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Robert S. Evans, ... , Elizabeth Turner, Margaret Bingham

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Research Article

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Normal red cells can be made equally resistant to C' hemolysis by incubation with cold agglutinin and normal serum at temperatures which allow transient reactions between the red cells and cold agglutinins. The development of resistance to C' hemolysis was related to increasing susceptibility to agglutination in anti- β_{1c} - and anti- β_{1e} -sera and by increasing uptake of ^{131}I activity from labeled anti- β -globulin serum containing antibodies for both globulins. There was decrease in the adsorption of ^{131}I -labeled cold agglutinin during the development of resistance to C' hemolysis and reduced susceptibility to agglutination by cold agglutinins.

Since cold agglutinins have been demonstrated to dissociate from the red cell, leaving fractions of C' globulin attached, it is postulated that repeated transient reactions produce the accumulation of incomplete C' complexes. Steric hindrance by the adsorbed C' complexes is probably responsible for the inhibition of the reaction with cold agglutinin. There is evidence that the adsorbed C' complexes also interfere with the hemolytic action of C' even when cold agglutinin has become reattached to the red cells.

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Chronic Hemolytic Anemia due to Cold Agglutinins: the Mechanism of Resistance of Red Cells to C' Hemolysis by Cold Agglutinins *

ROBERT S. EVANS, ELIZABETH TURNER, AND MARGARET BINGHAM

(From the Medical Service of the Veterans Administration Hospital and the Department of Medicine of the University of Washington School of Medicine, Seattle, Washington)

Abstract. The red cells of patients with chronic hemolytic anemia due to cold agglutinins are agglutinated by antiglobulin serum in a nongamma reaction due to the coating of β -globulins, C'4 and C'3. The red cells of such patients are abnormally resistant to C' hemolysis by cold agglutinin.

Normal red cells can be made equally resistant to C' hemolysis by incubation with cold agglutinin and normal serum at temperatures which allow transient reactions between the red cells and cold agglutinins. The development of resistance to C' hemolysis was related to increasing susceptibility to agglutination in anti- β_{1c} - and anti- β_{1e} -sera and by increasing uptake of ^{131}I activity from labeled anti- β -globulin serum containing antibodies for both globulins. There was decrease in the adsorption of ^{131}I -labeled cold agglutinin during the development of resistance to C' hemolysis and reduced susceptibility to agglutination by cold agglutinins.

Since cold agglutinins have been demonstrated to dissociate from the red cell, leaving fractions of C' globulin attached, it is postulated that repeated transient reactions produce the accumulation of incomplete C' complexes. Steric hindrance by the adsorbed C' complexes is probably responsible for the inhibition of the reaction with cold agglutinin. There is evidence that the adsorbed C' complexes also interfere with the hemolytic action of C' even when cold agglutinin has become reattached to the red cells.

The accumulation of C' complexes by cold agglutinins appears to be the most important factor in the abnormal resistance to C' hemolysis exhibited by the patient's red cells. Other factors, such as the heterogeneity within a population of normal cells, appear to be of minor significance.

Introduction

The circulating red cells of patients with chronic hemolytic anemia caused by cold agglutinins are abnormal in two respects: they give a positive "nongamma" Coombs' test because of coating by C' globulins, and they possess an unusual degree of

resistance to C' hemolysis by cold agglutinin (1, 2). The first abnormality can be explained by the observation that radioiodinated cold agglutinins dissociate from the red cell, leaving fractions of C' globulin adherent to the cell surface (3, 4). Evidence is presented in this report that the second abnormality is due largely to the accumulation of C' globulin by repeated reactions with cold agglutinin. Normal red cells develop resistance to C' hemolysis equal to that of the patients' cells during incubation with cold agglutinin in the presence of C' (5). We postulate that similar reactions falling short of hemolysis occur in vivo

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Address requests for reprints to Dr. Robert S. Evans, Chief, Medical Service, Veterans Administration Hospital, 4435 Beacon Avenue South, Seattle, Wash. 98108.

and that the accumulation of C' globulins on the red cells interferes with further reaction with cold agglutinin and C'.

Serologic material. The sera of two patients, E.M., a 60 yr old white male, and A.J., an 80 yr old white female, with the syndrome of chronic hemolytic anemia and acrocyanosis were used in these studies. Both sera contained high titers of cold agglutinins of anti-I specificity. Normal group-O I-positive red cells were agglutinated at 5°C in dilutions varying from 1:64,000 to 1:500,000.

