Fixation of the First Component of Complement (C'1a) by Human Antibodies

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ABSTRACT The fixation of the first component of complement (C'1a) by human antibodies and human cells has been studied by the use of the C'1a fixation and transfer test (C'1a FT test) of Borsos and Rapp.

Cold agglutinin antibodies appear to require no more than one antibody molecule to fix one molecule of C'1a.

Most warm agglutinin antibodies are IgG in immunoglobulin type and require at least two molecules of antibody to fix a molecule of C'1a. Donath-Landsteiner antibody has the same requirements for C'1a fixation. A single example of a warm agglutinin antibody which appears to require one molecule of antibody for the fixation of C'1a was found.

Antibodies of the Rh system do not fix significant amounts of C'1a in the absence of anti-antibody when antiserum of a single Rh specificity was used. However, when three antisera at different specificity are present, C'1a may be fixed. Under these conditions cells from a patient with paroxysmal nocturnal hemoglobinuria may be lysed when fresh serum is added to provide the other components of complement.

The presence of IgG antibodies could be detected by the use of anti-IgG^{Hu} antiserum and a one-to-one relationship between the concentration of antiserum in the reaction and the amount of C'1a fixed could be established.

The effect of temperature, ionic strength, papainization of the red cells, and repeated washing of the red cell–antibody aggregates on the amount of C'1a fixed was investigated. Conditions of maximal C'1a fixation were established for each class of antibodies.

Globulins present in normal isologous or autologous serum are absorbed in small amounts to normal red cells in a manner analogous to warm agglutinin antibody. Their presence is detectable by the C'1a fixation and transfer test only with antiglobulin antiserum.

Within certain limits, the C'1a fixation and transfer test provides a quantitative measure of the reaction of human red cells and antibodies to antigens on their surface.

INTRODUCTION

The fixation by antibody of complement components to red cells of patients with immune hemolytic anemia is, in certain circumstances, an important event leading to the destruction of those cells. In some instances, complement may damage the cell by making small "holes" in the cell membrane (1). When this occurs, intravascular hemolysis results with consequent hemoglobinemia and hemoglobinuria. In other instances, the presence of complement components on the surface of the circulating red cell appears to render the cell abnormal and to lead to the sequestration and destruction of the cell by the reticuloendothelial system (2). Hence, information about the fixation of complement by human antibodies is important in understanding the events leading to hemolysis in patients with immune hemolytic anemia.

The first step in the fixation of complement by antibody is the fixation of the first component, C'1 (3).[1] Once this component is attached by

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[1] The following symbols are commonly used in discussing the reactions of complement: E = a red blood

antibody, the remainder of the components will interact sequentially if all are available and cation and temperature requirements are met. Borsos and Rapp (4) and Ishizaka, Ishizaka, Borsos, and Rapp (5) have studied the fixation of C'1a by a variety of rabbit antibodies and by human isoimmune anti-A antibodies. They have shown that with these antibodies, a single IgM antibody molecule is sufficient for the fixation of a molecule of C'1a whereas two IgG antibody molecules in juxtaposition are required.

In the present investigation we have studied the fixation of C'1a by "autoimmune" and isoimmune human antibodies to human red cells, using the C'1a fixation and transfer test of Borsos and Rapp (6). By this test, the number of molecules of C'1a fixed by antibody on each red cell may be calculated and the relationship of this to the amount of antibody present may be determined. This technique not only provides a method for determining the way in which C'1a is fixed but also provides a quantitative measure of the amount of antibody attached to the red cell.

METHODS

A. Buffers and complement components

Buffer. Veronal-buffered saline (VBS) was made according to the formula given in reference 3. When buffers of reduced ionic strength were required, this buffer was mixed with appropriate amounts of a buffered isotonic sucrose solution made according to the formula given in reference 7. VBS-sucrose buffer refers to a solution of 40% VBS–60% isotonic sucrose ($\mu = 0.065$) unless otherwise stated.

EDTA solution. A 0.1 M solution of EDTA (ethylenediaminetetraacetate, disodium salt) pH 7.4 was made according to the formula given in reference 8. Lower concentrations of EDTA were obtained by diluting this isotonic solution with appropriate amounts of VBS.

Alsever's solution was made according to the formula given in reference 3.

Whole blood was obtained weekly from healthy sheep and was stored at 4°C in an equal volume of Alsever's solution.

Complement. Fresh frozen guinea pig serum, obtained from Suburban Serum Laboratories, Silver Springs, Md., was absorbed twice with a 1/10 volume of washed sheep cells, and was stored at −90°C.

C'1a. A solution containing a high concentration of C'1a and lacking C'2 and C'4 activity was prepared from guinea pig serum by the method of Borsos and Rapp (9). This solution contained at least 10^{12} molecules of C'1a/ml and was absorbed twice at 37°C and twice at 0°C with packed human red cells before use.

C'2. A solution containing a high concentration of C'2 and lacking C'1a, C'4, and "C'3" activity was prepared from guinea pig serum by the method of Borsos, Rapp, and Cook (10). This solution contained at least 5×10^{11} molecules of C'2/ml as determined by the method of Borsos and Rapp (11).

EAC'4 was made according to the method given in reference 3. In later experiments, the revised method of Borsos and Rapp, using EAC'1a and C'-EDTA, was used for making EAC'4 (11).

B. Human material

Human red blood cells (E^{Hu}). Blood was removed aseptically by venipuncture, placed with an equal amount of Alsever's solution, and stored at 4°C. At the time of use, the cells were incubated with a 1/10 volume of isotonic 0.1 M EDTA for 5 min, washed once in 0.01 M EDTA in VBS and three times in either VBS or VBS-sucrose. One part of packed cells was added to 18 parts buffer and this suspension was adjusted so that a 1 in 25 dilution in 0.01 M NH_4OH had an optical density in a spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) of 0.210 at a wavelength 541 mμ in a 1.000 cm cuvette. Such a suspension contains about 2.2×10^8 red blood cells/ml.

Human red cells were treated with papain as follows. Equal volumes of a standard suspension of E^{Hu} in VBS and of a 1% papain solution in VBS were mixed and incubated for 30 min at 37°C. The cells were washed three times with VBS and suspended in the original volume of VBS or VBS-sucrose.

Antisera. Serum from patients with hemolytic disease due to cold-reacting antibodies was obtained from blood drawn with a warmed syringe and kept warm until the clot had separated. After centrifugation at 37°C, the serum was heated to 56°C for 30 min and was stored at −20°C until use.

Serum from patients with hemolytic disease due to warm-reacting antibodies was obtained from blood collected at room temperature and allowed to clot at room temperature. This serum was stored and used as outlined above. Eluates were made from the red cells of pa-

cell (usually from sheep unless otherwise indicated), A = antibody to that red cell (usually made in rabbits unless otherwise indicated), and C' = complement taken as a whole. The individual components are denominated by numbers 1–9 and the activated form of the component is indicated by the letter "a" (e.g. C'1a = the activated form of the first component of complement). Cellular intermediates in which some but not all of the components of complement are present at the cell surface are denominated by symbols such as EAC' 1a, 4. The sequence of reactions of complement components is thought to be as follows: $E + A \rightarrow EA + C'1 \rightarrow EAC'1a + C'4 \rightarrow EAC'1a, 4 + C'2 \rightarrow EAC'1a, 2 \rightarrow EAC'1a, 4, 2a + C'3 \rightarrow EAC'1a, 4, 2, 3 + C'5 \rightarrow EAC'1a, 4, 2, 3, 5 \ldots + C'9 \rightarrow EAC'1a, 4, 2, 3, 5, 6, 7, 8, 9 \rightarrow$ lysis.

