COMPLEMENT FIXATION BY A TWO-COMPONENT ANTIBODY SYSTEM: IMMUNOGLOBULIN G AND IMMUNOGLOBULIN M ANTI-GLOBULIN (RHEUMATOID FACTOR)

PARADOXICAL EFFECT RELATED TO IMMUNOGLOBULIN G CONCENTRATION*

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(Received for publication 7 May 1970)

Immunoglobulin M antibody bound to erythrocyte membranes fixes complement more effectively on a molar basis than immunoglobulin G antibody (1, 2). However, the addition of IgM\(^1\) antigen (rheumatoid factor [RF]) to IgG complexed with cell-surface antigens actually reduces the amount of complement fixed below the amount observed with IgG alone (3, 4, 5). This finding initially led to the belief that the IgM in question, human rheumatoid factor, lacked the capacity to fix complement, an observation subsequently disproven when it was shown that IgM-RF like most other IgM molecules did indeed possess this ability (6, 7, 8). Therefore, the decreased effect observed when IgM is reacted with IgG complexed with cell membranes must be explained on other grounds.

This report presents evidence that complement fixation in this two component antibody system is critically dependent upon the concentration of IgG. The addition of purified IgM-RF to high concentrations of IgG antibody on erythrocytes reduces the amount of complement fixation compared to that observed with IgG alone. Addition of IgM-RF to low concentrations of IgG antibody produces enhancement of complement fixation.

**Materials and Methods**

*Rheumatoid Factor Preparation.*—High-titered sera from patients with rheumatoid arthritis were absorbed by sheep cells and peglobulin fractions obtained by dilution with 14 volumes of

*Supported in part by research grant AM 11513, U. S. Public Health Service, and by grants from The Medical Research Council, United Kingdom.

\(^1\) Abbreviations used in this paper: BDB-HGG, bis-diazotized benzidine-human gamma globulin (Cohn Fraction II); C1q, the 11S unit of the first component of complement; R1q, reagent deficient in C1q but containing the remaining components of complement; RF, rheumatoid factor; SCAT, sheep red cell agglutination test for rheumatoid factor.

673
distilled water, the pH being brought to 6.0 by careful addition of dilute HCl. The precipitate was suspended in a volume of phosphate-buffered saline, pH 7, equal to that of the original serum, centrifuged at 4°C to float off lipid material, and kept in small portions at -20°C. Purified RF was obtained either from such euglobulin preparations or from whole serum by adsorption onto insoluble human gamma globulin cross-linked by bisdiazotized benzidine (9), and elution by 0.3 M acetate buffer, pH 4.0. In later experiments, the acid eluates were further purified by sucrose density ultracentrifugation employing gradients of 10–40% sucrose in acetate buffer, the 19S fraction being dialyzed against phosphate buffer pH 7.0. These preparations had a protein content of 1.0–1.5 mg/ml and gave a single IgM line on immunoelectrophoresis and a single 19S peak on analytical ultracentrifugation. After analysis, bovine serum albumin was added to a concentration of 10 mg/ml and stored at -20°C. Such purified preparations maintained their potency for up to 12 months.

The agglutinating ability of RF was assayed by a microtiter technique against sheep cells sensitized with one quarter the amount of rabbit anti-sheep erythrocyte serum needed to cause agglutination. Sera or fractions being tested were first absorbed with sheep cells. The agglutinating titer was expressed as the reciprocal of the highest dilution causing maximal agglutination.

Hemolytic Antibodies.—Rabbit antiserum to sheep red blood cells was either obtained commercially (Markham Laboratories, Chicago, Ill. and Burroughs Wellcome, London, England) or prepared by immunization with sheep red blood cell stromata (10). Immunoglobulin G antibody was separated from IgM: IgG by elution of the breakthrough peak from diethylaminoethyl-cellulose in 0.01 M phosphate buffer, pH 8.0, and IgM by one or two passages through a column of Sephadex G200. Completeness of separation was assessed by immunoelectrophoresis using commercial antisera against rabbit serum and an antibody preparation against rabbit IgM kindly supplied by Doctor Parker Small. Bovine serum albumin, 10 mg/ml, was added to samples of the purified antibodies which were stored at -20°C.

Determination of Complement Levels.—Whole serum was assayed for its complement titer (CH50 units/ml) by the method of Kabat and Mayer (10) except for a reduction to 40% of the volume of reagents employed. Results, however, are expressed in the text and figures in terms of the standard 7.5 ml volume of reagents. Sheep erythrocytes were sensitized with an amount of either IgM or IgG hemolysin just sufficient to give optimal lysis in the presence of 1–2 CH50 units of human serum previously absorbed with sheep cells to remove natural hemolytic antibody. Such cells were designated as sensitized with 1.0 unit of antibody. Traces of self-agglutination were observed at this concentration with IgG hemolysin but not with IgM hemolysin. These cells were added to dilutions of the sera to be tested in 13 X 100 mm tubes, incubated for 60 min at 37°C with periodic swirling of the contents, centrifuged, and the supernatant fluid read for hemolysis at 541 m# in a spectrophotometer. Determination of CH50 was made on probit graph paper from lines determined by three or more points (11).

In later experiments, before using red cells, we washed them twice with ethylenediaminetetraacetate, pH 7.0, 0.01 M in saline to remove trace amounts of the first component of complement (1). Results with such cells were similar to those obtained without this step.