Red cells from a group-O I-positive donor were used in the studies related to the development of resistance to C' hemolysis by cold agglutinin and will be designated as normal red cells. Other normal red cells will be designated as to blood group and I or i type.

Methods

The methods used for observing C' hemolysis by cold agglutinin and for preparing and employing radioiodinated cold agglutinin have been previously described (2).

Preparation of anti-human globulin serum with activity for β_{1c} - and β_{1e} -globulins. Serum with high titers of antibodies for human C' globulins was obtained by the method of Gold and Lockyer (6). Activity for β_{1c} -globulin (C'3) and β_{1e} -globulin (C'4) was demonstrated by precipitin bands in immunoelectrophoretic preparations (7, 8). This reagent will be referred to as anti- β -globulin serum. Less prominent bands for immunoglobulins G and A were also present. Activity of these antibodies was inhibited by the addition of human immune globulin.¹ Activity of anti- β_{1e} -globulin antibodies was demonstrated by agglutination of red cells interacted with cold agglutinin and human serum that had been depleted of both C'3 and C'2 (9, 10). Red cells reacted with C'1 and C'4 when washed three times at 37°C were agglutinated to a dilution of 1:160. Activity for β_{1e} was adsorbed by the C'14-coated cells. The adsorbed antiglobulin serum was retested for β_{1e} -activity by the agglutination of red cells remaining after partial hemolysis by cold agglutinin and whole serum. Such residual cells, when washed at 37°C, were agglutinated by a 1:320 dilution of the adsorbed serum. This was interpreted as evidence of anti- β_{1e} -activity.

Additional antisera with activity for β_{1c} - or β_{1e} -globulins prepared by injection of these individual fractions of β -globulins into rabbits were available and confirmed the activity of the anti- β -globulin serum described above.²

Preparations of ^{125}I -labeled anti- β -globulin serum. The method of Horejsi and Smetana (11) was used to frac-

tionate the rabbit anti- β -globulin serum. The gamma globulin so obtained was dialyzed against 0.15 M NaCl and 0.20 M borate buffer, pH 8.0, overnight at 5°C. The concentration of protein was determined by the biuret method. Trace amounts of ^{125}I were incorporated by the iodine monochloride technique (12). Precipitation of samples of the radioiodinated globulin by 10% trichloroacetic acid removed 92-96% of the ^{125}I activity.

The ^{125}I -labeled anti- β -globulin serum agglutinated cells coated with C' complex by the action of cold agglutinin and R3 serum to dilutions of 1:640. The adsorption of ^{125}I activity by coated cells was compared to the uptake of radioactivity by normal cells. Nonspecific adsorption was reduced by the addition of bovine serum albumin to the anti- β -globulin serum (13). 0.02 ml of packed red cells was suspended in a solution of 0.5 ml of the radioiodinated anti- β -globulin serum, 0.2 ml of 30% BSA, and 0.3 ml of a 1:5 saline dilution of human immune globulin. The suspension was incubated for 15-20 min at 37°C before washing three times in 20 volumes of cold buffered saline. Microhematocrits were done in duplicate and the adsorbed radioactivity was expressed in counts per minute/0.1 ml of cells. The accuracy of microhematocrit determinations was confirmed by determinations of hemoglobin concentration after complete lysis. Determinations of osmotic fragility were also used to detect change in red cell volume during incubation. Since the radioactivity of anti- β -globulin preparations varied with intensity of labeling and decay of ^{125}I , determinations done at different times were expressed as the uptake of radioactivity of cells coated with C' globulin divided by the background uptake of normal red cells.

Accumulation of C' globulins by normal human red cells. Red cells were incubated with cold agglutinin and human C' as follows: zymosan-adsorbed human serum (R3) or normal serum was mixed with the heat-inactivated patients' serum, the pH was adjusted to 7.3, and normal red cells were added at 37°C. The suspension was kept at 25°C until agglutination was observed and then warmed to 37°C until agglutinated cells had dispersed. The cooling and rewarming was repeated six times each hour. At the end of 1 and 2 hr, the cells were sedimented and the supernatant was replaced by a new mixture of the serum and cold agglutinin. Samples were removed at 0, 1, 2, and 3 hr, washed three times at 37°C, and tested for adsorption of ^{125}I -labeled anti- β -globulin serum and ^{125}I -labeled cold agglutinin, as well as for susceptibility to C' hemolysis. Hemolysis did not occur during the incubation of cells with cold agglutinin and R3 serum. Cells so treated were designated as having C'142 coating, although it is recognized that some β_{1c} -globulin was present in R3 serum and participated in the reaction as C'3 (14). Control suspensions of red cells in which EDTA was used to block the action of C' during incubation were also prepared.