TABLE I

Brief Clinical Summary of Patients with Immune Hemolytic Anemia

Patients	Age	Sex	Duration of disease	Lowest recorded hemoglobin	Reticulocyte count	Direct Coombs test: anti-IgG	Direct Coombs test: anti-C′	Treatment	Other disease
	yr		*yr*	*g/100 ml*	%				
Cold-reacting antibody Agglutinin									
M. G.	63	F	13*	6.3	10.0	−	+	Steroids,‡ 6-MP§	Carcinoma of colon
T. B.	60	M	8.0	7.2	14.0	−	+	?	
A. D.	58	F	0.5*	5.8	22.0	−	+		Carcinoma of colon
A. P.	60	F	0.4*	4.2	5.0	−	+	Steroids	Carcinoma of lung Polycythemia rubra vera
J. M.	26	M	0.3*	4.8	2.0	−	+	Steroids, 6-MP, vincristine	Acute lymphocytic leukemia
Donath-Landsteiner									
J. J.	24	M	0.3	6.0	18.5	±	±	Steroids	Hypergammaglobulinemia
Warm-reacting antibody									
R. G.	66	F	9.0	4.6	56.0	+	+	Steroids, splenectomy	Systemic lupus erythematosus
M. P.	36	F	1.8	6.1	22.0	+	+	" "	Systemic nocardiosis
A. G.	23	F	5.0	4.8	60.0	+	−	" "	Thalassemia minor
J. S.	72	M	1.0*	5.4	32.0	+			
A. P.	42	F	1.5	7.6	5.6	+	+	"	Systemic Lupus
B. B.	36	M	1.4	6.7	24.0	+		"	
A. B.	72	F	1.5*	6.8	12.0	+	−	"	Chronic lymphocytic leukemia
E. B.	63	F	1.0	3.8	52.0	+	+	"	Systemic lupus
L. W.	56	M	0.7	10.3	9.3	+	−	"	
C. F.	1.5	F	1.0	6.3	15.0	+	−	"	Multiple infections
J. B.	62	M	0.5	5.5	17.0	+	−	"	Chronic lymphocytic leukemia

* Patient subsequently deceased.
‡ Usually prednisone.
§ 6-mercaptopurine.

tients with warm-reacting antibodies by the method of Rubin (12). A brief summary of the clinical status of these patients is given on Table I.

Isoimmune antibodies of the Rh system were obtained from Ortho Laboratories, Raritan, N. J. or from patients sensitized during the course of pregnancy. These antibodies were stored at −20°C until use and did not appear to lose their ability to react as antibodies over a period of 18 months.

Human complement. Human serum for complement was obtained from blood collected by venipuncture from normal donors and placed immediately into cooled tubes which were centrifuged at once for 4 min at 10,000 rpm in a refrigerated centrifuge. The plasma was removed and allowed to clot. The fibrin clot was removed with applicator sticks and the serum was stored at −90°C until use.

C. Antisera to human immunoglobulins

Anti-human globulin. Rabbit antiserum against whole human serum (anti-WS^{Hu}) was obtained from Ortho Laboratories. This antiserum was found on immunoelectrophoresis to react with IgG, IgA, and IgM immunoglobulins, B_{1C} (C′3), and serum albumin. The anti-IgG^{Hu} antibodies were found to be IgG in type by chromatography on Sephadex G-200 column.

Goat anti-human immunoglobulin antisera. Antibodies against human IgG (IgG^{Hu}), IgM (IgM^{Hu}), and IgA (IgA^{Hu}) immunoglobulins were made by injecting individual goats with single purified immunoglobulin proteins according to the method outlined in footnote 2. These antisera were absorbed with both type I and type II Bence Jones protein as well as mixed normal human red cells of all major blood groups. These antisera were monospecific as determined by immunoelectrophoresis and hemagglutination inhibition.

Rabbit anti-human immunoglobulin antisera. Antibody to IgG^{Hu}, IgM^{Hu}, and IgA^{Hu} were made by injecting single purified human immunoglobulins mixed with Freund's complete adjuvant into the foot pad of rabbits twice weekly for 2 weeks. The 0.5 ml of the immunoglobulin-containing solution was then injected subcutaneously three times weekly for 6 more weeks. Blood was obtained at weekly intervals thereafter. The serum was removed after clotting, heated to 56°C for 30 min, and absorbed successively with mixed human red cells and Bence Jones proteins. The anti-IgG^{Hu} was absorbed with purified IgM^{Hu} and IgA^{Hu} protein.

The anti-IgM^{Hu} was absorbed with human fetal serum

[2] Buckley, E. E., III, and R. Hornsby. Personal communication.

and the anti-IgA^{Hu} with serum from a patient with macroglobulinemia whose serum was deficient in IgA.

D. Procedures

Separation of proteins on Sephadex columns was performed according to the method given in reference 13. Sephadex G-200 was boiled 5 min, allowed to settle several times while the finer granules were removed, and was put on a 100 cm × 1.0 cm column. The effluent was collected in 3.5–4.0 ml fractions and analyzed for protein content by determining the optical density at 278 mμ using a Beckman DU spectrophotometer.

The C'1a fixation and transfer test (C'1a FT test) of Borsos and Rapp was performed as outlined in reference 6 with minor modifications. The first part of the test involved the fixation of C'1a by E^{Hu} and antibody, the removal of the excess C'1a, and the release of C'1a from the cell-antibody aggregates. For cold-reacting antibodies, the following procedure was used. 0.1 ml of a standard suspension of E^{Hu} was mixed in a siliconized test tube at 0°C with 0.1–0.4 ml of antiserum and allowed to incubate for 60 min in an ice bath. If a low concentration of serum was used, 0.3 ml C'1a solution was added at the onset of the incubation. If a high concentration of serum was used, the cells and antiserum were allowed to incubate 1 hr, were washed twice with ice-cold VBS-sucrose, and were incubated for a further 60 min with 0.3 ml of C'1a solution. At the end of this incubation, the cells were washed once in ice-cold VBS-sucrose, carefully poured over into a second set of siliconized tubes, and washed three times more with 8 ml of cold VBS-sucrose. At the end of the washing, the cells were suspended in 9 ml of VBS, appropriate dilutions were made, and the amount of C'1a in solution was determined by the method of Borsos and Rapp (9).

When warm-reacting antibodies were used, the 0.1 ml of a standard suspension of E^{Hu} was mixed with 0.1–0.4 ml of antiserum diluted appropriately and the mixture was incubated for 30 min at 30°C. The cells were washed twice with 8 ml of VBS-sucrose and 0.1–0.6 ml of antiglobulin antiserum was added if necessary for the experiment; the mixture was then incubated for 30 min at 30°C. and the cells again washed with 8 ml VBS-sucrose. 0.3 ml of C'1a solution was added and the mixture incubated for 30 min at 30°C. The cell-antibody-C'1a aggregates were treated as in the test for cold-reacting antibodies.

