The Detection of Cell-Bound Antibody on Complement-Coated Human Red Cells

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ABSTRACT This study sought to elucidate the mechanism by which human red cells, in a variety of clinical settings, become coated in vivo with autologous complement components in the absence of anti-red cell autoantibodies demonstrable by standard methods. By means of a newly developed complement-fixing antibody consumption test, previously undetectable red cell-bound γG globulin could be detected and quantified. By this technique, the complement-coated red cells of 13 of 16 patients were shown to carry abnormally high numbers of γG molecules per cell, which were nevertheless below the level for detection by the direct antiglobulin test. Eluates were made from the red cells of seven of these patients and each eluate, when sufficiently concentrated, was capable of sensitizing normal human red cells (with γG antibodies) to give a positive indirect antiglobulin test with anti-γG serum. In the presence of fresh normal serum, six of the eluates so tested were capable of fixing complement to normal human red cells. The antibodies in the red cell eluates did not exhibit Rh specificity and did not react with nonprimate red cells. When studied by sucrose gradient ultracentrifugation, the γG antibodies to human red cells in these eluates sedimented in the 7S region. It is concluded that in many patients in whom direct antiglobulin tests reveal only cell-bound complement, the complement fixation is mediated *in vivo* by small quantities of "warm-reacting" erythrocyte autoantibodies of the γG class.

INTRODUCTION

Red blood cells (RBC) showing positive direct antiglobulin tests with anti-complement serum and negative reactions with antisera to γG, γA, or γM immunoglobulins have been observed in patients with connective tissue disorders, lymphoproliferative diseases, or idiopathic acquired hemolytic disease (1–3). The mechanism by which complement is bound to autologous RBC in such cases has been elusive. Earlier attempts to demonstrate autoantibodies in eluates from the RBC of such patients were unsuccessful (4, 5). Likewise, efforts to detect in these patients' sera complement-fixing autoantibodies reactive with unmodified human red cells have yielded inconsistent or negative results (1, 2). Cold agglutinin titers have usually been normal (1). Since attachment of complement to RBC can be produced experimentally in vitro by mechanisms other than fixation by antibodies (6–8), similar mechanisms may conceivably be operative in clinical illnesses. Alternatively, it seemed possible that small amounts of RBC autoantibodies or antigen-antibody complexes (9), undetectable by conventional serological techniques, were responsible for complement binding to the RBC of these patients.

To investigate the latter possibilities, a more sensitive method for detection and quantification of γG globulin on the RBC was developed in our laboratory. This new method, a complement-fixing antibody consumption (CFAC) test, can detect as few as 20 molecules of γG per RBC. This test was applied to the study of 16 patients with complement (C′)–coated RBC in whom traditional serological studies had failed to indicate the mechanism of this in vivo C′ coating.

METHODS

Patients

The clinical diagnoses of the 16 patients studied were: systemic lupus erythematosus (10 patients), idiopathic acquired hemolytic disease (4 patients), lymphosarcoma (1 patient), and rheumatic heart disease (1 patient). Seven patients had mild anemia, but only one patient (H. M.) had an overt hemolytic process at the time of study. Seven patients were receiving corticosteroids. None had recently received blood transfusions. Cold agglutinin titers in all of

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the patients were 1:20 or less. Likewise, serum antibodies capable of sensitizing unmodified normal RBC with complement components at 37°C could not be detected by indirect antiglobulin tests with anti-complement serum.

Antisera

For all of the complement-fixing antibody consumption (CFAC) tests, a rabbit anti-human γG globulin serum prepared in our laboratory was used. The specificity of the antiserum was established by immunoelectrophoresis, Ouchterlony analysis, and by complement fixation tests. This antiserum diluted 1:3000 gave 95% complement fixation with γG globulin, 65% with F_c fragments, and less than 10% with F_{ab} fragments. This antiserum was diluted 1:4000 for use in CFAC tests.

The following antisera were used in direct antiglobulin tests: (*a*) polyvalent rabbit antiserum to whole human serum, (*b*) a rabbit anti-human γG globulin serum, and (*c*) a rabbit antiserum to human complement which had been absorbed with γG globulin. All RBC samples showing agglutination with the anti-complement serum were further tested with a rabbit antiserum specifically reactive with C'4 (β_{1E}) and C'3 (β_{1C}-β_{1A}) components of human complement. In all instances, the results with the two anti-complement sera were comparable. In addition, the RBC of 7 of the 16 patients had been tested with rabbit antisera to human γA and γM globulins, prepared as described elsewhere (10), and were found to be negative.[1] All of these antisera had been absorbed thoroughly with human RBC.