Complement Components.—C1q (the 11S unit of the first component of complement) was obtained from fresh human sera free of RF by the method of Morse and Christian (12) except that insoluble bis-diazotized benzidine-human gamma globulin (BDB-HGG) was used as an adsorbant rather than heat-aggregated globulin. Elution was accomplished by 0.3 M acetate buffer, pH 4.5, and the acid eluate further purified by column chromatography on G200 Sephadex; after this it was concentrated and dialyzed against phosphate buffer, pH 7.0. Such preparations were contaminated by trace amounts of IgG and occasionally by other proteins, as determined by immunodiffusion. On sedimentation-velocity ultracentrifugation, a major 11S peak was obtained. Protein concentrations ranged between 1.0 and 2.0 mg/ml. Bovine serum albumin was added to a concentration of 10 mg/ml and portions were stored at -20°C.
A reagent deficient in Clq (Rlq) was prepared from normal human serum (13) by use of insoluble BDB-HGG as an absorbant. Such preparations were unable to lyse optimally sensitized sheep blood cells but activity could be restored by addition of purified Clq.

**Complement Inhibition and Enhancement Assays.**—RF preparations or control materials to be tested for their effect upon hemolysis were added to dilutions of a standard normal human serum from which natural hemolytic antibody had been completely removed by two absorptions with sheep red blood cells at 0°C for 5–10 min each. A volume of 0.1 ml of purified RF or 0.4 ml of euglobulin preparation to be tested was used in the 40% assay system which would be equivalent to 0.25 ml and 1.0 ml respectively in the full-volume method (10). The mixture was then assayed for complement activity by the addition of cells coated with 1.0 unit of IgG antibody or of cells coated with lesser amounts (0.1–0.7 units) of IgG antibody. Parallel experiments were set up without the addition of RF preparations. The order of addition of reagents for these tests was as follows. The buffer, test material, and serum dilution used as a source of complement were added to 13 x 100 mm tubes kept at 0°C. Sensitized cells were then added and the mixture incubated at 37°C for 1 hr with repeated mixing of the contents at 10–15 min intervals. In other experiments, the order of addition was changed as indicated in the text. Removal of unbound antibody or RF by washing cell mixtures prior to the addition of complement serum did not alter the degree of lysis from that obtained without this step.

Results of these assays were calculated in two ways. In one, they were expressed as the change in the number of CH₅₀ units of complement detected after the addition of the test material. Inhibition of complement activity was reflected by the lesser number of CH₅₀ units observed, and enhancement by the greater number. By definition, CH₅₀ units can only be employed when using cells coated with optimal (1.0 unit) amounts of IgG hemolytic antibody (10). Therefore, data from experiments in which suboptimal amounts (less than 1.0 unit) of IgG antibody were used to coat cells are reported as the change in the percentage of lysis achieved in the presence of a fixed number of CH₅₀ units of complement. In this second expression, inhibition of complement activity was indicated by a lesser percentage of lysis, and enhancement by a greater percentage. With reduction in the amount of IgG antibody employed, larger amounts of complement were used to achieve hemolysis within the measurable range of 20–80% since an inverse hemolytic relationship exists for the amounts of sensitizing antibody and complement (Fig. 4).

**Kinetic Experiments of Complement Inhibition.**—Reaction mixtures were prepared in 50 ml stoppered Erlenmeyer flasks that were constantly agitated in an oscillating 37°C water bath. Materials were added in the order described in the text to a total volume of 15 ml. Zero time was recorded at the time of addition of the Rlq reagent. At timed intervals thereafter, one ml samples were withdrawn into iced tubes containing 2 ml of 0.005 M ethylene diamine tetraacetate in phosphate saline buffer, pH 7.0, and immediately centrifuged. Supernatant fluids were tested for degree of hemolysis as already noted.

**RESULTS**

*Effect of Rheumatoid Factor on Immune Lysis of Erythrocytes Coated with Optimal (1.0 Unit) Concentrations of IgG Hemolysin*

*Euglobulin Preparations from Rheumatoid Patients.*—Euglobulin preparations from six adults with rheumatoid arthritis and four normal individuals were tested for their ability to influence hemolysis using standard normal human sera absorbed with sheep erythrocytes as sources of complement (Table I). Normal sera alone showed similar complement activity with red cells optimally
sensitized with either IgM or IgG hemolysins, although slightly higher values were usually obtained with the IgM coat.

To differentiate between complement fixation occurring on the membrane where RF binds to IgG from a nonspecific reaction of the preparation with complement, the effect of euglobulin on the lysis of IgM-coated cells was tested. Since RF does not react with IgM, any reduction in lysis would be attributable to nonspecific complement consumption. When the euglobulin fraction was added to IgM-coated cells, variable but relatively minor degrees of inhibition or