The second part of the C'1a fixation and transfer test is the determination of the C'1a content in solution, using sensitized sheep red cells and guinea pig complement components. After appropriate dilution of the C'1a-containing solution (to achieve partial lysis of the sheep cells at the end of the experiment), 0.5 ml was mixed with 0.75×10^8 sheep EAC'4 contained in 0.5 ml of VBS. After allowing the reaction to proceed at 30°C for 15 min, 0.5 ml of C'2 solution containing at least 10^{10} molecules of C'2/ml in isotonic sucrose solution was added and allowed to react for 10 minutes. Following this, 5 ml of a 1/50 dilution of guinea pig serum in ice-cold 0.015 M EDTA was added and the cells placed in a 37°C water bath for 60 min. The remaining cells were then removed and optical density of the supernatant fluid at a wavelength of 412 mμ was determined in a spectrophotometer (Gilford Instrument Company, Oberlin, Ohio). Controls for these experiments consisted of: (*a*) a "cell blank" consisting of 0.5 ml of EAC'4 + 6.0 ml of VBS-sucrose, (*b*) a C' color blank consisting of 1.5 ml VBS-sucrose + 5.0 ml C'-EDTA, (*c*) a "complete blank" consisting of 0.5 ml EAC'4, 0.5 ml VBS-sucrose, 0.5 ml C'2, and 5.0 ml C'-EDTA, and (*d*) "a complete lysis control" consisting of 0.5 ml EAC'4 + 6.0 ml 0.01 M NH_4OH.

The net optical density of each sample (optical density less the optical density of the cell control and of the complement color control) was divided by the net complete lysis control (the optical density of the complete lysis control less the optical density of the cell control). This fraction was subtracted from 1.0, and the negative natural logarithm of the result was found (z). The value of z for the complete blank was subtracted and corrected value of z was multiplied by the dilution of the original sample. This yields the number of C'1a molecules fixed per E^{Hu}, assuming 80% efficiency in transfer and detection of C'1a molecules (6).

In all experiments, controls in which E^{Hu} were incubated with C'1a in the absence of antibody and (or) anti-globulin antibody were performed. The number of molecules of C'1a fixed per E^{Hu} was usually small (10–50) and this number was subtracted from all experimental values. All values represent the mean of two or more determinations of the C'1a content of the solution derived at the end of the first part of the C'1a FT test.

RESULTS

Characteristics of the antibodies

The cold-reacting antibodies. To characterize the molecular species of the antibodies contained in the serum of patients with cold agglutinin hemolytic anemia, 2-ml aliquots of serum were chromatographed on Sephadex G-200 and the effluent was analyzed for protein content and for antibody content as judged by ability to fix C'1a to red cells. In each case, the antibody activity was found in the first (excluded) peak of protein which contained macroglobulin proteins. The antibody in antiserum T. Bu. was further characterized by purification by ultracentrifugation as outlined in reference 1. The antibody activity was found to be in the macroglobulin fraction by this method as well.

Serum from patient J. J. containing a Donath-Landsteiner antibody was chromatographed on Sephadex G-200; the antibody activity was de-

tected using the C'1a FT test by reacting 0.2 ml of effluent with E^{Hu}, followed by 0.2 ml of anti-WS^{Hu} antibody. The antibody activity was found primarily in the second peak which contained IgG protein.

The warm-reacting antibodies. The antibodies in serum from patients with warm-reacting antibody hemolytic disease were characterized in two ways. (*a*) The immunoglobulin type of the antibodies was determined by affixing the antibody to E^{Hu}, reacting the E^{Hu}-antibody aggregates with specific anti-IgG^{Hu}, anti-IgM^{Hu} or anti-IgA^{Hu} antisera, and determining the amount of C'1a fixed by the E^{Hu}-antibody–antiantibody aggregates by the C'1a fixation and transfer test; the results are shown in Table II.

(*b*) Certain of the antisera were passed through Sephadex G-200 columns and the antibody activity of the effluents determined by sensitizing 0.1 ml of standard E^{Hu} with 0.2 ml of effluent, reacting the E^{Hu}-antibody aggregates with anti-WS^{Hu}, and determining the amount of C'1a fixed to the cells by this combination. With the exception of antisera from patients M. W. and M. P., the antibody activity was found in the second peak which contains the IgG immunoglobulins.

TABLE II

Fixation of C'1a by Human Antibodies in Combination with Heterologous Anti-IgG^{Hu}, Anti-IgM^{Hu}, and Anti-IgA^{Hu} Antibodies

Antibody	Dilution	Anti-antibody: Anti-IgG^{Hu}	Anti-IgM^{Hu}	Anti-IgA^{Hu}
		C'1a molecules fixed/E^{Hu}		
Cold-reacting Agglutinin				
T. Bu.	1/100	0	1238	16
Warm-reacting Autoimmune				
J. B.	1/1	155	0	0
E. B.		380	0	0
A. G.		230	6	2
J. S.		390	0	0
R. F.		370	0	12
M. P.		1355	20	100
Isoimmune				
anti-e		180	3	0
anti-c		170	3	30
anti-D		196	13	102
anti-C		95	13	21
Normal Serum				
J. P.	1/1	112	30	100

Immunological specificity of the antibodies

Cold-reacting antibodies. Cold agglutinins are usually classified by their reaction with so-called "I-" red cells, i.e., red cells from newborn infants and red cells from rare adults said to be lacking the "I" antigen (14). The ability of the cold agglutinins used in these studies to fix C'1a to normal group O red cells was compared to their fixation to group O cord cells. The results are reported in detail elsewhere [3] but are summarized in Table III. For each antibody, the variation in the amount of C'1a fixed by red cells from different donors was great in both groups but the mean value was lower in each case for the "I negative" cells, except for antiserum T. B.

Warm-reacting antibodies. Certain warm-reac-

[3] Rosse, W. F., and J. B. Sherwood. Cold agglutinin antibodies: differences in C'1a fixation by "I+" and "I-" red cells. Manuscript in preparation.

TABLE III

Fixation of C'1a by Cold Agglutinin Antibodies to Red Cells from Adult and Newborn Donors

Antiserum	Reciprocal of dilution	E^{Hu}-adult: No. of donors tested	C'1a molecules fixed/E^{Hu}: Mean	SD	E^{Hu}-newborn: No. of donors tested	C'1a molecules fixed/E^{Hu}: Mean	SD
V. L.	1600	14	6920	1930	9	3555	1115
M. G.	10	24	1470	1410	12	550	520
T. B.	100	16	1110	330	8	1340	1570
A. D.	100	6	3000	950	10	1620	930
A. P.	10	10	1320	485	14	190	80

ting antibodies occurring in "autoimmune" hemolytic anemia have been found to be specific for antigens of the Rh system (15), whereas others have been found to act with all cells possessing any antigens of this system but not with cells lacking all these antigens, the so-called Rh_{null} cells (16). The immune hemolytic antibodies used in the present experiments were tested with cells of known Rh phenotype. Only antibody of patient A. B. was found to have specificity for a defined Rh antigen, e (hr″).