Direct antiglobulin tests

RBC from fresh ethylenediaminetetraacetate (EDTA)-blood (see below) were washed three times at room temperature in 20 volumes of isotonic saline. 0.1 ml volumes of 2% washed RBC suspensions and of serial 2-fold dilutions of a given antiglobulin serum were mixed together in tubes. The tubes were immediately centrifuged at 200 *g* for 1 min and agglutination read macroscopically on a scale of 1+ to 4+.

Complement fixation tests

Complement fixation tests, as used in the CFAC test (see below), applied the method of Wasserman and Levine (11), with some modifications, to a human γG: rabbit anti-human γG system. All dilutions were made in isotonic Veronal buffer containing 0.0005 M $MgCl_2$, 0.00015 M $CaCl_2$, and 0.1% bovine serum albumin. Guinea pig complement was used in amounts of 1.2–1.8 $C'H_{50}$ units for each reaction mixture. Antigen (γG globulin), antibody (anti-γG), and complement were incubated for 18 hr overnight at 4°C. Sheep cells sensitized in a standard fashion with amboceptor (12) were then added, and the reaction mixture incubated at 37°C. When the complement control (complement plus sensitized sheep cells) was approximately 80–90% hemolyzed, the tubes were transferred to an ice bath. The time required for this degree of hemolysis varied with each test ranging from 25 to 35 min. The degree of hemolysis was read by spectrophotometer at 413 mμ. All tests were performed in duplicate using 16 × 100 mm tubes. The total reaction mixture per tube was 3.5 ml with antigen dilutions composing 0.5 ml, antibody 0.5 ml, guinea pig complement 0.5 ml, sensitized sheep RBC 0.5 ml, and diluent 1.5 ml. Antigen, antibody, and complement controls were run with each test (12).

[1] In several hundred direct antiglobulin tests performed in this laboratory on various patients' RBC over the past 7 yr, 84 positive tests have been encountered but none with anti-γA or anti-γM.

Complement-fixing antibody consumption (CFAC) test

This method employs the principle of the antiglobulin consumption test (13) but measures the quantity of unconsumed anti-γG antibody by quantitative complement fixation rather than by hemagglutination titers.

Collection and washing of RBC. Blood from patients or normal subjects was collected in $\frac{1}{10}$ volume of 1.5% Na_3 EDTA and immediately centrifuged at 900 *g* for 10 min. Plasma, buffy coat, and the upper 0.25 ml of RBC were removed. For the CFAC test, the RBC were washed with isotonic saline, once at room temperature and five times at 4°–5°C. Each washing was carried out with a volume of saline 80 times the packed RBC volume. After the final wash, the RBC were packed by centrifuging at 900 *g* for 20 min at 4°–5°C.

Absorption step. 4 ml of a 1:4000 dilution of rabbit anti-human γG globulin serum were added to approximately 1 ml of washed packed human RBC (tube A). The same quantity and dilution of antiserum was also added to a control tube containing no RBC (tube B). The tubes were incubated for 30 min at 4°C with frequent mixing. Tube A was centrifuged at 900 *g* for 20 min and the supernatant was removed.

Complement fixation step. The amount of anti-γG antibody remaining in the supernatants from tubes A and B was determined as follows. To 0.5 ml of each supernatant, 0.5 ml of guinea pig complement (1.2–1.8 $C'H_{50}$ units) and 0.5 ml of human γG globulin (0.02 μg N/ml) was added. The complement fixation test as described above was then carried out. The concentration of human γG globulin used in the complement fixation test was that which had been shown to be slightly in antigen excess in the standard complement fixation analysis with 1:4000 rabbit anti-γG serum. This was 0.02 μg N/ml of γG globulin, and it gave 60–90% complement fixation in repeated tests.

Controls included those for any slight nonspecific hemolysis contributed by the human RBC, as well as that from the sensitized sheep RBC; the combined values from these two sources were used as the zero blank for reading the experimental tubes. The extent of these combined hemolysis values was generally less than 10% of the total hemolysis in the system.

Quantification. The results of the antibody consumption test were expressed as a reduction of per cent complement fixation. This was calculated by subtracting the per cent complement fixation observed with rabbit anti-γG serum absorbed with human RBC (tube A) from that observed with the unabsorbed rabbit anti-γG serum (tube B). For example, if the complement fixation observed with the RBC-absorbed anti-γG serum was 40% and that observed with the unabsorbed anti-γG serum was 90%, the reduction of per cent complement fixation would be 50. When the reduction of per cent complement fixation was complete, the test was repeated with fewer RBC to provide a point on the standard curve (Fig. 1).