| Euglobulin donor | IgG coat (CH₅₀ units measured) | IgM coat (CH₅₀ units measured) | CH₅₀ change due to euglobulin | SCA* titer |
|-----------------|-------------------------------|-------------------------------|-----------------------------|-----------|
|                 | Standard serum | Standard serum + euglobulin | Change due to euglobulin | Standard serum | Standard serum + euglobulin | Change due to euglobulin | via cell-bound IgG coat |
| R.A. Patients   |                 |                              |                             |           |
| MOR             | 33.4            | 5                             | -28.4                       | 35.7      | 28.3                          | -7.4                           | -21                   | 1536       |
| PAR             | 33.4            | 5                             | -28.4                       | 35.7      | 32.6                          | -3.1                           | -25.3                 | 768        |
| WOR             | 32.3            | 13.7                          | -18.6                       | 32.3      | 26.3                          | -6                            | -12.6                 | 1024       |
|                 | 24              | 14                            | -10                         | 29        | 30                            | +1                            | -11                   |            |
| LIF             | 21.7            | 5                             | -16.7                       | 25.4      | 21.3                          | -4.1                           | -12.6                 | 512        |
| BAK             | 21.7            | 5                             | -16.7                       | 25.4      | 29                            | +3.6                           | -20.3                 | 64         |
| DEL             | 21.7            | 20                            | -1.7                        | 25.4      | 31.9                          | +6.5                           | -8.2                  | 16         |
| Normals         |                 |                               |                             |           |
| PEG             | 21.7            | 16.1                          | -5.6                        | 25.4      | 18.2                          | -7.2                           | +1.6                  | <4         |
| GLE             | 24              | 36                            | +12                         | 29        | 35                            | +6                            | +6                    | <4         |
|                 | 33.4            | 31.9                          | -1.5                        | 35.7      | 31.9                          | -3.8                           | +2.3                  |            |
| DAV             | 24              | 29                            | +5                          | 29        | 35                            | +6                            | -1                   | <4         |
|                 | 32.3            | 38.4                          | +6.1                        | 32.3      | 40                            | +7.7                           | -1.6                  |            |
|                 | 33.4            | 33.4                          | 0                           | 35.7      | 36.5                          | +0.8                           | -0.8                  |            |
| BAN             | 33.4            | 37.5                          | +4.1                        | 35.7      | 37.5                          | +1.8                           | +2.3                  | <4         |
|                 | 21.7            | 17.3                          | -4.4                        | 25.4      | 20.8                          | -4.6                           | -0.2                  |            |

Values refer to the number of CH₅₀ units measured per milliliter of the standard serum.

* Sheep red cell agglutination test for rheumatoid factor; reciprocal of maximum positive dilution.
augmentation of complement action were noted. However, with the IgG coat, marked inhibition occurred in the presence of rheumatoid euglobulin. Inhibition of complement activity specifically related to blockage of interaction at the antibody site on the cell membrane was computed by subtracting any comple-

| Preparation*                  | Change in $\text{CH}_{50}$ | $\text{CH}_{50}$ change due to preparation acting via cell-bound IgG coat | SCAT titer |
|-------------------------------|-----------------------------|--------------------------------------------------------------------------|------------|
|                               | IgG Coat                  | IgM Coat                  |                           |            |
| R.A. Patients                 |                            |                            |                           |            |
| MOR: eluted RF                | $-20.4$                    | $+2.8$                    | $-23.2$                  | $32$       |
| absorbed euglobulin           | $-11.5$                    | $-7$                      | $-4.5$                   | $<4$       |
| LIF: eluted RF                | $-19.7$                    | $+1.3$                    | $-21$                    | $32$       |
| absorbed euglobulin           | $-7.8$                     | $-2.4$                    | $-5.4$                   | $<4$       |
| TOB: eluted RF                | $-15$                      | $+6.1$                    | $-21.1$                  | $16$       |
| absorbed euglobulin           | $-4.4$                     | $+6.1$                    | $-10.5$                  | $4$        |
| PAR: eluted RF                | $-8.5$                     | $+4.4$                    | $-12.9$                  | $8$        |
| absorbed euglobulin           | $-30$                      | $-32$                     | $+2$                     | $<4$       |
| WOR: eluted RF                | $-1.2$                     | $+2.8$                    | $-4$                     | $4$        |
| absorbed euglobulin           | $+1.3$                     | $+2.8$                    | $-1.5$                   | $<4$       |
| DEL: eluted RF                | $+10$                      | $+2.8$                    | $+7.2$                   | $<4$       |
| absorbed euglobulin           | $-3.4$                     | $0$                       | $-3.4$                   | $<4$       |
| Normals                       |                            |                            |                           |            |
| CAM: eluted RF                | $+1.3$                     | $+1.3$                    | $0$                      | $<4$       |
| absorbed euglobulin           | $-6.2$                     | $-5.4$                    | $-0.8$                   | $<4$       |
| PEG: eluted RF                | $+4.4$                     | $+2.8$                    | $+1.6$                   | $<4$       |
| absorbed euglobulin           | $-6.2$                     | $-4.4$                    | $-1.8$                   | $<4$       |

* RF preparations in this particular experiment only were not further purified by sucrose density ultracentrifugation.

† Standard human serum alone contained 32 $\text{CH}_{50}$ units/ml in assays with erythrocytes coated with optimal amounts of either IgG or IgM rabbit hemolysin.

§ Sheep red cell agglutination test for rheumatoid factor; reciprocal of maximum positive dilution.

Effect of Rheumatoid Factor Purified from Euglobulin Preparations on Complement-Mediated Immune Hemolysis. Euglobulins were Absorbed with Insoluble Human Gamma Globulin (BDB-HGG) and the Rheumatoid Factors Eluted with Acid

Purified Rheumatoid Factor.—The essential role of RF in this inhibition on the
cell membrane was confirmed by using a purified preparation rather than the euglobulin fraction. A series of eight euglobulins, six from patients with rheumatoid arthritis and two from normal individuals, were absorbed onto insoluble BDB-HGG. The supernatant fluids from such mixtures as well as the material eluted from the BDB-HGG were tested for their effect upon hemolysis using cells optimally coated with IgM and IgG hemolysin (Table II). The eluates which were positive in the sheep cell agglutination test inhibited lysis in the

![Diagram](image)

**Fig. 1.** Complete recovery in the supernatant fluid of complement activity not fixed by rheumatoid factor—IgG system. Incubation I: Cross-hatched bars indicate number of CH₅₀ units of complement consumed after 5 min incubation at 37°C by sheep erythrocytes optimally sensitized by IgG or IgM hemolysin in the presence or absence of rheumatoid factor. Incubation II: Open bars indicate number of CH₅₀ units of complement remaining in the supernatant fluids after the above incubation when tested by sheep erythrocytes optimally sensitized by IgM hemolysin.

