 were all labeled with ^{125}I in order to measure the amounts bound to cells or antibody. The chloramine T method as described by McConahey and Dixon (13) was used for this purpose. IgG and C3 retained full antibody and immune adherence activity respectively when labeled by this technique with 1 mCi ^{125}I/mg protein. IgM, however, was found to be very labile upon iodination. Part of the difficulty was traced to the excess of reducing agent, Na₂S₂O₅, added to stop the iodination reaction. By using only just sufficient Na₂S₂O₅ to neutralize the amount of chloramine T added, reduction of IgM no longer occurred. In addition it was found desirable to reduce the amount of ^{125}I to 0.05 mCi ^{125}I/mg IgM and to keep the reaction time to 1 min at room temperature. The number of atoms of carrier-free ^{125}I bound per molecule protein was about 5×10^{-3} to 1×10^{-2} for IgM and IgG and 5×10^{-2} to 1×10^{-1} for C3. The immune adherence titer per milligram of β_{1c} of each C3 preparation labeled with ^{125}I was always within one to twofold dilution of that of the original serum from which the preparation was isolated.

Binding of antibody and complement to cells. Addition of antibody and complement components to erythrocytes was carried out in pH 7.5 barbital buffer, containing 0.1% gelatin, relative NaCl 0.075 M. During addition of antibody, EDTA, 0.01 M, was present. Calcium and magnesium were present in the buffer used for addition of C. Cells plus IgG antibody were incubated 30 min at 37°C followed by 30 min at 0°C. After washing, the EA were incubated with human IgM for 30 min at 37°C and then washed with buffer containing Ca^{++} and Mg^{++}. After addition of C1 the cells were incubated at 0°C for 1 hr and again washed. Components C4, C2, and C3 were added sequentially with incubation at 30°C for 30 min after each component and washing at 0°C between each step. The number of molecules of antibody bound to sheep cells was determined by incubating 0.5 ml of a series of dilutions of rabbit ^{125}I-labeled IgG with 0.5 ml of sheep cells, 1.0×10^7/ml. After washing three times with
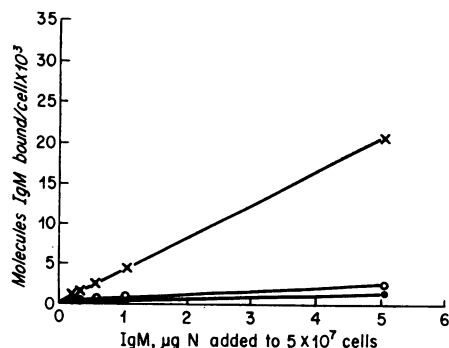

FIGURE 2 Binding of normal and rheumatoid human ^{125}I-labeled IgM to sheep erythrocytes coated with 34,000 molecules IgG antibody per cell (EA). (X), rheumatoid IgM bound to EA; (○), rheumatoid IgM bound to E; (●), normal IgM bound to EA.

the buffer the cells were counted in a Packard autogamma scintillation counter (Packard Instrument Co., Downers Grove, Ill.). Counts in tubes not containing cells were subtracted. These "glass binding" counts were always less than 5% of the counts added. The determinations of bound RF and C3 were made by the same technique except that the buffer used for C3 did not contain EDTA. Appropriate controls for nonspecific binding by E, EA, or $EAC_{1,4}$ were performed as well as controls of normal, nonimmune rabbit IgG, or human IgM. Binding of Cl to EAC was determined by the Cl transfer test of Borsos and Rapp (11).

Phagocytosis. We employed the method of Gigli and Nelson (14) in which sheep erythrocytes coated with antibody and complement were phagocytized by rabbit neutrophils. Nonphagocytized cells were measured by lysis with distilled water since the erythrocytes within granulocytes were protected. A portion of appropriately coated sheep erythrocytes, 0.5 ml containing 2.5×10^{9} cells in 0.075 M relative NaCl barbital buffer, was incubated with 3.0 ml of rabbit granulocytes containing $2-3 \times 10^{7}$ cells in Hanks' solution, and the mixture tumbled for 1 hr at 37°C. Then 5.0 ml of distilled water was added, the cells centrifuged, and the OD of the supernates determined at 415 nm in a spectrophotometer. Appropriate controls of granulocytes alone and uncoated erythrocytes plus granulocytes were prepared as well. The number of cells phagocytized was calculated as:

$$\frac{OD \text{ (test mixture)} - OD \text{ (granulocytes)} - OD \text{ (EA, spontaneous lysis)}}{OD \text{ (EA} + H_2O) - OD \text{ (EA, spontaneous lysis)}} \times F$$

where

$$F = \frac{1}{OD \text{ of } 1 E}.$$

RESULTS

Binding of antibody and RF to cells. Figs. 1 and 2 show representative examples of the binding data obtained for rabbit IgG antibody to E and rheumatoid IgM to EA. The very low blank values obtained for normal IgG and IgM with E and EA respectively establish the validity of the method for the measurement of anti-sheep cell antibody and RF. In subsequent experiments reference curves such as those shown were used in calculating the amount of unlabeled antibody or RF bound to cells.