The standard curve relating reduction of complement fixation to quantity of γG globulin bound to RBC was prepared with group A_1 RBC coated with known amounts

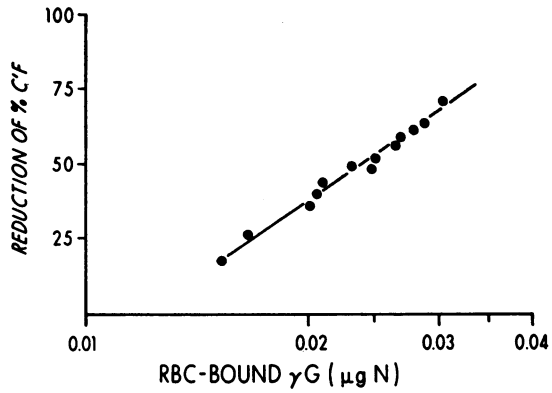

FIGURE 1 Standard curve relating RBC-bound γG (μg N) to reduction of per cent complement fixation (% C'F). Points on the curve represent the results of multiple complement fixing antibody consumption (CFAC) tests performed on RBC sensitized with 50, 100, 200, 400, 800, and 1600 molecules of γG anti-A per red cell.

of γG anti-A antibody. To obtain γG anti-A, a normal subject of blood group A was hyperimmunized with A substance (14), resulting in a 1:512 titer of anti-A as measured by indirect antiglobulin test. γG globulin was separated from this serum on a diethylaminoethyl (DEAE)-cellulose column using 0.01 M phosphate buffer, pH 8.0. The eluted γG globulin fraction was concentrated by negative pressure dialysis through a colloidon membrane from a volume of 180 ml to 18 ml, and then shown to have no other immunoglobulins in it by Ouchterlony analysis. Precipitin analysis (15) of this preparation with soluble A substance [2] revealed the concentration of anti-A to be 28.6 μg N/ml.

Group A RBC were incubated at 37°C for 1 hr and at 4°C overnight with quantities of the γG anti-A preparation adjusted to contain 50, 100, 200, 400, 800, and 1600 molecules of γG anti-A antibody per RBC respectively. The supernatants recovered from each of these mixtures were concentrated 30- to 50-fold by negative pressure dialysis through collodion membranes. Residual anti-A could not be demonstrated by saline agglutination or indirect antiglobulin tests in any of the supernatants. From this, the assumption can be made that more than 90% of the γG anti-A in each mixture had been bound to the RBC, giving a corresponding limit to the possible error in constructing the standard curve. The RBC were sufficiently sensitized to give the macroscopic antiglobulin reactions shown in Table I.

Complement-fixing antibody consumption (CFAC) tests were performed with the RBC from each of the variously sensitized preparations described above. A straight line relationship was found between reduction of per cent complement fixation (C'F) and the log of the quantity of human γG globulin calculated to be present on the absorbing RBC (Fig. 1). This curve was not affected by the intensity of the γG globulin sensitization per RBC through the ranges tested (50–1600 molecules per RBC), i.e., the absorption of anti-γG globulin as shown by reduction of C'F was responsive to the total cell-bound γG globulin.

For measuring the γG globulins on an unknown RBC sample, the reduction of per cent C'F was obtained using a measured number of RBC. By reference to the standard curve, the reduction in per cent C'F was obtained using a measured number of the RBC. By reference to the standard curve, the reduction in per cent C'F can then be expressed as μg γG globulin per RBC or as molecules of γG per RBC. The calculation of the number of γG molecules in all of such experiments has been based on a molecular weight of 160,000 for human γG globulin. RBC counts were done microscopically on a standard hemocytometer. In experiments for preparation of the standard curve, the average of eight separate counts was used. All other RBC counts were based on the average of two separate determinations.

Preparation of RBC eluates

The method of Kochwa and Rosenfield (16) was followed with some minor modifications. Eluates were prepared from 100–250 ml of the patient's blood collected in 1.5% EDTA (nine parts blood to one part EDTA). These volumes of whole blood represented packed RBC volumes from 40 ml to 120 ml. The RBC were thoroughly washed with 20 volumes of isotonic saline, once at room temperature and five times at 4°–5°C, and then were made up to a 10% suspension in saline. For preparing RBC stromata, the RBC were lysed by adding 0.5 ml of a 0.5% solution of digitonin for each 10 ml of the 10% RBC suspension. RBC stromata were washed five times with large volumes of cold isotonic saline (4°–5°C). 0.1 M glycine buffer pH 3 was added to the RBC

[2] Pfizer Diagnostics, New York.