IgG system but had no such comparable effect upon the IgM system. In contrast, the absorbed euglobulin preparations were often anti-complementary in the IgM system and the difference in effect observed between IgG- and IgM-coated cells was markedly lower than that obtained with the purified RF preparations. This was particularly evident with the supernatant resulting from absorption of the euglobulin from patient PAR which almost completely inhibited lysis of both IgM- and IgG-coated erythrocytes.

Further evidence that inhibition of complement activity by RF was due to blockage of its attachment to the antibody site on the cell membrane and not to the result of its destruction or utilization in the liquid phase was shown in the
following experiment. Using a standard normal serum absorbed by sheep cells, approximately 40% of complement activity was consumed after incubation with either IgG- or IgM-coated erythrocytes, the remaining 60% being detected in the supernatant when subsequently incubated at 37°C for 60 min with IgM-coated cells (Fig. 1). When RF was added, about 40% of complement was again consumed by IgM-coated cells but only 16% by IgG-coated cells. The remaining complement in each supernate was completely recovered when tested with IgM-coated cells. The initial incubation time had to be shortened to 5 min since no measurable complement could be subsequently demonstrated in any of the supernates if this period was extended to 15 min or longer.

**Relationship between Agglutinating Titer and Inhibitory Activity of Rheumatoid Factor.**—A direct relationship was found to exist between the agglutinating titer of RF and its effectiveness in blocking complement-dependent lysis of optimally sensitized sheep cells. A total of 22 RF preparations from 16 different patients had been derived from either the euglobulin fraction or whole serum...
by absorption with BDB-HGG and elution, and were compared (Fig. 2). Agglutination titer correlated well with ability to inhibit complement fixation ($r = 0.91; P < 0.001$). In another experiment, the inhibitory effect of three RF preparations was studied over a wider range of concentrations using erythrocytes coated with two different amounts of IgG hemolysin. At optimal (1.0 unit) levels of sensitization, increasing quantities of RF led to greater degrees of inhibition; at levels of sensitization two-thirds optimal, a similar but slightly greater inhibitory pattern was demonstrated. Complete inhibition of lysis was not achieved even with the maximal amounts of RF used (Fig. 3).

![Graph](image)

**Fig. 3.** Progressive inhibition of immune lysis of IgG-coated red cells by increasing concentrations of three purified rheumatoid factors. Individual rheumatoid factors are indicated by different symbols. Red cells sensitized with 1.0 unit of antibody for preparations □, ○, and □ sensitized with 0.67 unit of antibody for preparation ●.

**Effect of Rheumatoid Factor on Immune Lysis of Erythrocytes Coated with Suboptimal (<1.0 Unit) Concentrations of IgG Hemolysin**

**Euglobulin Preparation from a Rheumatoid Patient.**—Erythrocytes which were coated with one-tenth of an optimal amount of IgG hemolysin measured just 2.3 CH50 units out of the 24 actually present in a standard normal serum (Table III). Unexpectedly, the addition of a euglobulin preparation from a rheumatoid patient increased the amount of complement activity detected while that from a normal individual did not. When erythrocytes were sensitized with 0.4 unit IgG antibody, enhancement by rheumatoid euglobulin no longer could be demonstrated. Rather, at this level of antibody and at optimal or 1.0 unit antibody concentration as noted previously, inhibition occurred.
**Purified Rheumatoid Factor.**—Constant amounts of seven RF preparations obtained from the sera of four patients were evaluated for their effect upon complement-dependent hemolysis using erythrocytes that had been sensitized with varying amounts of IgG hemolysin. Barely detectable degrees of hemolysis with unsensitized cells were noted for several of the RF preparations indicating that a trace amount of natural hemolytic antibody still persisted after two prior absorptions with sheep cells (Table IV). Both at 0.1 and 0.2 units of antibody sensitization, the addition of each RF strikingly enhanced the degree of hemolysis noted. This enhancement became more marked as the number of units of complement was increased from a minimal quantity of 1.75 up to 8.5 CH₅₀ units. At 0.5 units of antibody sensitization, slight inhibition of lysis at minimal amounts, and slight enhancement at maximum amounts of complement were noted. At optimal (1.0 unit) levels of antibody sensitization, inhibition of lysis could easily be demonstrated, confirming earlier results. Although not indicated in the table, none of the RF preparations influenced the degree of lysis observed with control cells coated with optimal amounts of IgM hemolysin nor, in the case of a few preparations, with lesser amounts of IgM hemolysin ranging downward to levels barely adequate to generate lysis.