Effect of IgM RF on phagocytosis. Fig. 3 shows the effect of rheumatoid IgM on phagocytosis of cells coated with 195,000 molecules IgG antibody per cell, an amount which represents the optimum needed for complement fixation. Human IgM was added to the cells after antibody and before Cl. Little inhibition of phagocytosis was produced by normal IgM, even at a concentration of 10 µg protein N. Rheumatoid IgM on the other hand, at a concentration as low as 0.5 µg N (resulting in 2500 molecules bound per cell), produced striking inhibition of phagocytosis to the base line level. When the amount of IgG antibody was reduced by 85% to 34,000 molecules bound per cell, a similar inhibitory effect was seen
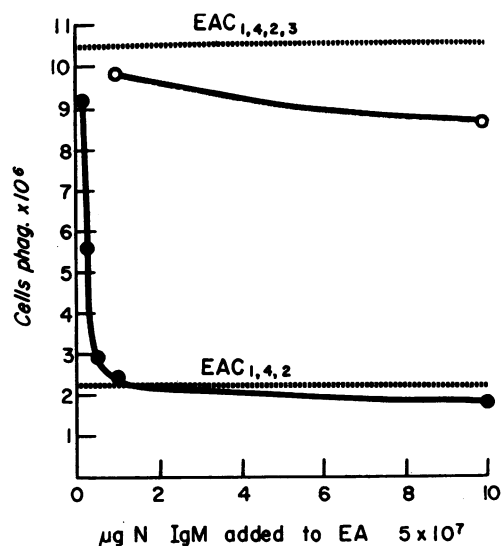

FIGURE 3 Inhibition of phagocytosis by rheumatoid IgM of EA coated with 195,000 molecules IgG antibody per cell. Complement components used per cell were Cl, 100 EM; C2, 75 EM; C4, 100 EM; C3 (β_{1c}) 280 molecules. (O), normal IgM; (●), rheumatoid IgM.

as shown in Fig. 4. About twice as much rheumatoid IgM, 1.0 µg N, was needed to produce comparable inhibition, but this amount added actually resulted in the same amount of bound RF, 2400 molecules per cell. The results shown were obtained with 9500 molecules of C3 (β_{1c}) per cell, a larger amount than was employed in Fig. 3. However, very similar results were obtained when a smaller amount of C3 (β_{1c}) was employed, 280 molecules per cell.

Role of complement components in inhibition of phagocytosis (high input of IgG antibody). In order to determine whether the inhibitory effect of rheumatoid IgM on phagocytosis which we had observed was mediated through an effect on the complement system, we examined the fixation of complement components to cells coated with 195,000 molecules of antibody per cell and treated with rheumatoid IgM before addition of complement components. Fig. 5 demonstrates that inhibition of phagocytosis (Fig. 3) was almost exactly paralleled by inhibition of binding of ^{125}I-labeled C3 (Fig. 5). To answer the question of whether inhibition of C3 binding reflected a direct effect on C3 binding or inhibition of an earlier step in the complement sequence, Cl transfer tests on EAC_1 made with normal and rheumatoid IgM were carried out. Fig. 6 shows that when a high input of rabbit IgG antibody was employed (195,000 molecules per cell) Cl uptake was almost completely inhibited by as little as 0.5 µg N of rheumatoid IgM, the same amount required to block complement-dependent phagocytosis and C3 uptake completely (Figs. 3 and 5).
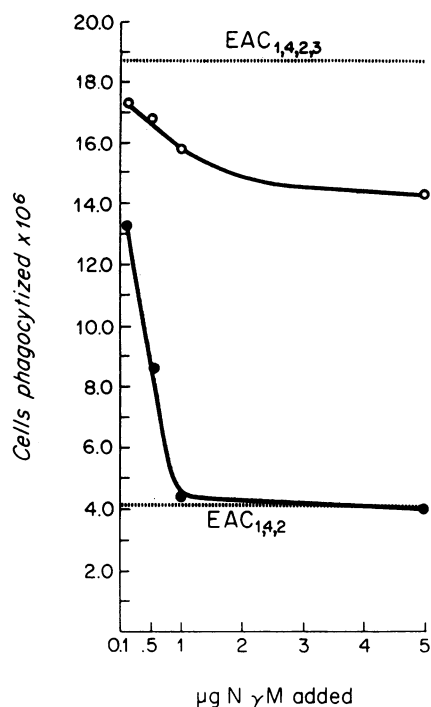
FIGURE 4 Inhibition of phagocytosis by rheumatoid IgM of EA coated with 34,000 molecules IgG antibody per cell. Complement components used were same as in 3 except C3 (β_{1c}) 9500 molecules. (O), normal IgM; (●), rheumatoid IgM.