TABLE I

Antiglobulin Tests on Group A RBC Sensitized with γG Anti-A*

Estimated number of molecules of γG anti-A per RBC	Dilution of anti-γG antiglobulin serum						
	1:10	1:20	1:40	1:80	1:160	1:320	1:640
Zero	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0
100	0	0	0	0	0	0	0
200	0	0	0	0	0	0	0
400	±	0	0	0	0	0	0
800	0	tr.‡	+	tr.	0	0	0
1600	+	+	2+	+	tr.	0	0

* Macroscopic tube test (see Methods).

‡ tr. = trace.

stromata and mixed thoroughly. The stromata were immediately centrifuged in a refrigerated Sorvall RC2-B centrifuge at 10,000 *g* for 20 min. The supernatants were removed and dialyzed at once against phosphate-buffered saline pH 7.4. Three to five cycles of elution were done on the RBC stromata. The combined eluate volumes from the five cycles, ranging from 50 to 140 ml, were concentrated to approximately 1 ml using negative pressure dialysis through colloidon membranes.

Testing of eluates

Anti-RBC antibody activity. RBC from normal human donors or from animals were washed three times with isotonic saline and adjusted to a 2.5% suspension. One volume of 2.5% RBC was added to an equal volume of serial dilutions of eluate, and incubated for 1 hr at 37°C with frequent mixing. The RBC were washed three times with saline (4°–5°C) and resuspended to 2% in room temperature saline. Antiglobulin tests with anti-γG serum and, in some cases, with anti-γM serum were then performed on these RBC, using the grading system for agglutination as described above.

Complement fixation to normal RBC. Eluates were diluted either 1:2 or 1:3 with pH 7.3 triethanolamine-buffered saline containing Ca^{++} 1.5×10^{-4} mole/liter and Mg^{++} 5×10^{-4} mole/liter (TBS). A 5% RBC suspension made in a 1:5 dilution of fresh autologous serum in TBS was prepared. Equal volumes of each were mixed together and were incubated for 1 hr at 37°C. The RBC were washed once with 37°C saline and twice with room temperature saline, suspended to 2%, and tested with anti-γG and anti-complement sera. As a control, eluates were tested using the same procedure except that the 5% RBC suspension was made in a 1:5 dilution of heat-inactivated serum (56°C, 30 min).

Sucrose density gradient ultracentrifugation

The sedimentation behavior of the antibodies in the eluates was determined by sucrose density gradient centrifugation using catalase (11S) and aldolase (8S) markers (17). The gradient was continuous, ranging from 10 to 40% sucrose in 0.15 saline and had a volume of 4.1 ml. The samples were centrifuged at 35,000 rpm for 18 hr at 4°C in a Spinco model L ultracentrifuge using an SW 50 rotor. The gradient was then divided by puncture from below into 32 fractions consisting of 10 drops each. Indirect antiglobulin tests with anti-γG serum were performed on the respective fractions.

RESULTS

Comparison of the CFAC test and the antiglobulin test in detection of anti-Rh isoantibodies. 4% cell suspensions of Rh_o(D)-positive human RBC were sensitized with equal volumes of increasing 2-fold dilutions of a potent anti-Rh_o (D) serum. Dilutions of the anti-Rh serum as high as 1:8000 were able to sensitize RBC to give a positive macroscopic antiglobulin test. By the complement-fixing antibody consumption (CFAC) test, RBC sensitized by the same anti-Rh serum at 1:128,000 still showed clear-cut specific reduction of complement fixation. CFAC tests on RBC sensitized with greater dilutions of anti-Rh serum did not show significant reduction of complement fixation.

The quantity of detectable γG globulin on normal human RBC. When 1.05×10^{10} RBC or greater (equivalent to approximately 1 ml or more packed RBC) did not produce reduction of complement fixation of 25 in the CFAC test, it could be calculated that the total cell-bound γG globulin was less than 0.0155 μG N and that the number of γG molecules per RBC was less than 35. The RBC of 23 normal subject, 12 males and 11 females with a median age of 28 yr were shown to have less than 35 identifiable molecules of γG per RBC by this criterion. In 16 of these subjects, the test was performed with greater numbers (up to 1.7×10^{10}) of RBC so as to give a sufficient reduction of per cent C'F (> 25) to allow an estimation of quantity of γG globulin present when it was less than 35 molecules per RBC. The number of γG molecules per RBC ranged from 24 to 34 in these normal subjects.