The ability of autoimmune and isoimmune antibodies to fix C'1a to Rh_{null} cells in the presence of anti-IgG^{Hu} antibody was tested (Table IV). All autoimmune antibodies fixed nearly the same amount of C'1a to Rh_{null} cells as to normal cells when cell-antibody aggregates were reacted with anti-WS^{Hu} antiserum. Isoimmune antibodies of the Rh system did not fix C'1a to these cells under the same experimental conditions.

TABLE IV

Fixation of C'1a by Autoimmune and Isoimmune Warm Reacting Antibodies to Rh-Containing and Rh_{null} (---/---) Cells in combination with Anti-WS^{Hu} Antiserum

Antiserum or eluate	Phenotype	Molecules C'1a fixed/E^{Hu}	
		Test cells having Rh antigens	Test cells having Rh_{null} (---/---) phenotype
Autoimmune			
R. F.	CC D ee	3710	3150
M. P.	"	640	1070
A. G.	"	3240	3020
L. S.	"	2340	1800
A. P.	"	755	730
B. B.	"	425	550
A. B.	CC D ee	920	18
	Cc D Ee	510	
Isoimmune			
Anti-c	cc d ee	860	4
	Cc D ee	1290	
Anti-C	CC d ee	1110	22
	Cc D ee	270	
Anti-D	CC D ee*	580	0
	Cc D ee‡	970	
Anti-E	cc d EE	4020	79
	cc d Ed	2200	
Anti-e	cc d ee	810	0
	cc d Ee	430	

* Presumed genotype CDe/CDe from family studies.
‡ Daughter of above; presumed genotype CDe/cde from family studies.

TABLE V

Effect of the Temperature of Reaction of E^{Hu} and Antibody of the Fixation of C'1a

A. Temperature constant during first stage of C'1a FT test.

Antibody	Dilution	Temperature			
		0°C	17°C	30°C	37°C
		C'1a molecules fixed/E^{Hu}			
Cold agglutinin					
V. L.	1/1600	9300	2560	—	0
T. Bu.	1/100	5770	1410	—	0
M. G.	1/10	4830	254	—	0
J. M.	1/10	230	—	—	157
A. D.	1/100	2950	510	—	0
A. P.	1/10	2220	1520	—	0
Donath-Landsteiner					
J. J.	1/5	4140	—	—	137*
		390	—	—	18
Warm agglutinin*					
R. F.	1/4	4020		2060	
M. P. eluate	1/1	3160		2330	
J. S. serum	1/1	760		850	
M. W. eluate	1/8	1090		910	
Normal serum*					
J. P.		160		180	

B. Temperature changed during first stage of C'1a FT test.

Antibody	Incubation temperature		Molecules of C'1a fixed/E^{Hu}
	1st incubation	2nd incubation	
M. G.	0°	0°	1090
	0°	37°	0
	37°	37°	0

* E^{Hu}-antibody aggregates incubated with anti-IgG^{Hu} before C'1a was added.

The effect of alteration of the test conditions on the fixation of C'1a by human antibodies

Temperature. The fixation of C'1a by human antibodies and E^{Hu} at different temperatures was tested. The results are shown in Table V, part A. Cold-reacting antibodies showed a marked decrease in the amount of C'1a fixed at higher temperatures whereas the warm-reacting antibodies showed a less marked difference or no difference at all.

To determine whether C'1a fixed at 0°C by cold agglutinin antibody remained attached to the cell when warmed to 37°C, we prepared three tubes containing 0.1 ml of a standard suspension

of $E^{Hu\text{-}adult}$, 0.1 ml of a 1/50 dilution of antiserum M. G. containing cold agglutinin antibody, and 0.3 ml of C'1a solution. The first tube was incubated at 0°C for 30 min, the second at 0°C for 30 min and then at 37°C for 30 min, and the third was incubated at 37°C for 60 min. The amount of C'1a fixed per E^{Hu} was determined at the end of the incubations by the C'1a fixation and transfer test. The results are shown on Table V, part B. C'1a was fixed by the antibody in the cold but was released during incubation at 37°C.

Ionic strength. The effect of ionic strength on the reaction of antibody and E^{Hu} was tested by reacting red cells and antibody in either VBS ($\mu = 0.15$) or VBS-sucrose ($\mu = 0.065$), washing the antibody-cell aggregates in the respective buffer, and determining the amount of C'1a fixed by the C'1a FT test. When warm-reacting antibodies were used, anti-IgG^{Hu} in VBS or VBS-sucrose was added to the cell-antibody aggregates and the cells were washed twice with the same buffer before the addition of C'1a. The results are shown in Table VI; in each instance, the amount of antibody fixed was increased by the reduction of the ionic strength by isotonic sucrose.

Repeated washing of the antibody-E^{Hu} aggregates. The effect of repeated washing of the antibody–red cell aggregates was assessed by mixing 0.1 ml of E^{Hu} and 0.1 ml of antiserum or eluate in a series of tubes. These were incubated at 30°C for 30 min and the cell-antibody aggregates were washed two, three, four, and six times, respectively. C'1a fixation was estimated by the C'1a FT test. The results are shown on Table VII.

TABLE VI

The Effect of Ionic Strength on the Fixation of C'1a by Antibody

		Molecules of C'a fixed/E^{Hu}	
Ionic strength of reaction mixture...		$\mu = 0.15$	$\mu = 0.065$
Antibody			
Cold agglutinin			
T. B.	1/100	580	715
M. G.	1/10	130	210
Warm agglutinin*			
R. F.	1/1	630	1040
Normal serum*			
	1/1	170	150
Control‡			
Cold		50	57
Warm		60	73

* Anti-WS^{Hu} antiserum added.
‡ No antiserum added.

TABLE VII

Effect of Repeated Washing of E^{Hu}-Antibody Aggregates on the Amount of C'1a Fixed

		No. of times washed			
Antibody	Dilution	2	3	4	6
		C'1a molecules/E^{Hu}			
Cold agglutinin					
T. Bu.	1/100	660	520	620	640
M. G.*	1/10	155	93	21	—
Warm agglutinin					
R. G.‡	1/4	1570	1700	1700	1700
Normal serum					
J. P.‡	1/1	150	120	60	50

* No C'1a present during incubation of E^{Hu} and antibody (see Reference 27).
‡ Anti-WS^{Hu} antiserum added to washed E^{Hu}-antibody aggregates.

Papainization of the E^{Hu} before reaction with antibody. To assess the effect of papainization of the red cell on the fixation of antibody, we performed parallel C'1a fixation and transfer tests using either unpapainized cells or papainized cells and several human antibodies. The results are shown on Table VIII.

The fixation of C'1a in the presence and in the absence of antibody to human immunoglobulins.