Role of complement components in inhibition of phago-cytosis (low input of IgG antibody). When the above experiments were repeated with cells coated with only one-sixth as much IgG antibody it was found that whereas C3 uptake was similarly inhibited by RF, C1 uptake was not. Table I demonstrates that C1 uptake as measured by transfer test was only very slightly inhibited by addition of RF at low levels of C1 input (100 EM per cell) and not at all at high C1 input (1000 EM per cell). In additional experiments (Table II) in which

TABLE I
Effect of RF on C1 Uptake by Cells Having Low Input of IgG Antibody

Order of addition	EM C1 added per cell	EM C1 bound per cell
EA + C1	100	42
EA + RF + C1	100	34
EA + C1 + RF	100	34
EA + C1	1000	914
EA + RF + C1	1000	1000
EA + C1 + RF	1000	914

EA coated with 34,000 molecules IgG antibody per cell.
RF = rheumatoid IgM, 1.0 μg N, added to 5 $\times$ 10^7 EA.

TABLE II
Effect of RF on Binding of C2

Cells	EM C2 per cell in supernate	EM C2 bound per cell	C2 bound
			%
None	(96)		
EA + C2	88	0	0
EA + RF + C2	84	4	5
EA + RF + C1 + C4 + C2	68	20	22
EA + C1 + RF + C4 + C2	64	24	25
EA + C1 + C4 + RF + C2	39	49	56
EA + C1 + C4 + C2	38	50	57

34,000 molecules IgG antibody per cell.
RF = rheumatoid IgM, 1.0 μg N, added to 5 $\times$ 10^7 cells. C1, C4, and C2, 100 EM of each added per cell.

RF was added at different steps of the complement sequence, it was found that addition of RF after C4 did not affect C2 uptake as measured by titration of residual C2. Nor did addition of RF after C2 affect uptake of C3 as determined by binding of ^{125}I-labeled C3. Table III, however, demonstrates that binding of C4 by EA was
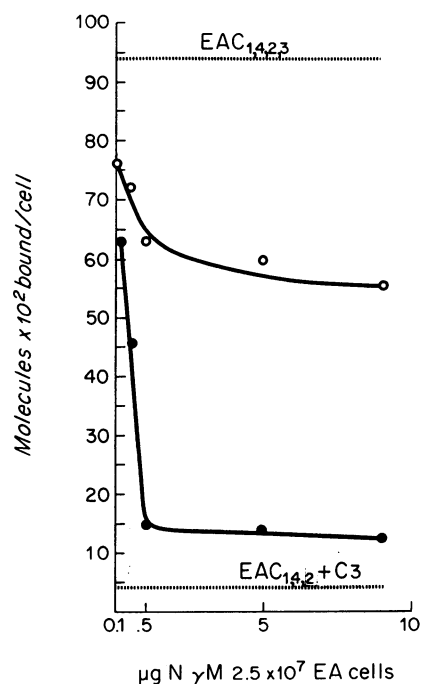

FIGURE 5 Effect of RF on binding of C3 (β_{1c}). Antibody and complement components same as in Fig. 4. Line labeled EAC$_{1,4}$ + C3 represents nonspecific binding of C3 to EAC$_{1,4}$. Line labeled EAC$_{1,4,2,3}$ represents specific binding of C3 to EAC$_{1,4,2}$. (O), binding of C3 (β_{1c}) to EAC$_{1,4,2,3}$ to which normal IgM had been added; (●), binding of C3 (β_{1c}) to EAC$_{1,4,2,3}$ to which rheumatoid IgM had been added.

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impaired, even when RF was added after C1. Thus although EAC, not treated with IgM, bound 66 molecules C4 per cell, addition of RF after C1 reduced C4 binding to 26 molecules per cell, a result suggesting interference by RF of binding of C4 to antibody.

Effect of human C1. The above experiments were performed with partially purified guinea pig C1 as a source of the first component of complement. Zvaifler and Bloch (26) found that RF inhibited uptake of guinea pig complement at 4° and 37°C yet was able to fix human complement at 37° but not 4°C. We therefore examined the effect of RF on phagocytosis of cells first coated with human C1 at 37°C. The results of Table IV show that RF added before or after human C1 inhibited phagocytosis of such cells about equally as well as cells made with guinea pig C1 (compare Fig. 4).