To investigate the possibility that our estimates of RBC-bound γG globulin were due to loss of rabbit anti-human γG activity by dilution with solute trapped in the packed RBC, 10 μl of ^{125}I-labeled human serum albumin were added to the anti-human γG serum. The loss of radioactivity in this antiserum after absorption with RBC was measured. It was determined that the amount of solute trapped in 1 ml of packed RBC was 0.1 ml or less, and that the actual effective dilution of our standard (1:4000) rabbit anti-γG globulin was, at most, 1:4100. This highest possible dilution of the standard anti-γG was not sufficient to induce a detectable change in per cent of C'F when the standard amount of γG globulin (0.02 μg N) was added.

Nonspecific adsorption of rabbit anti-human γG to human RBC appeared to be a most unlikely explanation of our data. CFAC tests were performed with another antigen-antibody system standardized in a fashion similar to the anti-γG:γG globulin system described above. Rabbit anti-egg albumin "absorbed" with 1 ml of packed human RBC gave no reduction of C'F with a standard quantity of egg albumin as antigen. In addition, 1.5 ml (5.3×10^{10}) of packed goat RBC did not produce any significant reduction of the ability of our standard anti-human γG serum to fix complement with the standard amount of human γG as antigen.

Since it was not possible to prepare packed RBC entirely free of white blood cells (WBC) and platelets, the possibility was considered that the WBC or platelets could have a sufficiently heavy coating of γG globulins to account for reduction of per cent C'F in our tests. To investigate this, CFAC tests were initially attempted on isolated WBC preparations. The anticomplementarity which resulted made results uninterpretable. The following method was found to circumvent this problem.

TABLE II

Number of γG Molecules per RBC in Patients with Complement-Coated RBC

Patient	Diagnosis	Direct antiglobulin test		Number of γG molecules/RBC‡
		Anti-γG	Anti-C'*	
H. M.	IAHD§	0	4+	470
H. C.	Lymphosarcoma	0	2+	228
R. G.	IAHD	0	+	168
W. B.	SLE§	0	+	126
R. K.	SLE	0	+	98
I. R.	SLE	0	+	92
F. A.	SLE	0	+	87
D. H.	IAHD	0	+	>86
H. A.	SLE	0	+	68
B. F.	SLE	0	+	67
A. C.	SLE	0	+	66
G. K.	SLE	0	+	65
C. D.	SLE	0	+	61
R. B.	SLE	0	+	<35
P. F.	RHD§	0	+	<35
J. M.	IAHD	0	+	<35
Normals (23)	—	0	0	<35

* Degree of agglutination with anti-C' antiglobulin serum diluted 1:10. Other dilutions of antiserum were tested but reactions were equal to or weaker than those with 1:10. Anti-γG globulin serum was also employed in a range of dilutions, with entirely negative results. Similarly, anti-γM and anti-γA sera gave negative reactions (not shown) in all seven cases tested. See footnote 1.

‡ Determined by CFAC test (see text).

§ Abbreviations: IAHD = idiopathic acquired hemolytic disease; SLE = systemic lupus erythematosus; RHD = rheumatic heart disease.

The buffy coat of blood from a normal subject was washed six times with large volumes of isotonic saline. Known numbers of the WBC, so obtained, together probably with whatever platelets were present, were mixed with 1 ml aliquots of washed packed goat RBC. The addition of washed goat RBC to the washed human WBC prevented anticomplementarity. No reduction of per cent C'F was observed even with cell mixtures containing 4×10^7 WBC. This number of WBC was more than 10 times that usually found in 1 ml of washed packed normal human RBC used in CFAC tests. These results indicate that the WBC and also, in all likelihood, the platelets found in the final packed RBC preparations from the normal subjects played no role in the reduction of per cent C'F.

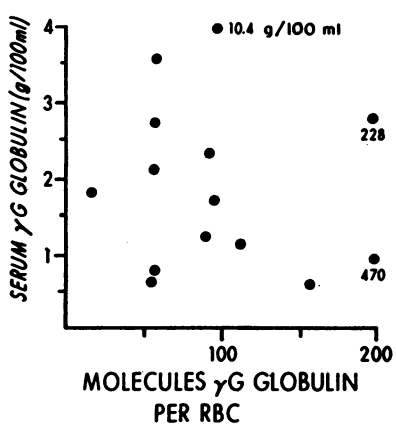

FIGURE 2 Serum γG globulin levels measured by Hyland immunoplates. Determinations were not performed in two patients (P. F. and J. M.) whose RBC had less than 35 molecules of γG per RBC.