### TABLE III

| Degree of IgG sensitization | IgG coat (CH₅₀ units measured) | IgM coat (CH₅₀ units measured) | CH₅₀ change due to euglobulin | SCAT* titer |
|----------------------------|--------------------------------|--------------------------------|-------------------------------|------------|
|                            | Standard serum                 | Standard serum + euglobulin    | Change due to euglobulin     |            |
| R.A. Patient (WOR)         |                                |                                |                               |            |
| 1.0                        | 24 14                          | 29 30                          | +1 -11                        | 1024       |
| 0.4                        | 8.3 5.3                        | 5.3 5.3                        | -3 (768)                      |            |
| 0.1                        | 2.3 5.3                        | 5.3 5.3                        | +3 (48)                       |            |
| 0                          | 0 0                            | 0 0                            | 0 (<6)                        |            |
| Normal (DAV)               |                                |                                |                               |            |
| 1.0                        | 24 29                          | 29 35                          | +6 -1                         | <4         |
| 0.4                        | 8.3 5.3                        | 5.3 5.3                        | -3 (<6)                       |            |
| 0.1                        | 2.3 0.7                        | 0.7 5.3                        | -1.8 (<6)                     |            |
| 0                          | 0 0                            | 0 0                            | 0 (<6)                        |            |

Values refer to the number of CH₅₀ units measured per milliliter of the standard serum.

* Sheep red cell agglutination test for rheumatoid factor; reciprocal of maximum positive dilution. Values in parentheses obtained using cells sensitized with IgG antibody at the same concentration used for respective complement assay.
Absorption Studies of a Rheumatoid Factor Preparation.—The essential role of RF in these reactions was confirmed by absorption studies of a potent preparation by BDB-HGG. Removal of agglutinating activity was associated with loss of both the inhibiting and enhancing effects of the preparation (Table V).

### TABLE IV

**Effect of Purified Rheumatoid Factors upon Complement-Mediated Lysis of Sheep Erythrocytes Coated with Varying Amounts of IgG Rabbit Hemolysin**

| Rheumatoid factor SCAT* | Relative IgG hemolysin concentration |
|------------------------|-------------------------------------|
|                        | 0 | 0.1 | 0.2 | 0.5 | 1.0 |
| 1.75 CH50 units        |   |     |     |     |     |
| HAL—43                 | 2048 | 1  | 12  | 22  | 32  | 35  |
| WEG—42                 | 2048 | 1  | 3   | 8   | 24  | 51  |
| VAN—41                 | 2048 | 1  | 4   | 8   | 24  | 47  |
| None                   |     | 0   | 0   | 0   | 40  | 85  |
| 5.8 CH50 units         |   |     |     |     |     |
| KIS                    | 2048 | 0  | 7   | 27  | 55  |
| WEG                    | 1024 | 0  | 1   | 12  | 54  |
| VAN                    | 128 | 0  | 1   | 6   | 84  |
| None                   |     | 0   | 0   | 0   | 94  |
| 6.0 CH50 units         |   |     |     |     |     |
| HAL                    | 256 | 4  | 78  | 86  | 82  |
| None                   |     | 0   | 24  | 92  | 98  |
| 8.5 CH50 units         |   |     |     |     |     |
| HAL—43                 | 2048 | 3  | 76  | 89  | 100 | 100 |
| WEG—42                 | 2048 | 2  | 31  | 74  | 100 | 100 |
| VAN—41                 | 2048 | 3  | 29  | 65  | 100 | 100 |
| None                   |     | 0   | 8   | 41  | 90  | 100 |

Values refer to the percentage lysis observed.
* Sheep red cell agglutination test for rheumatoid factor; reciprocal of maximum positive dilution.

### TABLE V

**Effect of Absorption of a Rheumatoid Factor Preparation by BDB-HGG upon its Ability to Influence Complement-Mediated Lysis of Erythrocytes Coated with Varying Amounts of IgG Hemolysin**

| Rheumatoid factor (KIS) | SCAT* | Relative IgG hemolysin concentration |
|-------------------------|-------|-------------------------------------|
|                         |       | 0 | 0.1 | 0.2 | 1.0 |
| Unabsorbed              | 480   | 0 | 3   | 20  | 63  |
| Absorbed                | <15   | 0 | 0   | 0   | 93  |
| None                    |       | 0 | 0   | 2   | 100 |

Values refer to the percentage of lysis observed in the presence of 5.5 CH50 units of complement.
* Sheep red cell agglutination test for rheumatoid factor; reciprocal of maximum positive dilution.
In the experiments recorded in Table IV, some of the RF preparations were able to induce definite but insignificant degrees of lysis of unsensitized cells even though each preparation had been twice absorbed with sheep cells prior to use. This raised the possibility that trace amounts of residual natural hemolytic antibody in the preparation might potentiate the degree of sensitization.

**TABLE VI**

*Effect of Five Successive Absorptions with Sheep Erythrocytes upon the Ability of a Rheumatoid Factor Preparation to Influence Complement-Mediated Lysis of Erythrocytes Coated with Varying Amounts of IgG Hemolysin*

| No. of absorptions* | Absorbed supernate | IgG concentration | Absorbing cells |
|---------------------|---------------------|-------------------|-----------------|
|                     | SCAT§               | 0     | 0.1 | 0.2 | 0.4 | 1.0 |
| Unabsorbed          | 512                 | 0     | 27  | 41  | 47  | 89  | 2    |
| No RF§              | 1                   | 2     | 13  | 81  | 100 | 3    |
| 1                   | 512                 | 1     | 26  | 41  | 43  | 90  | 0    |
| No RF               | 1                   | 2     | 11  | 79  | 99  | 4    |
| 2                   | 256                 | 1     | 26  | 41  | 41  | 91  | 0    |
| No RF               | 2                   | 2     | 13  | 80  | 99  | 5    |

Values refer to the percentage lysis observed in the presence of 3.1 CH₅₀ units of complement.