The effect of the addition of heterologous antibodies to the human immunoglobulins on the amount of C'1a fixed by various human antibodies to human red cells was determined by C'1a fixation and transfer tests. In each two tubes, 0.1 ml of a standard suspension of E^{Hu} was mixed with antiserum or eluate and incubated at either 0°C or 30°C. The cell-antibody aggregates were washed and 0.1 or 0.2 ml of anti-WS^{Hu} was added to one of the cell pellets. After incubation for 30 min the cell-antibody-antiantibody aggregates were washed and C'1a was added to both tubes. The amount of C'1a fixed was determined by the C'1a fixation and transfer test. The results are shown in Table IX.

The four groups of antibodies differ in their fixation of C'1a in the absence of anti-globulin antiserum. The cold agglutinin antibodies fix large amounts of C'1a even at high dilutions of antiserum

TABLE VIII

Effect of Papainization of the Test Red Cells on the Fixation of C'1a by Human Antibodies

Antibody	Dilution	Molecules of C'1a fixed/E^{Hu}		Ratio of papainized nonpapainized
		Non-papainized	Papainized	
Cold-reactive agglutinin				
V. L.	1/1600	980	1915	1.95
T. B.	1/100	490	1720	3.51
M. G.	1/10	67	560	8.3
A. D.	1/100	100	250	2.5
Donath-Landsteiner				
J. J.*	1/8	410	980	2.4
Warm-reactive autoimmune*				
C. F.	1/1	885	840	0.95
R. F.	"	2070	2250	1.1
A. G.	"	260	2304	8.9
M. P.	"	3240	2440	0.75
J. S.	"	1840	6230	3.4
M. W.	"	1160	1560	1.35
Normal serum*				
	"	71	149	2.1

* Anti-IgG^{Hu} used in tests involving those antibodies.

TABLE IX

Fixation of C'1a by "Autoimmune" Antibodies in the Presence and in the Absence of Anti-Globulin Antibodies

Antiserum	Neat serum or eluate		Dilution	Diluted serum or eluate	
	No anti-WS^{Hu}	With anti-WS^{Hu}		No anti-WS^{Hu}	With anti-WS^{Hu}
	molecules of C'1a fixed/E^{Hu}			*molecules of C'1a fixed/E^{Hu}*	
Cold-reactive agglutinin					
V. L.			1/1600	380	1150
Donath-Landsteiner					
J. J. serum			1/5	540	7970
Warm-reactive Autoimmune					
R. F. serum	380	1230	1/8	21	1260
eluate	175	4940			
J. S. serum					
eluate	41	3490	1/4	10	2000
M. W. serum	23	1410			
eluate	70	2475	1/8	6	1270
E. B. serum	160	370			
eluate	77	1370			
A. B. serum	14	845			
M. P. serum	890	2100			
eluate	250	850	1/2	108	270
Normal serum	21	150			

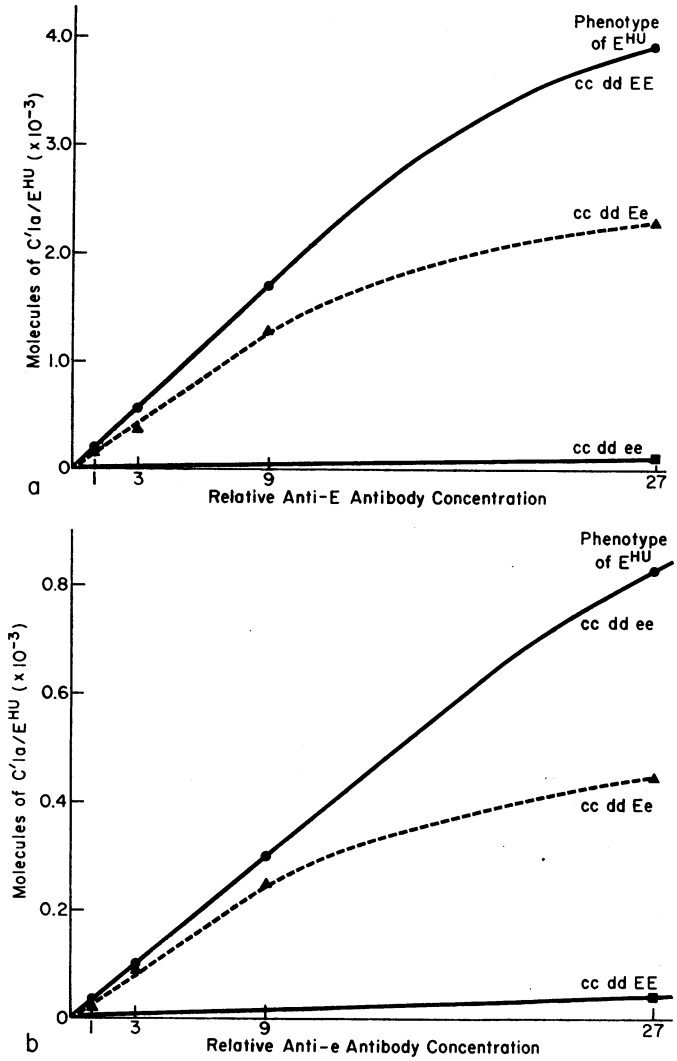

FIGURE 1 (*a*) The fixation of anti-E (anti-rh") by red cells homozygous, heterozygous, and lacking the E (rh") antigen. The relative antibody concentration is plotted on the abscissa, the number of molecules of C'1a fixed in the presence of anti-globulin antiserum is plotted on the ordinate; (*b*) The same as *a*, using the anti-e (anti-hr") antiserum.

but only somewhat more is fixed in the presence of anti-antibody (cf. Tables II, III, and IX). The Donath-Landsteiner antibody fixes a considerable amount of C'1a but this is markedly increased by the addition of anti-human globulin antibodies. Most autoimmune warm agglutinin antibodies fix little or no C'1a in the absence of anti-human globulin antibodies but fix large amounts in their presence.

Antibodies of the Rh system fix insignificant amounts of C'1a in the absence of anti-globulin^{Hu}. In the presence of anti-antibody, C'1a is fixed and the amount varies, depending upon the antibody used and the antigenic composition of the cell with which it is reacted (see Table IV). In the case of the antibodies anti-E and anti-e, about 1/2–2/3 as much C'1a is fixed at maximal antibody concentration by cells homozygous for the specific antigen as by cell heterozygous for that antigen (see Fig. 1). However, no such simple relationship is apparent for other antibodies of the Rh system.

The effect of the presence of more than one specific antibody of the Rh system on the red cell at a time on the ability to fix C'1a was tested. Red cells were incubated successively with one, two, or three antibodies of the Rh system and the amount of C'1a fixed in the presence and absence of anti-antibody was measured. Little or no C'1a was fixed by single antibodies or combination of 2 antibodies whereas a large amount of C'1a was fixed when all three antibodies are present on the red cell simultaneously. (See Table X.)

TABLE X

Fixation of C'1a and the Lysis of PNH Red Cells (Phenotype Cc D ee) by Rh Antibodies Alone and in Combination

Antisera	Molecules of C'1a fixed/E		% Lysis by C' (whole fresh serum)	
	No anti-IgG^{Hu}	With anti-IgG^{Hu}	No anti-IgG^{Hu}	With anti-IgG^{Hu}
Anti-c	0	2220	0	30.3
Anti-D	27	2550	0	37.9
Anti-e	21	1220	0	12.9
Anti-c + Anti-D	105	4770	0	33.0
Anti-c + Anti-e	55	3440	0	23.4
Anti-D + Anti-e	45	3890	0	39.0
Anti-c + Anti-D + Anti-e	2810	4090	41.3*	41.6

* 42% of the cells were complement-sensitive (see reference 17).