When normal serum was used in the preparation of C'-coated cells hemolysis occurred during incubation; this was most marked during the 1st hr. The cells lost

¹ Antipolio immune globulin was used.

² Antisera for β_{1c} - and β_{1e} -globulins were obtained through the courtesy of Dr. John Boyer, Cleveland.

through C' hemolysis were measured by hematocrit and hemoglobin determinations before and after incubation. Cells treated in this way were designated as having C'1423 coating, indicating adherence of β_{1c} -globulin. Again, it is recognized that other fractions of the third component were also present (15). The behavior of the residual cells was compared with that of suspensions of control cells in which EDTA was used to block the action of C' during incubation. In some experiments the temperature of incubation was alternated between 31° and 37°C, while in others the temperature of incubation was constant at 33° or 37°C.

Resistance to C' hemolysis. Normal red cells and red cells coated with C' complexes were tested for susceptibility to C' hemolysis by incubation in 4 volumes of the serum of E.M. or A.J. and in 16 volumes of normal serum. The pH of the serum was adjusted to 7.2 or 6.8 with 0.1 N HCl before addition of the cells at 37°C. The suspensions were incubated at 25°C for 30 min. The cells were then sedimented and the supernatant hemoglobin compared with that produced by 100% hemolysis.

Complement titers were determined by the methods of Ecker, Pillemer, and Seifter and Pillemer and coworkers (16, 17).

Heterogeneity of normal red cells. 5 ml of washed normal red cells was suspended in 40 volumes of a 1:20 dilution of the serum of E.M. (titer 1:500,000) in buffered isotonic saline. The suspension was left at 25°C for 1 hr while the agglutinated cells sedimented. The supernatant suspension of cells was removed and the volume of these cells was computed from the microhematocrit. The agglutinated cells were sedimented three times at 37°C, the supernatant removed, and the cells resuspended in 40 ml of a 1:80 dilution of the serum of E.M. The suspension was again left for 1 hr at 25°C and separated as before. The process of separating agglutinated from unagglutinated cells was repeated with 1:320, 1:1280, and 1:5120 dilutions of the serum.

5 ml of a suspension of normal cells from the same donor was also subjected to separation into agglutinated fractions by first suspending it in a 1:5120 dilution of the serum of E.M. and then incubating it at 25°C for 1 hr. In this instance the agglutinated cells were removed and their volume determined. The unagglutinated cells were sedimented at 37°C, the supernatant was removed, and the cells were resuspended in the next higher concentration of cold agglutinins and again incubated at 25°C.

Populations of cells removed in both experiments were washed three times at 37°C and 0.05 ml of each was suspended in 0.5 ml of the serum of E.M. and 1 ml of normal serum at pH 7.0. After 1 hr at 25°C, the per cent hemolysis was determined by comparing the supernatant hemoglobin with 100% lysis.

Complement hemolysis of reticulocytes. Whole blood collected in EDTA was separated into a reticulocyte-rich and reticulocyte-poor layer by centrifugation at 800 rpm for several 15-min periods following the method of Kan and Gardner (18). Reticulocyte counts were done with red cells from the top and bottom layers and uncentri-

TABLE I
Comparison of normal red cells with red cells of patients E.M. and A.J.

Red cells	Hemolysis at 25°C, pH 7.2	¹³¹ I-labeled cold agglutinin adsorption at 25°C, pH 7.2	¹³¹ I-labeled anti- β -globulin activity adsorption: patients' cells/normal cells*
	%	cpm/min	
Normal†	9-52	6000-91,000	1.0(0.95-1.05)
E.M.	0	3000	4.5-5.0
A.J.	0	2700	4.0-4.7

* Red cells from normal subjects did not vary more than 10% in the "nonspecific" uptake of ¹³¹I activity with any one preparation of labeled anti- β -globulin serum. The adsorption of anti- β -globulin activity by the patient's red cells is recorded as the multiple of the background adsorption by normal red cells.

† Normal red cells from 200 adults were examined for susceptibility to C' hemolysis by cold agglutinin and uptake of ¹³¹I-labeled cold agglutinin.

fused whole blood. 0.1 ml of a 50% cell suspension from both layers and from uncentrifuged cells were added to a mixture of 10 volumes of serum of A.J. and 10 volumes of normal serum at pH 6.8 and incubated for 30 min at 25°C. The per cent hemolysis was determined and reticulocyte counts were done on the unhemolyzed cells of each suspension.