* 1.6 ml rheumatoid factor preparation absorbed with five successive portions of 5 × 10⁶ sheep red cells. Each sample of absorbing cells tested for lysis using 3.1 CH₅₀ units of complement.

§ Sheep red cell agglutination test for rheumatoid factor; reciprocal of maximum positive dilution.

§§ Control without added RF.

of cells coated with suboptimal concentrations of IgG hemolysin. Therefore, RF was absorbed five times with sheep erythrocytes. Each sample of cells used in absorption, when subsequently tested, was unable to generate lysis in the presence of complement (Table VI). No significant fall in SCAT (sheep red cell agglutination test) titer was noted in the absorbed RF preparation and, when tested after the last absorption it was still able to either inhibit or enhance lysis depending on the IgG antibody concentration.

*Relationship between Agglutinating Titer and Enhancing Activity of Rheuma-
toid Factor.—A positive correlation existed between the degree of enhancement of immune lysis and the agglutinating titer of the RF. Such a tendency was suggested in a previous experiment in which three RF preparations, each with a different titer (Table IV at 5.8 CH<sub>50</sub> units), caused increasing amounts of hemolysis with increasing agglutinating titer. In a further experiment, three other preparations caused progressively greater lysis with each fourfold increase of agglutinating titer (Table VII). Two of these three equipotent agglutinating preparations gave almost identical degrees of hemolysis but the third (HAL-43) had a considerably greater hemolytic effect. The lysis of IgM-coated cells was unaffected.

**TABLE VII**

| Rheumatoid factor | SCAT*   | Relative rheumatoid factor concentration |
|-------------------|---------|-----------------------------------------|
|                   | 0.0  | 0.25 | 1.0 | 4.0 |
| 0.1 IgG hemolysin |       |      |     |     |
| HAL—43            | 2048  | 2    | 62  | 74  | 82  |
| WEG—42            | 2048  | 2    | 12  | 28  | 34  |
| VAN—41            | 2048  | 2    | 15  | 27  | 43  |
| 0.01 IgM hemolysin|       |      |     |     |
| HAL—43            | 2048  | 5    | 1   | 1   | 1   |
| WEG—42            | 2048  | 5    | 1   | 1   | 1   |
| VAN—41            | 2048  | 5    | 1   | 1   | 0   |

Values refer to the percentage of lysis observed in the presence of 8.5 CH<sub>50</sub> units of complement.

* Sheep red cell agglutination test for rheumatoid factor; reciprocal of maximum positive dilution. SCAT titer refers to strength of the preparation at 1.0 concentration.

**Effect of Complement and its C1q Component on the Degree of Hemolysis Observed in the Presence of Rheumatoid Factor**

Complement.—The interaction between complement and the hemolysin and RF components on the erythrocyte membrane was studied over the range of 0.1–1.0 unit IgG antibody sensitization to further define the complement requirements of the system. The results obtained with 0.2 and 1.0 unit of hemolysin are illustrative of the trend observed (Fig. 4), with enhancement at the lower level and inhibition at the higher level of sensitization.

When complement activity is expressed as a percentage of the maximum observable with optimally sensitized cells, this paradoxical effect of RF on complement consumption is readily seen (Fig. 5). At approximately one-third optimal IgG hemolysin concentration on the erythrocyte, RF exerted no measurable effect on complement activity; to achieve a given degree of complement activity...
above that point required a substantially greater amount of IgG hemolysin in the presence of RF, while below that point required less IgG hemolysin.

Effects of Sequential Addition of C1q and Rheumatoid Factor.—Kinetic experiments on the inhibition of complement fixation by RF showed that the over-all

![Figure 4](image-url)

Fig. 4. Plot of the number of CH50 units of complement required to lyse red cells coated with optimal (1.0) and suboptimal (0.2) amounts of IgG antibody in the presence of rheumatoid factor. RF preparations 25 (■) and 43 (○) had sheep cell agglutination titers of 1/512 and 1/2048 respectively. —— without rheumatoid factor; —— with rheumatoid factor.

![Figure 5](image-url)

Fig. 5. Effect of rheumatoid factor preparations upon complement activity achieved with varying concentrations of IgG hemolysin. Data plotted from the type of experiment illustrated in Fig. 4 utilizing three preparations, numbers 25 (SCAT titer 1/512), 41, and 42 (both with SCAT titers 1/2048).
sigmoid shape of the curve was not grossly altered but that the rate of cell lysis was reduced and in the period from 45 to 90 min there was only a slight increase in hemolysis (Fig. 6). The effect of varying the order of addition of C1q and RF was studied. In one case RF was added to the sensitized cells 10 min before a C1q preparation, the reaction system being completed after a further 10 min by addition of an R1q reagent, while another reaction mixture was set up with C1q first, followed 10 min later by RF plus the R1q reagent. The rate of cell lysis obtained with prior addition of C1q was comparable to that seen in the absence of RF over the initial 15 min, but thereafter inhibition became evident.

![Diagram showing the effect of sequence of addition of C1q and rheumatoid factor on the rate of lysis of sensitized cells in the presence of R1q reagent.](image)

**Fig. 6.** Effect of sequence of addition of C1q and rheumatoid factor on the rate of lysis of sensitized cells in the presence of R1q reagent. Sequence of addition of reagents to sensitized cells: ••; Clq, -10 min; R1q + RF, 0 min. ●●; RF, -20 min; Clq, -10 min; R1q, 0 min. ○○; Clq, -10 min; R1q, 0 min.