When cells from a patient with paroxysmal nocturnal hemoglobinuria were used in such experiments, the ability of such cells to be lysed by C' was tested by adding fresh human serum to the cells after sensitization with 1, 2, or 3 Rh antibodies with and without anti-antibody. The results are shown in the table. Lysis of about 40% of the cells (all the complement-sensitive cells present (17) occurred only in the presence of anti-antibody or, in the absence of anti-antibody only if all three Rh antibodies were present on the cell. These studies indicate that Rh antibodies are intrinsically capable of fixing C'1a. The ability to fix C'1a and amount of C'1a fixed varies with different cells and different antibodies. Studies on the combinations of antigens and antibodies necessary for the fixation of C'1a are in progress.

The relationship between the amount of antibody present and the amount of C'1a fixed in the absence of anti-antibody.

Cold agglutinins. C'1a FT tests were performed in which the amount of antibody reacting with E^{Hu} was reduced by twofold serial dilution. In the range of limited antibody concentration, the number of molecules of C'1a fixed is directly proportional to the amount of cold agglutinin–containing antiserum in the reaction mixture (Fig. 2). If the logarithm of the relative antibody concentration is plotted against the logarithm of the number of molecules of C'1a fixed, a straight line results, the slope of which is about 1.0 for all cold agglutinin–containing antisera tested (see Fig. 3, Table II). This indicates that no more than one antibody molecule is required for the fixation of a C'1a molecule.[4] The number of C'1a molecules fixed by cold agglutinin antibody is therefore a direct measure of the amount of antibody present on the cell.

Warm agglutinin and Donath-Landsteiner antibodies. When the amount of warm agglutinin or Donath-Landsteiner antibody reacting with E^{Hu} is reduced serially, the amount of C'1a fixed is not directly related to the relative concentration of the antibody (Fig. 4). The logarithmic plot of the relative concentration of antibody against the number of molecules of C'1a fixed per cell is a straight line, the slope of which is 1.7–2.1 (see Fig. 5, Table II). This indicates that more than one antibody molecule is required for the fixation of a molecule of C'1a.

[4] The importance of the slope of the logarithmic plot of relative antibody concentration against the number of molecules of C'1a-fixed E^{Hu} is derived from the following considerations: The relationship between the concentration of reactant and product is given by the equation

$$[\text{product}] = K[\text{reactant}]^n$$

where n = the number of molecules of a reactant which, reacting together, produce the product and K is a proportionality constant. If the logarithm of both sides of the proportionality are taken,

$$\log [\text{product}] = n \cdot \log [\text{reactant}] + \log K$$

which is the formula of a straight line of slope n. Hence, plotting the logarithm of the reactant concentration against the logarithm of the product concentration yields a straight line of slope n. The numerical value of n gives the mean number of reactant molecules yielding a molecule of the product, i.e., the number of antibody molecules required to fix a molecule of C'1a.

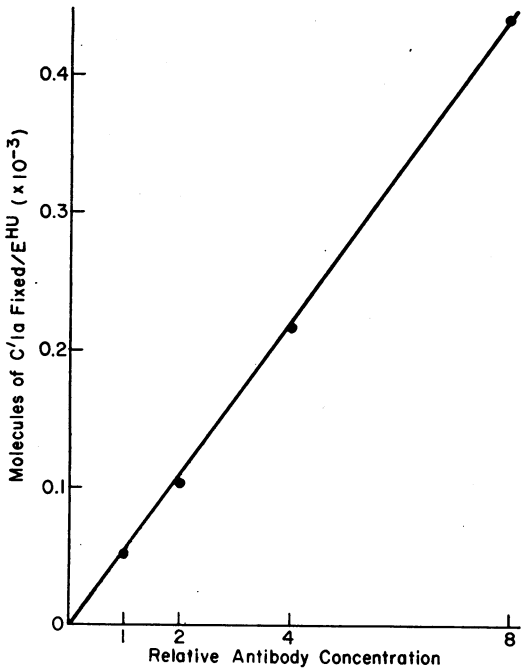

FIGURE 2 Titration of IgM (autoimmune cold agglutinin) antibody with C'1a. The relationship between the amount of cold agglutinin antiserum (V. L.) in the reaction mixture and the amount of C'1a fixed as determined by the C'1a FT test.

The dose response curve of antibody M. P. was consistently different than that of the other warm agglutinin antibodies when this antiserum was reacted with E^{Hu} in the absence of anti-antibody. In the range of limited antibody, the plot of the logarithm of the relative concentration of antibody against the logarithm of the number of molecules of C'1a fixed per cell was a straight line, the slope

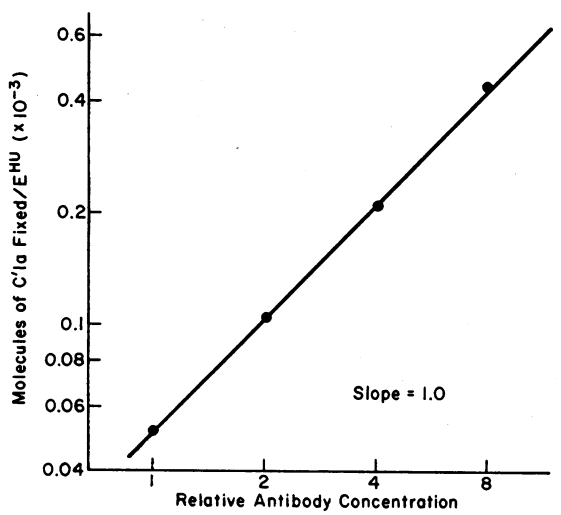

FIGURE 3 The data shown in Fig. 2 regraphed as the logarithm of the relative concentration of antiserum against the logarithm of the number of molecules of C'1a fixed per cell.

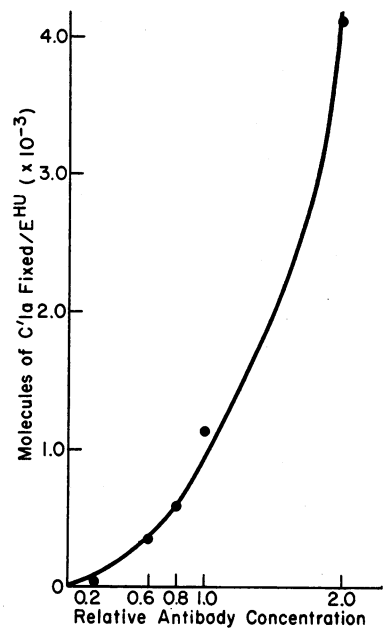

FIGURE 4 Titration of IgG (autoimmune warm agglutinin) antibody with C'1a. The relationship between the amount of warm agglutinin (R. F.) present in the reaction mixture and the amount of C'1a fixed per red cell in the absence of anti-globulin antibodies.