Results

Resistance of patients' cells to C' hemolysis by cold agglutinin. The red cells of E.M. and A.J. were resistant to C' hemolysis by the cold agglutinin and adsorbed less ¹³¹I-labeled cold agglutinin than normal cells. The adsorption of activity from ¹³¹I-labeled anti- β -globulin serum, based on multiple determinations during the course of the patients' chronic hemolytic anemia, was four to five times that adsorbed by normal red cells (Table I).

Development of resistance by normal red cells to C' hemolysis by cold agglutinin during incubation with cold agglutinin and R3 serum. Three aliquots of normal group-O I-positive red cells were each suspended in a mixture of 2 volumes of patient's serum and 6 volumes of R3 serum. The results of observations made on cells removed at intervals during the 3 hr of incubation appear in Fig. 1. The decrease in susceptibility to hemolysis when incubation was carried out at pH 7.2 and 6.8 is shown. The adsorption of ¹³¹I-labeled cold agglutinin by the red cells before and during incubation is presented as the percentage decrease during the 3 hr period. The adsorption of ¹³¹I-labeled anti- β -globulin serum is expressed

as the number of times the incubated cells exceeded the background adsorption by the normal cells. Agglutination of the cells in dilutions of anti- β -globulin sera is shown in Table II.

The above findings were typical of many other experiments with normal red cells, cold agglutinin,

and R3 serum. Since hemolysis of the red cells during incubation did not occur, it is evident that the entire cell population acquired resistance to C' hemolysis. The reduced adsorption of ^{131}I -labeled cold agglutinin in this and other experiments was reflected in some decrease in susceptibility of C'

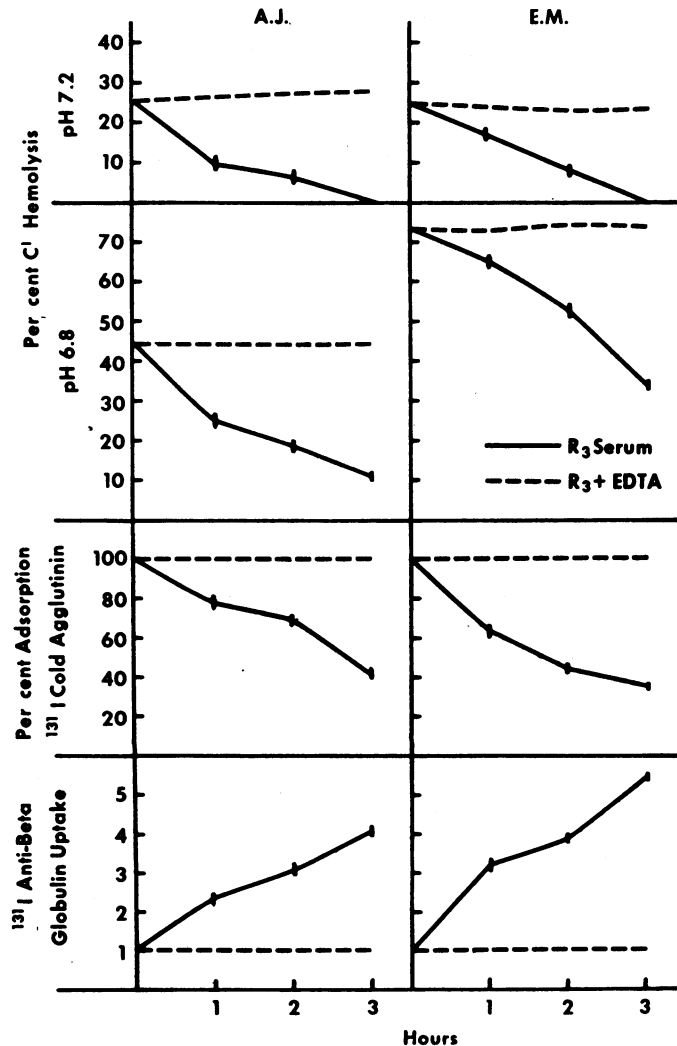


FIG. 1. THE DEVELOPMENT OF RESISTANCE TO C' HEMOLYSIS BY COLD AGGLUTININ DURING INCUBATION OF THREE ALIQUOTS OF NORMAL RED CELLS IN A MIXTURE OF R3 SERUM AND COLD AGGLUTININ AT TEMPERATURES ALTERNATING BETWEEN 25° AND 37°C. Vertical bars indicate the limits of variation in the determinations. Top two frames show the percentage of C' hemolysis by cold agglutinins after each hour of incubation at pH 6.8 and 7.2. Third frame down shows the per cent reduction in ^{131}I -labeled cold agglutinin adsorption. In the lower frame, the adsorption of ^{131}I -labeled anti- β -globulin activity by the C' coated cells is expressed as the multiple of the background adsorption by normal cells. A suspension to which EDTA has been added to block the action of C' served as a control.