The quantities of γG globulins on complement-coated RBC from patients giving negative direct antiglobulin tests with rabbit anti-human γG serum.

CFAC tests were performed on the complement-coated RBC of 16 patients. 13 of these 16 patients had RBC exhibiting 61–470 molecules of γG per RBC (Table II). The numbers of γG molecules per RBC were significantly greater than those found on the RBC from 23 normal subjects all of whom showed less than 35 molecules of γG per RBC (Table II). The RBC of three patients with C'-coated RBC (R. B., P. F., and J. M.) had numbers

TABLE III

*Indirect Antiglobulin Reactions of Concentrated Eluates with Normal Human RBC**

Source of eluate	Dilution of eluate‡							
	Neat	1:2	1:4	1:8	1:16	1:32	1:64	1:128
H. M.		3+	3+	3+	2+	2+	+	0
R. G.			3+	2+	2+	2+	+	+
I. R.		4+	3+	2+	2+	+	0	0
H. C.	+	tr.	0	0	0	0	0	0
W. B.	2+	+	tr.	0	0	0	0	0
R. K.	+§							
C. D.	+§							
Normals (8)	0§							

tr. = trace agglutination.

* Anti-γG antiglobulin serum used in a 1:40 dilution.

‡ Dilution of eluate used to sensitize normal human RBC.

§ Tested only neat.

of γG molecules per RBC in the same range as the normals.

Since 6 of the 12 patients with increased quantities of γG globulin on their RBC had elevated serum γG globulin levels, the question was raised whether the increased numbers of γG globulin molecules on the RBC merely reflected nonspecific absorption of γG globulin from the plasma. However, CFAC tests performed on the RBC of two γG myeloma patients with serum γG globulin levels of 3.5 and 6 g/100 ml respectively showed less than 35 molecules of γG per RBC in each case. Furthermore, Fig. 2 shows the lack of relationship between serum γG globulin levels and the numbers of γG molecules per RBC in the 16 patients with complement-coated RBC.

Elution of γG antibodies from complement-coated RBC. Highly concentrated eluates were prepared from the RBC of seven of the patients listed in Table II whose RBC had shown (*a*) positive direct antiglobulin reactions with anti-complement serum, (*b*) negative direct antiglobulin reactions with anti-γG serum, and (*c*) increased numbers of RBC-bound γG globulin by the CFAC test. All seven of these eluates, when incubated with normal human RBC at 37°C for 1 hr, were able to sensitize these RBC with γG globulin so that they gave a positive antiglobulin reaction with anti-γG serum (Table III). Five eluates so tested gave negative indirect antiglobulin reactions with an anti-γM serum. Eluates prepared from comparable or greater numbers of RBC from eight normal subjects, when similarly tested, gave negative results.

The specificities of five of these RBC eluates were tested (Table IV). All five failed to sensitize detectably either sheep or rabbit RBC. Two of three eluates reacted positively with rhesus monkey RBC (Table IV). Three eluates were able to sensitize human Rh_{null} RBC to the same degree as human R^2R^2 RBC, indicating a "non-Rh" specificity of the antibodies in these eluates. Inadequate amounts of eluate in the other four patients precluded testing with Rh_{null} RBC.

The RBC eluates from six patients were tested for their abilities to fix human complement to normal RBC, as determined by the agglutinability of such cells by specific anti-C′ serum. As shown in Table V, when five of the six eluates were incubated with human RBC in the absence of a C′ source (heated serum), the test RBC nonetheless acquired some reactivity with the anti-C′ serum. This phenomenon has been encountered in other studies (18, 19) and can be attributed to antibody-complement complexes in eluates derived entirely from the patients' RBC.[3] On the other hand, when fresh normal human serum was provided as a source of new C′ during the in vitro incubation, the subsequent reactions of

TABLE IV

Reactions of Eluates with Animal RBC and Human Rh_{null} RBC

Source of eluate	Indirect antiglobulin reaction with anti-γG serum*				
	Sheep RBC	Rabbit RBC	Rhesus monkey RBC	Human Rh_{null} RBC	"Normal"‡ human RBC
H. M.	0	0	2+	3+	3+
R. G.	0	0	0	3+	3+
I. R.	0	0	+	3+	3+
W. B.	0	0	n.t.	n.t.	2+
C. D.	0	0	n.t.	n.t.	+

n.t. = not tested.