In all stages of the reaction the degree of hemolysis was only marginally greater than that found when RF had been added first.

**Effect of Red Blood Cell Agglutination on Hemolysis**

During the incubation period in which lysis occurred, macroscopic agglutination of red cells developed in mixtures containing RF, small aggregates sometimes persisting despite periodic shaking of the mixture during incubation. This was not evident at suboptimal levels of antibody sensitization. The possibility was therefore entertained that agglutination per se either inhibited the release of hemoglobin from cells or did not allow ready access of complement components to available sites through close packing of the cells. To test this possibility, hemolysis was determined in the presence of phytohemagglutinin, an
agglutinators that act directly on the red cell (Table VIII). When degrees of macroscopic agglutination were achieved similar to those found in the rheumatoid system, barely detectable interference with lysis occurred. Only when the cells agglutinated to form a single mass was it possible to inhibit lysis to a significant degree. It should be mentioned that the degree of agglutination which occurred in the RF system became evident only after 15-20 min incubation so that adequate time would be available for reacting complement components to have access to the cell membrane. Indeed, in the presence of excess complement, complete lysis would still be achieved despite the presence of strong agglutination.

**TABLE VIII**

_Effect of Macroscopic Agglutination Caused by Phytohemagglutinin M (PHA-M) on the Susceptibility of Optimally Sensitized Sheep Erythrocytes to Immune Lysis_

| Incubation time of PHA-M and RBC prior to addition of complement | Degree of macroscopic agglutination | Class of hemolysin | CHa units assayed |
| --- | --- | --- | --- |
| (min) | | | PHA-M absent | PHA-M present |
| 10 | None | IgM | 30.3 | 28.6 |
| 15 | Moderate | IgM | 27.8 | 25.0 |
| 15 | " | IgM | 35.7 | 37.5 |
| 23 | " | IgG | 33.4 | 30.0 |
| 35 | " | IgM | 30.3 | 28.6 |
| 90 | Solid | IgM | 33.3 | 21.3 |

**DISCUSSION**

These studies demonstrate that IgM rheumatoid factor, after its interaction with IgG antibody on the erythrocyte membrane, causes a paradoxical response in complement fixation—enhancement at lower concentrations of IgG and inhibition at higher concentrations. The reaction in either direction is accentuated by increased concentrations of RF.

In assessing the influence of RF on this system, it is important to distinguish between the effect caused by nonspecific complement-consuming reactions in the fluid phase in contrast to the specific binding of complement to antibody on the cell surface. A competing fluid phase reaction must be considered since RF was first eluted from an immunoabsorbant of human gamma globulin and then bound to rabbit IgG hemolysin on the erythrocyte. That portion of the RF with affinity for human but less affinity for rabbit IgG (14) might therefore be available to bind to the aggregated IgG conceivably present in the system. Any fluid phase reaction, however, was controlled in the design of the present study by using red cells coated with IgG hemolysin to which RF binds and with IgM...
hemolysin to which it does not. RF caused no significant changes in complement fixation by cells coated with either optimal or suboptimal amounts of IgM hemolysin whereas striking changes were noted with IgG-coated cells. As noted below, earlier studies investigating the interaction of RF and complement did not always exclude this fluid-phase effect with their euglobulin preparations. Furthermore, when red cells coated with IgG hemolysin and RF were washed to remove unbound reactants, they still behaved in the same fashion as cells not washed to which complement was added. Finally, when RF inhibited the hemolysis of cells coated with optimal amounts of IgG, complement was spared and could be recovered completely in the fluid phase.

That IgM rheumatoid factor, rather than some other constituent in rheumatoid serum, was the effective agent on the cell membrane was evident from both the activity of purified RF and the loss of activity after absorption with insoluble gamma globulin. In addition, there was a good correlation between the agglutinating titer of purified RF and the degree to which it influenced complement fixation.

It is highly unlikely in the face of this evidence that other substances in human serum could have been active in conjunction with or in place of RF. Significant amounts of natural or heterophile antibody against sheep cells found in almost all human sera were removed by prior absorption procedures, and trace amounts, if any, that might have been left behind would lyse the control unsensitized erythrocytes. Subthreshold amounts of these antibodies in conjunction with IgG hemolysin might have the potential of binding complement but no fixation could be detected by even more rigorous absorption procedures carried out on a single RF preparation. Although rheumatoid sera contain immunoconglutinin (15) and this might have contaminated the RF preparations, it seems unlikely that the effects observed on the IgG hemolysin system could be attributed to this protein since comparable changes should have been observed with IgM-coated erythrocytes. Heimer et al., using erythrocytes sensitized with whole rabbit-hemolytic serum, attributed inhibition of complement fixation to a macromolecule other than rheumatoid factor (3). Its nature was not established but it lacked the ability to agglutinate globulin-coated particles and resided in a slightly different region of the peak eluted from DEAE-cellulose by 0.3 M salt solutions than did RF. However, in the present report, the active material isolated from serum fulfilled the generally accepted criteria for RF (16).