DISCUSSION

Phagocytosis of microorganisms, erythrocytes, or other particles by polymorphonuclear granulocytes is markedly enhanced by the addition of antibody and complement. Indeed whether antibody alone in the absence of complement is capable of promoting phagocytosis is not entirely certain. Although a specific binding site for IgG antibodies has been clearly demonstrated on the surface of the macrophage (15) it has been more difficult to establish the presence of such a site on granulocytes. Messner and Jelinek have presented evidence for such a specific receptor site for the Fc portion of γG-globulin on the surface of human neutrophils (16). However these authors were only able to achieve binding of Rh-positive erythrocytes to neutrophils by using a particular hyperimmune complement-fixing anti-Rh (Ripley) and not with ordinary anti-Rh sera. Even when purified IgG and thoroughly washed granulocytes are used in phagocytic assays it is difficult to exclude participation of small amounts of complement bound to leukocytes. Golden and McDuffie (17), for example, found that inhibition of lupus erythematosus cell formation could be achieved with anti-complement antiserum in a phagocytic system in which complement could not be detected by ordinary means. Although factors other than complement may be responsible for the enhancing effect of fresh serum on phagocytosis none has as yet been unequivocally identified. Gigli and Nelson (14) showed that addition of C1, C2, or C4 had no effect on phagocytosis whereas addition of C3 produced a marked enhancement, not affected by addition of the subsequently reacting components, C5-C9.

Thus it is not surprising that IgM RF which blocks complement fixation by IgG antibodies is able to inhibit phagocytosis of particles coated with antibody and complement. Messner, Lasdal, Quie, and Williams (5) have previously shown that IgM RF isolated from patients with subacute bacterial endocarditis (SBE) was able to
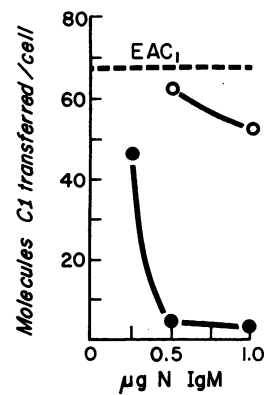

FIGURE 6 Effect of RF on binding of C1 to EA coated with 195,000 molecules of antibody per cell. 100 EM C1 added per cell. (O), normal IgM; (●), rheumatoid IgM.

inhibit ingestion by polymorphonuclear leukocytes of bacteria coated with IgG opsonin. If RF was added to antibody-coated bacteria before addition of fresh normal serum the effect was more pronounced than if it was added after fresh serum. The present experiments clearly demonstrate that this effect is mediated through inhibition of C1 and C4 uptake by antigen-antibody complexes. Presumably the inhibition of C1 uptake is the result of competition between RF and C1q for the same site or adjacent sites on the Fc portion of IgG (18, 19).

We cannot explain why inhibition of C1 fixation by RF was so much more pronounced when cells heavily coated with antibody were used. Aho, Harboe, and Leikola (20) found that the agglutination by RF of sheep cells coated with small amounts of IgG could be inhibited

TABLE III

Effect of RF on C4 Uptake by Cells Having Low Input of IgG Antibody

	100 EM C4 added per cell		
Order of addition	EM C4 per cell in supernate	EM C4 bound per cell	C4 bound
			%
EA	82	0	0
EA + C4	82	0	0
EA + RF + C4	82	0	0
EA + C1 + C4	16	66	80
EA + C1 + RF + C4	56	26	32
EA + RF + C1 + C4	74	8	10

EA coated with 34,000 molecules IgG antibody per cell. RF = rheumatoid IgM, 1.0 μg N, added to 5×10^7 cells. C1, 100 EM per cell. 100 EM C4 added per cell and after centrifugation residual C4 determined in the supernate by hemolytic titration. Bound C4 determined by subtraction of appropriate controls.

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TABLE IV

Effect of RF on Phagocytosis (Human C1)

Order of addition	Cells phagocytized $\times 10^6$	Cells phagocytized
		%
EAC$_{1,4,2}$	1.25	5
EAC$_{1,4,2,3}$	12.0	48
EA + N IgM + C1,4,2,3	9.3	37
EAC$_1$ + N IgM + C4,2,3	9.8	39
EA + RF + C1,4,2,3	2.5	10
EAC$_1$ + RF + C4,2,3	7.0	28

34,000 molecules IgG antibody per cell. EM added per cell were 100 C1, 75 C4, and 50 C2. 200 molecules C3 (β_{1c}) added per cell.