* Anti-γG antiglobulin serum used in a 1:40 dilution.

‡ RBC from normal group O donor with common Rh phenotype (R^2R^2).

[3] Leddy, J. P., and J. H. Vaughan. In preparation.

TABLE V
Capacity of Eluates to Fix Complement to Normal Human RBC

Source of eluate	Indirect antiglobulin reaction with anti-complement serum*	
	Eluate incubated with RBC in the presence of	
	Fresh‡ serum	Heated§ serum
H. M.	4+	2+
R. G.	3+	+
I. R.	3+	+
R. K.	2+	+
W. B.	+	0
H. C.	+	+
Normals (2)	0	0

* Anti-complement antiglobulin serum used 1:10. Other dilutions were used in the tests but are not shown here because they were the same or weaker.
‡ Fresh normal human serum from RBC donor used as a source of complement.
§ Fresh normal human serum from RBC donor was heat inactivated at 56°C for 30 min.

the test RBC with anti-C′ serum were distinctly and reproducibly stronger in every case but one (Table V). This augmentation is taken to mean active fixation of C′ components to the RBC surface, over and above that which was carried over passively in complex with autoantibody. In similar experiments, RBC eluates from two normal subjects did not fix complement to normal RBC after incubation in either fresh or heat-inactivated serum.

Sucrose gradient ultracentrifugation was performed on three eluates (H. M., I. R., and R. G.). The results of the run on eluate I. R. are shown in Fig. 3: eluates H. M. and R. G. gave comparable results. The peak of γG anti-RBC antibody activity was found to sediment slightly slower than the peak of aldolase enzyme activity

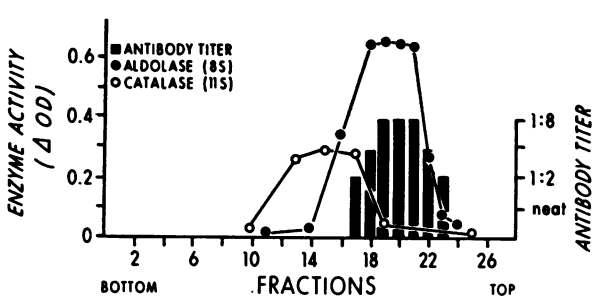

FIGURE 3 Sucrose gradient ultracentrifugation on eluate I. R. (0.2 ml volume). Antibody titer represents dilution of fraction used to sensitize normal RBC as revealed by indirect antiglobulin tests with anti-γG 1:40. Enzyme activities are expressed as Δ OD per 10 μl of fraction.

serving as the 8S marker. An eluate from normal RBC sensitized in vitro by serum containing a high titer of γG anti-Rh isoantibodies was run along with the above eluates. The antibody activity in this control eluate sedimented in the same region.

DISCUSSION

The complement-coated RBC of 13 of 16 patients were shown by a new complement-fixing antibody consumption (CFAC) technique to have greater amounts of cell-bound γG globulin per RBC than do RBC from normal subjects. The quantities of γG globulin measured on these RBC were below the threshold for detection by macroscopic direct antiglobulin tests. Although the demonstration of increased amounts of γG globulin on the RBC of these patients strongly suggested that the RBC had been sensitized in vivo with either autoantibodies or antigen-antibody complexes, the possibility existed that we were merely demonstrating γG globulin nonspecifically absorbed to the RBC surface. Other investigators have shown that normal RBC, when incubated in vitro with either autologous or homologous ^{131}I-labeled γG globulin preparations, do absorb γG globulin which remains on the RBC through numerous washings with saline (20–23). RBC coated in this manner with as many as 10^5 molecules of ^{131}I γG per RBC failed, however, to give a positive antiglobulin test, implying that the arrangement, location, or binding of nonspecifically bound γG on the red cell differs from that of γG antibody. The major evidence that the abnormal levels of γG per RBC in our cases represent cell-bound antibodies is (*a*) the ability of the concentrated eluates from these RBC to sensitize normal human RBC to give positive antiglobulin tests with anti-γG (Table III), (*b*) the specificity of these reactions for human RBC (Table IV), and (*c*) their ability to fix complement (C′) to unmodified human RBC at 37°C (Table V). One of these serologically active eluates was prepared from a sample of red cells which had shown only 61 identifiable molecules of γG per RBC by the CFAC test. Eluates similarly prepared from normal subjects' RBC and concentrated at least 100-fold before exposing to homologous normal RBC did not produce positive antiglobulin tests with either anti-γG serum or anti-C′ serum despite the presence in such eluates of appreciable (presumably nonspecific) γG globulin measured by an independent method (24).