The inhibitory effect of RF is presumably linked to its ability to sterically inhibit the reactions of bound C1q or more probably to its competitive displacement of C1q from the IgG heavy chain. The loose binding of C1q to antibody, recognized for intact C1 complex by other workers (17), was demonstrated in our kinetic experiments. C1q was added to cells sensitized with high concentrations of IgG prior to the addition of IgM-RF; the reaction was then initiated
by the introduction of the remaining complement components. The initial rate of lysis approximated that seen in the absence of RF, but after a period of approximately 15 min inhibition became apparent and the speed of the reaction fell to that obtained in the system in which RF had been added before C1q. This changeover in reaction rate would support the view that RF displaced C1q from its binding to IgG.

Earlier reports emphasized an inhibitory effect of RF on the ability of complement to fix to IgG coated onto particulate carriers. These included studies on the binding of complement by IgG on sheep red cells (3), latex particles (18, 19), and mitochondrial subfractions (20) where the concentration of the immunoglobulin coat was not a limiting factor. The inhibition observed in the present work using sheep cells coated optimally with IgG is fully consistent with these findings. The paradoxical enhancing effect of RF seen with suboptimal IgG hemolysin coats can probably be accounted for in terms of direct fixation of complement by the RF itself, as has been independently demonstrated by others (6, 8). At low concentrations, the IgG antibody fixes complement poorly but is still capable of binding RF, as evidenced by agglutination reactions. At high concentrations of complement by this bound RF, although of a relatively weak order, is greater than that obtained with the IgG antibody alone. On the other hand, the red cells coated with optimal amounts of IgG hemolysin fix considerably more complement and this is reduced by addition of RF; on the simplest view RF blocks the complement-binding activity of IgG and substitutes its own relatively weak reactivity. Parallel studies using IgG reactant pretreated with 2-mercaptoethanol accord with this interpretation. In other words, the RF interaction with IgG generates only a limited ability to fix complement; when contrasted to the fixation at suboptimal concentrations of IgG alone, the net result appears as enhancement but when contrasted with that at an optimal coat of IgG, the net result is inhibition (Figs. 5 and 7).

Since RF influences complement action in vitro, the possible involvement of this effect in biologic systems is of interest. Evidence has accumulated that complement fixation occurs within the synovial fluids of patients with seropositive rheumatoid arthritis (21, 22). Soluble IgG complexes of such fluids, possibly containing anti-globulins of IgG class, fix complement in vitro and are associated with low levels in vivo (23). The number of cellular inclusions in polymorphonuclear phagocytes of joint fluids from seropositive patients are related to high titers of IgM-RF and to lowered joint fluid complement levels (24). If purified IgM-RF is added to synovial fluids of patients with seronegative rheumatoid disease, cellular inclusions form within polymorphonuclear cells (25), perhaps in the same manner in which IgM-RF increases the phagocytosis of aggregated IgG in vitro (26). Less is known of the effects of RF in tissues.

Schmid, F. R., and M. J. Rocha. Unpublished observations.
outside the joint but several clinical situations suggest a possible role (27). Recently, in the experimental model of nephrotoxic nephritis, RF appeared to enhance the degree of renal inflammation (28). Thus, the likelihood must be entertained that RF is an active agent in the pathogenesis of tissue inflammation.

Whether the contrary circumstance of inhibition of complement fixation occurs in vivo remains to be explored. In a tissue culture system complement-dependent cytolysis of human thyroid cells was shown to be partially inhibited by IgM-RF (4). Gough and Davis have suggested that IgM-RF might trap immune complexes in joint fluids, thereby excluding them from the vascular compartment and protecting other organs of the body, for example, the kidney, from the damaging effects of circulating soluble complexes (29).

It may be that in vivo as well as in vitro RF plays a dual role. Where local circumstances favor its ability to enhance complement fixation, perhaps in the joint or when bound to membranes coated with suboptimal amounts of antibody, it potentiates the inflammatory response. On other cell membranes sensitized by larger amounts of IgG antibody to which RF binds, it is possible that a
less effective site for complement fixation is offered, thereby blunting the intensity of the inflammatory response.

SUMMARY

Complement-mediated lysis of sheep erythrocytes coated with optimal concentrations of rabbit IgG hemolysin was inhibited by euglobulin fractions from the sera of patients with seropositive rheumatoid arthritis. That this was due to direct interaction with the IgG coat on the red cell rather than a nonspecific reaction with complement in the fluid phase was confirmed by controls using cells coated with IgM hemolysin. The inhibitory activity was recovered in purified IgM rheumatoid factor preparations and could be absorbed out with insoluble aggregated human IgG. The inhibitory potency of the rheumatoid factors correlated well with their sheep cell agglutination titers. Inhibition was not the result of physical aggregation of the erythrocytes by rheumatoid factor. Kinetic studies were consistent with the view that rheumatoid factor displaces C1q from its binding to IgG. Paradoxically, at suboptimal sensitizing concentrations of IgG hemolysin, rheumatoid factor enhances the fixation of complement. These results can be interpreted on the basis of the blockage of complement fixation by IgG and its replacement by a relatively weak direct fixation by the IgM rheumatoid factor. Thus, the interaction of RF with IgG generates only a limited ability to fix complement which, when contrasted with the fixation at suboptimal concentrations of IgG hemolysin alone, appears as net enhancement; when this is contrasted with fixation occurring with optimal concentrations of IgG, it appears as net inhibition.

The authors wish to express their appreciation to Mr. Abdul Ghaffar for his expert technical help and to Mrs. Sharon Kunitz and Gladys Stead for their careful assistance in the preparation of the manuscript. Also, we thank the American Red Cross for kindly providing supplies of human gamma globulin. Finally, we thank Doctors H. J. Rapp and T. Borsos for their helpful advice during the course of these studies.

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