1.0 μg N RF or normal IgM per cell.

by native IgG in serum, but agglutination of heavily coated cells could only be inhibited with aggregated IgG such as that in an immune precipitate. Whether the greater affinity of RF to heavily coated cells reflects only the involvement of more binding sites (out of a possible total of five on the IgM molecule) or a configurational change occurring on the IgG antibody after its reaction with red cell antigens cannot be answered. C1q is also multivalent, and C1 binds to EA more strongly as the number of IgG molecules on the cell surface is increased (21). A similar mechanism of competition may account for the inhibition of C4 uptake which we observed at lower levels of antibody input, since C4 is known to bind to an as yet unidentified site on antibody (22). Since C2 binds directly to C4 to form a complex which acts in turn to activate or bind C3, failure of RF to inhibit binding of C2 when added after C4 is not surprising (23). Although C3 or β_{1c} may bind directly to immunoglobulin (24) it may also attach to the surface of sensitized erythrocytes (25), a phenomenon which may explain failure of RF to inhibit C3 binding when added to EAC$_{1,4,2}$.

IgM RF has previously been shown to interfere with fixation of complement by IgG antibodies (26, 27). Zvaifler and Schur (28) found that the complex formed between RF and mercaptoethanol-treated aggregated IgG (which reacts with RF but does not itself fix complement) did not consume human or guinea pig complement when the complex was added to fresh rheumatoid serum. Large amounts (> 1.0 mg) or preformed complexes of RF and mercaptoethanol-treated IgG were, however, able to fix human but not guinea pig complement. In our experiments we found RF equally inhibited phagocytosis of cells coated with either human or guinea pig C1. Schmid, Roitt, and Rocha (29) found that, whereas RF inhibited complement fixation of sheep cells optimally sensitized with IgG antibody, some complement fixation by RF

occurred when cells coated with suboptimal amounts of antibody were used. In the present experiments, however, reduction in the amount of IgG antibody used to coat sheep cells to 15% of the optimal amount did not significantly reduce the complement uptake nor the amount of phagocytosis observed. The complement-fixing activity of RF must be weak at best, even under carefully controlled conditions.

The mechanism by which RF inhibits complement fixation remains obscure. No significant structural differences between IgM RF and other human IgM globulins have so far been detected (30, 31). Yet IgM antibodies of most species fix complement and promote phagocytosis more efficiently than IgG antibodies (32–34). MacKenzie, Creevy, and Heh (35) have reported that some IgM myeloma globulins bind C1q to a much lesser extent than normal. Possibly RF contains a high proportion of such antibodies since we found that inhibition of C1 fixation was virtually complete when optimally sensitized cells were pretreated with RF. The explanation for the inhibitory effect on C4 binding is not apparent from our data although it has been found that IgM antibodies are less efficient than IgG antibodies in the titration of C4 (36). Human IgM antinuclear antibodies in our experience also appear to be deficient in their ability to fix complement and to promote phagocytosis (37). Most of the previous work on inhibition of complement fixation by RF has employed inhibition of immune hemolysis as an indicator so that no one has excluded the possibility that RF may fix complement at a position sufficiently distant from the cell surface so that, in spite of activation of the components, cell lysis may not occur. The present experiments clearly show that activation and binding of the first four steps of the complement sequence are actually prevented by RF.

These results imply that circulating IgM RF does not play a significant role in the inflammation of vessels and tissues in RA, since the phlogistic effects of IgM antibodies are probably mediated largely through the complement system. Though immune complexes appear to be responsible for the low levels of complement in synovial fluid (38), pleural fluid (39), and serum[2] of rheumatoid patients, IgM RF itself does not seem to be the major antibody in these complexes. Winchester, Agnello, and Kunkel (38) found only IgG and not IgM in the high molecular weight material in rheumatoid synovial fluid which they were able to relate to complement depletion. Horwitz, Garrett, and Davis (40) found that rheumatoid IgM inhibited rather than enhanced release of complement-derived chemotactic activity. In experiments with passive immune synovitis in the rabbit knee joint we have not found any consistent enhancing effect of

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[2] Hunder, G. G., and F. C. McDuffie. Hypocomplementemia in rheumatoid arthritis. Unpublished observations.

RF on the inflammatory reaction, slight inhibition or no effect being the usual result.[3] On the other hand Tesar, Schmid, and Suarey (41) have pointed out that RF vary in avidity for antigen and that those of high avidity fix complement better than those of low avidity. Thus it is possible that a small proportion of RF of particularly high avidity may be contributing to tissue damage.

ACKNOWLEDGMENTS

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