Efforts by others to demonstrate C′-fixing red cell antibodies in eluates from C′-coated red cells have heretofore been unsuccessful (4, 5). The most likely explanation for these previous failures is that elution was made from an insufficient number of red cells and that the eluate was insufficiently concentrated. In our patients, RBC eluates were prepared from as many as

120 ml of packed red cells and were concentrated at least 100-fold to a final volume of 1 ml.

Sucrose gradient ultracentrifugation studies have shown that the γG antibodies to human RBC in these eluates sediment in the 7S region. This would indicate that the γG eluted from the RBC was not in the form of complexes of antibody and macromolecular antigen. The possibility cannot be totally excluded that the γG antibody might be bound to a small molecular weight antigen. Shulman has demonstrated that certain complexes of drug with γM antibody were capable of fixing complement to human RBC (9). However, none of our patients was currently receiving drugs such as stibophen, quinidine, or quinine which are the only drugs that have been shown to form haptene-antibody complexes reactive in this manner.

The failure of the RBC eluates to sensitize sheep and rabbit RBC is in accordance with the known observation that "warm-type" RBC autoantibodies commonly found in acquired hemolytic disease do not react with nonprimate species (25). Furthermore, the capacity of the RBC eluates to fix human complement to normal RBC is a known characteristic of many RBC autoantibodies. The finding that the autoantibodies in the eluates reacted with Rh_{null} RBC is also consistent with the observation that complement-fixing RBC autoantibodies usually do not show specificity for Rh antigens (26, 2).

The present observations are in keeping with earlier studies on human RBC apparently coated with complement alone. Gilliland and Turner (27) had found that when the C′-coated RBC of certain rheumatoid arthritis patients were incubated at 37°C with normal RBC in the presence of fresh normal serum, the normal RBC acquired C′ on their surfaces. However, when incubation of the RBC was performed in complement-inactivated serum or in saline, the normal RBC did not acquire complement components. These results suggested that during in vitro incubation of the patient's complement-coated RBC with normal RBC in the presence of fresh normal serum, small quantities of antibody transferred from the patient's RBC to the normal RBC, resulting in fixation of C′ on the normal RBC surface.

Jenkins and Eyster (19) reported that eluates prepared by acid elution from RBC stromata of C′-coated RBC fixed complement to normal RBC. Although they were unable to demonstrate immunoglobulins on such cells or in these eluates, they showed that the property of these eluates to fix complement to normal RBC resided in the γG area obtained from Sephadex G-200 chromatography.[4]

The in vivo fixation of sufficient amounts of complement to give positive antiglobulin reactions by quantities of γG antibody insufficient to induce anti-γG antiglobulin reactions is not surprising. As shown by Müller-Eberhard, Polley, and Calcott (28), several hundred molecules of C′3 are bound to the RBC membrane through the action of one C′4 2a site, which in turn may be created by only two closely placed γG antibody molecules (29), or possibly even one γG antibody molecule (30, 31), bound to the RBC surface. The present observation that readily detectable in vivo complement fixation to RBC can apparently be mediated by minute quantities of γG antibody implies a remarkable efficiency in C′ fixation compared to other γG RBC autoantibodies. By present knowledge, this capability would require not only a suitable γG subclass (32, 33) but also the existence of favorably distributed antigenic sites on the RBC surface (29, 30). Further studies on these aspects are planned. Alternatively it must be noted that we may measure by the CFAC method only the more avid antibodies on the patient's RBC. Less avid antibodies capable of fixing complement to the RBC surfaces may dissociate either in vivo or in vitro (during the washing of the RBC). While low avidity is the explanation for the lack of immunoglobulin on complement-coated RBC of cold agglutinin disease, in which the autoantibody can readily be demonstrated in the serum (34, 35), this seems less likely for hemolytic disease of the "warm-type," in which free serum antibody is usually absent or difficult to demonstrate.

In conclusion, from these observations an entirely reasonable explanation can be offered for the previously unexplained occurrence of C′-coated RBC in *many* patients with connective tissue disorders, lymphoproliferative diseases, and idiopathic acquired hemolytic disease: a low concentration of "warm-reacting" γG autoantibody with highly efficient C′-fixing activity. Our failure to detect cell-bound γG antibody in 3 of the 16 cases studied could be due to either the dissociation of low affinity antibody (see above), the presence of antibody belonging to another immunoglobulin class (36, 37), or the operation of other mechanisms in some patients.

[4] Jenkins, D. E., Jr. Personal communication.

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