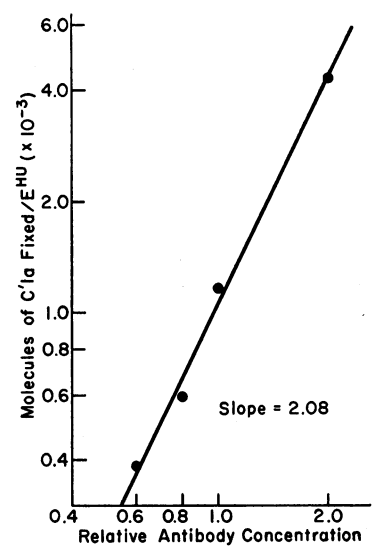

FIGURE 5 The data shown in Fig. 4 regraphed as the logarithm of the relative concentration of antiserum against the logarithm of the number of molecules of C'1a fixed per red cell.

of which was about 1. At high concentrations of antiserum, the slope approached 2.

The relationship between the amount of antibody present and the amount of C'1a fixed in the presence of anti-antibody.

Warm agglutinin antisera and eluates were diluted in twofold falling dilutions and the amount of C'1a fixed by 0.1 ml of a standard suspension of E^{Hu} and equal volume of graded dilutions of warm agglutinin antisera or eluates in the presence of a constant amount of anti-human globulin was determined by the C'1a fixation and transfer tests. The amount of C'1a fixed was proportional to the amount of autoimmune or isoimmune antibody present (in the range of limited antibody). The plot of the logarithm of the relative concentration of autoimmune or isoimmune antibody against the logarithm of the number of molecules of C'1a fixed yields a straight line, the slope of which is about 1 (Table II). The effect of variation in the amount of anti-antibody on the amount of C'1a fixed and the slope of the dose-response curve is discussed elsewhere.[5]

TABLE XI

Slope of Logarithmic Graph of Relative Antibody Concentration vs. Molecules of C'1a-Fixed/E^{Hu} for Autoimmune Antibodies in the Presence and Absence of Anti-Immunoglobulin Antibodies

Antiserum	Amount of anti-antibody	
	0 ml	0.1–0.2 ml
A. Cold-reacting		
Agglutinin		
V. L.	1.06	—
M. G.	0.95	—
T. B.	1.1	—
D. M.	1.0	—
A. P.	1.05	—
J. M.	0.98	—
Donath-Landsteiner		
J. J.	1.7	1.0
B. Warm-reacting		
Autoimmune		
R. F.	1.9	0.95
M. P.	0.9–2.0*	1.2
A. G.	—	1.1
M. W.		1.0
E. S.	2.1	—
E. B.	1.8	1.0
Isoimmune		
Anti-D	—	1.1
Anti-c	—	1.1
Anti-C	—	1.2
Anti-e	—	0.9
Anti-E	—	1.0
Normal serum		
J. P.	—	1.0

* See text.

The absorption of globulin in normal serum to normal E^{Hu}

Globulins present in normal serum appear to be adsorbed to normal red cells in a manner analogous in some respects to the adsorption of antibody. This adsorption is demonstrable in the reaction of red cells and serum from the donor of the cells or from different normal donors. The adsorption of this material is increased by reduction of ionic strength of the reaction mixture and by papainization of the red cells prior to reaction, and is reduced by repeated washing of the cell-serum aggregates. The material reacts with anti-IgG^{Hu} and to a lesser extent with anti-IgM^{Hu} and anti-IgA^{Hu}. About 80–100 molecules of C'1a are fixed per E^{Hu} by 0.2 ml of normal serum in the presence of anti-WS^{Hu} antiserum, whereas much less C'1a fixation occurs in the absence of anti-antibody. The amount of material on the cells detected by anti-WS^{Hu} antiserum is directly proportional to the amount of normal human serum present.

DISCUSSION

The fixation of complement by antibodies from patients with autoimmune hemolytic anemia is in part determined by the immunoglobulin type of the antibody. Borsos and Rapp (4) and Ishizaka et al. (5) have shown that only one molecule of IgM antibody appears to be necessary for the fixation of a molecule of C'1a whereas at least two molecules of IgG antibody in a "doublet" are required. In the present studies, the cold agglutinin antibodies were found to be IgM in immunoglobulin type, as has been previously described (18, 19); these antibodies were found to require no more than one molecule of antibody to fix a molecule of C'1a. The Donath-Landsteiner antibody and most

[5] Rosse, W. F. The fixation of the first component of complement (C'1a) by heterologous anti-human immunoglobulin antibodies in combination with human antibodies to erythrocyte antigens. Manuscript in preparation.

of the warm agglutinin antibodies appeared to be IgG in type and to require at least two molecules of antibody to fix a molecule of C'1.

In a previous publication, we have proposed an hypothesis to explain this difference in molecular requirement for C'1a fixation deduced from known properties of the structure of IgG and IgM molecules and the study of inactivation of the hemolytic activity of IgG and IgM rabbit anti-Forssman antibodies by ionizing radiation (20). The IgM molecule consists of at least five subunits, each about the size of an IgG molecule (21). When IgG antibodies were irradiated, only one radiosensitive target was found per molecule whereas a mean of 3.2 targets was found in IgM antibodies. Using this data, we have suggested that in the IgM molecule, two adjacent subunits are able to act together to fix C'1a in the manner of a doublet of IgG molecules.

This difference among different antibodies in the requirements for C' binding is important in determining differences in the manifestations of the hemolytic disease. The antibodies in cold agglutinin disease probably have only a transitory attachment to the red cell as the blood passes to the cooler skin and extremities during circulation. Therefore, the alteration on the surface leading to destruction is not due primarily to antibody but to the complement components fixed to the surface, some of which are detected in the "non-γ" Coombs test or as β_{1C} and β_{1E}. The requirement of only one molecule of antibody to initiate a complement sequence means that such sequences can be initiated efficiently during the passage through the cooled extremity. The present data and the data of Boyer (22) suggest that as the cold reacting antibody dissociate, C'1a leaves the cell as well. However, the human C' system is probably analogous to the classic guinea pig C' system, and in that system, once the sequence has proceeded through the addition of C'4 to the cell surface and the activation of C'2 at the site of reaction, the remainder of the reactions can proceed in the absence of C'1 (3).

Complement rarely causes direct intravascular lysis of the cell but brings about sequestration of the cell in the reticuloendothelial system. The work of Mollison (2) suggests that cells coated with C' components are removed from the circulation by all organs of the reticuloendothelial system, including both liver and spleen, and that the number of cells removed by the liver is greater due to its greater content of reticuloendothelial elements. Hence, splenectomy would be expected to be of little value in treating cold agglutinin disease and seldom is (23).

On the other hand, the antibodies in warm agglutinin disease are attached very efficiently at body temperature whereas complement fixation, because of the requirement for spatially aligned antibody doublets, is very much less efficient. Hence, the major cause of alteration of the red cell surface leading to destruction of the cell is probably due to the presence of antibody. Cells coated with IgG antibody are preferentially sequestered in the spleen (3), probably by attachment to macrophages there (24). Hence, splenectomy would be expected to be of value in treatment of this disease, and often is (23).

The Donath-Landsteiner antibody is anomalous. Although it is cold-reacting and apparently IgG in immunoglobulin type and hence requires doublet formation for C'1a fixation, it is a potent hemolytic antibody in vivo in causing hemolysis by the direct lytic action of C'. Certainly part of this potency is due to the very large amount of antibody usually present in the serum but other factors undoubtedly play a role. In other studies, we have compared th amount of lysis of normal or PNH red cells induced by limited concentration of complement in the presence of Donath-Landsteiner antibody, "Anti-I," anti-P, or anti-shigella antibody and adsorbed shigella antigen (24). In this test ("the complement-lysis sensitivity test") only the Donath-Landsteiner antibody induced an increased sensitivity of normal cells to lysis by C', suggesting that this antibody was able to use small amounts of C' more efficiently in bringing about the lysis of the normal cell than the other antibodies tested. The reasons for this difference are currently under investigation.

Antiserum M. P. exhibited several peculiar properties. The antibody appeared to be warm-reacting but, unlike most warm-reacting antibodies, considerable amounts of C'1a were fixed in the absence of anti-antibody. The logarithmic dose response curve showed a slope of 1 at low concentrations of antibody but approached 2 at higher concentrations. Reactions with the antisera specific for immunoglobulin types showed IgG antibodies to be present. However, on Sephadex gel filtration,

antibody activity was found in both the excluded peak and in the peak containing 7S proteins. Those antibodies in the excluded peak readily fix C'1a without anti-antibody and are probably responsible for the slope of 1 on the logarithmic dose-response curve at low concentrations of antiserum, whereas those in the 7S peak do not fix C'1a readily except at high concentrations of antiserum where they are probably responsible for the slope of 2. Studies are in progress to determine the nature of the antibody in the excluded peak.

Immunoglobulin type is not the only factor determining the efficiency of fixation of C'1a. The IgG isoimmune (or autoimmune) antibodies of the Rh system do not appear to fix C'1a even when present in maximal amounts on the cell. This is not due to the lack of a C'-fixing site on the antibody molecule since in combinations of several Rh antibodies, C'1a is fixed. The inability of antibodies of a single Rh specificity to fix C'1a may be due to sparse distribution of antigen sites or to peculiar alignment of the antigen sites. Since the Rh antibodies used here are IgG in immunoglobulin type, two molecules in juxtaposition are needed to fix a molecule of C'1a. If the antigen sites of a given specificity were situated at a distance greater than the critical "juxtaposition" distance, C'1a could not be fixed. If antigen sites of other specificity were near enough, then the simultaneous presence of antibody molecules of different specificities could lead to the fixation of C'1a. These possibilities are being investigated.

Since the cold agglutinin antibodies require only one molecule of antibody for the fixation of C'1a, the C'1a FT test provides a direct relative measure of antibody concentration on the cell surface. Whether it provides an absolute measure of antibody concentration is less certain. If more than one C'1a molecule were fixed per antibody molecule, then the C'1a FT test would overestimate the number of antibody molecules. However, the findings of Borsos and Rapp with IgM anti-Forssman antibody suggest that only one molecule of C'1a is fixed per molecule of antibody (25). On the other hand, the number of antibody molecules on the cell would be underestimated if all antibody molecules were not capable of fixing C'1a either because of "steric hindrance" or structural differences among molecules. Preliminary reports by Hoyer, Borsos, Rapp, and Vannier suggest that such heterogeneity may be present in some IgM antibody preparations (26) but is probably not present in others (25).

The relationship between the number of antibody molecules on the cell and the number in solution will depend in large part upon the dissociability of the antibody-antigen complex. Since the cold agglutinin antibodies tend to be easily dissociated (some, such as in antiserum M. G. (27), much more than others) the C'1a FT as performed in these studies will tend to underestimate the number of antibody molecules in solution.

The dissociation of cold agglutinin antibodies may be increased or decreased by several alterations in the conditions of the test. Probably the most important factor increasing the association of antibody and erythrocyte is decreased temperature. Because of this, all procedures designed to estimate the maximal fixation of cold-reactive antibody are performed at the lowest practical temperature.

In previous studies (27) we have shown that the fixation of C'1a by erythrocyte-antibody complexes enhances the fixation of the antibody to the red cell. Thus, with highly dissociable antibodies (antiserum M. G.) very much less antibody is fixed if C'1a is absent during the reaction of cells and antiserum than if it is present. The amount of antibody fixing to red cells is also increased by the prior treatment of the red cells by papain; this is probably due to an alteration in the equilibrium constant of the reaction between antigen and antibody rather than an "uncovering" of antigen sites (28). Finally the association of antibody and antigen can be increased by reduction of the ionic strength of the reaction mixtures.

Using these facts, the conditions under which C'1a is fixed can be designed so that the least amount of dissociation of antibody occurs. At any event, the amount of the underestimation due to dissociation will be the same fraction of the total antibody concentration in solution on each determination, provided the conditions of the test are the same. Hence, the C'1a FT test provides a direct estimate of the number of cold agglutinin antibody molecules which are capable of fixing C'1a at the red cell surface and a relative estimate of the number of molecules of antibody in solution.

The problems in quantitating the amount of IgG warm agglutinin on the cell surface or in solution

are different from those encountered with cold-reacting IgM antibodies. These antibodies are generally less dissociable than the cold agglutinin antibodies but require a "doublet" of molecules for the fixation of C'1a. Many single antibody molecules will be undetected by the C'1a FT test when anti-antibody is not present. However, if heterologous antibodies directed against the primary antibody are added, C'1a is fixed. By proper adjustment of the amount of anti-antibody, a direct proportionality between the amount of antibody in the reaction mixture and the amount of C'1a fixed can be established. The constraints upon this method are discussed elsewhere.[5]

As with the cold agglutinin antibodies, the method probably underestimates the number of antibody molecules in a solution. This underestimation due to dissociability of the antigen-antibody complex is probably less than with cold reactive antibodies since these antibodies are, in general, less dissociable. Papainization of the red cells and decrease in the ionic strength of the reaction mixture will increase the amount of these antibodies attached to the cells. Again, the proportion of antibody molecules either not attached to the red cell or washed from it during the testing procedure will be constant regardless of antibody concentration.

The finding that the incubation of human red cells with autologous serum results in the attachment of material which can be detected by its reaction with antiserum against the proteins of human serum is not particularly surprising. Gamma globulins are loosely attached to the membrane surface of human red cells and are very difficult to wash away (29). Either these proteins do not act as antibodies or their concentration is insufficient for the body to declare the cell abnormal since there is no evidence that the process of random destruction (destruction without regard to cell age) seen with antibody-coated cells occurs under normal circumstances (30). The finding that undiluted autologous serum and rabbit anti-whole human serum antiserum can bring about the fixation of a small amount of C'1a indicates that results of serum assays yielding low titers of C'1a fixation and transfer must be interpreted with caution with regard to content of specific antibody.

If these constraints upon the estimation of antibody number on the cell and in the solution are taken into consideration, the C'1a fixation and transfer test provides a quantitative measure of immunologic reactions occurring on human red cells. Its use in the quantitation of the severity of immune hemolytic disease and in the response of the disease to therapy is the subject of further study.

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