TABLE II
*Agglutination in anti-β-globulin sera of red cells incubated with R3 serum and the serum of A.J. or E.M.**

Antiglobulin sera	Time	Reciprocal of dilution of antisera													
		Serum of A.J.							Serum of E.M.						
		10	20	40	80	160	320	640	10	20	40	80	160	320	640
Anti-β-globulin	hr														
	1	3	2	1	1	0	0	0	3	2	2	1	1	0	0
	2	4	4	3	2	1	0	0	4	4	3	3	1	1	0
Anti-β _{1c} -globulin	1	4	4	3	2	1	0	0	4	3	3	2	1	1	0
	2	4	4	3	2	2	1	0	4	4	3	2	2	1	0
	3	4	4	4	4	3	2	1	4	4	4	3	2	2	1
Anti-β _{1c} -globulin	1	1	1	1	0	0	0	0	2	2	2	1	1	0	0
	2	3	2	1	1	0	0	0	2	2	2	2	1	1	0
	3	3	3	2	2	1	0	0	4	3	2	2	2	1	0

* Temperatures were alternated between 25 and 37°C six times per hour. At the end of each hour of incubation the serum mixture was replaced.

coated cells to agglutination in dilutions of cold agglutinin at 25°C. Control cell suspensions incubated with EDTA remained unchanged to all agglutination reactions.

Comparison of zymosan-adsorbed serum (R3) and whole serum in the development of resistance by normal red cells to C' hemolysis by cold agglutinin. Normal red cells were suspended in one part of the patient's serum and three parts of R3 prepared from serum of a normal donor. A duplicate suspension was prepared with the whole serum of the same normal donor. The two suspensions were mixed at 37°C and then divided. One set of suspensions was incubated at temperatures alternating between 25° and 37°C. The duplicate suspensions were incubated at temperatures alternating between 31° and 37°C. The susceptibility of the red cells to C' hemolysis by cold agglutinin, adsorption of ¹³¹I-labeled cold agglutinin and the adsorption of ¹³¹I-labeled anti-β-globulin serum are presented in Fig. 2.

In both experiments whole serum was more effective than R3 serum in producing inhibition of C' hemolysis by cold agglutinins. Red cells reacted with whole serum adsorbed more ¹³¹I-labeled anti-β-globulin serum than cells incubated with R3 serum. Such cells also showed greater reduction in adsorption of ¹³¹I-labeled cold agglutinin, with the single exception of a 3 hr determination with cells incubated with the cold agglutinin of E.M. The changes noted above were most marked with red cells incubated between 31° and 37°C

with the cold agglutinin of E.M. and between 25° and 37°C with the cold agglutinin of A.J.

The amount of C' hemolysis which occurred during each hour of incubation of the cells with cold agglutinin and whole serum is shown in Table III. There was relatively little hemolysis of normal cells during preparation of C'1423 cells with the serum of A.J. The serum of E.M. produced hemolysis of 70% of the normal cells during incubation at 31° and 37°C, the largest amount observed in any experiment. The surviving cells were completely resistant to C' hemolysis by cold agglutinin at pH 6.8.

The greater effectiveness of whole serum over R3 serum in producing the various changes depicted in Fig. 2 probably reflects the greater participation of C'3 (β_{1c}-globulin) in the reaction. In addition, zymosan is believed to reduce the levels of all fractions of C' in the preparation of R3 serum (19).

TABLE III
C' hemolysis during incubation of normal I-positive red cells in A.J. serum and normal serum or E.M. serum and normal serum

Time	Per cent hemolysis			
	A.J. + normal sera		E.M. + normal sera	
	25-37°C	31-37°C	25-37°C	31-37°C
hr				
1	7.5	3.5	28.0	43.5
2	4.0	1.0	15.5	16.5
3	1.5	0	6.0	9.5

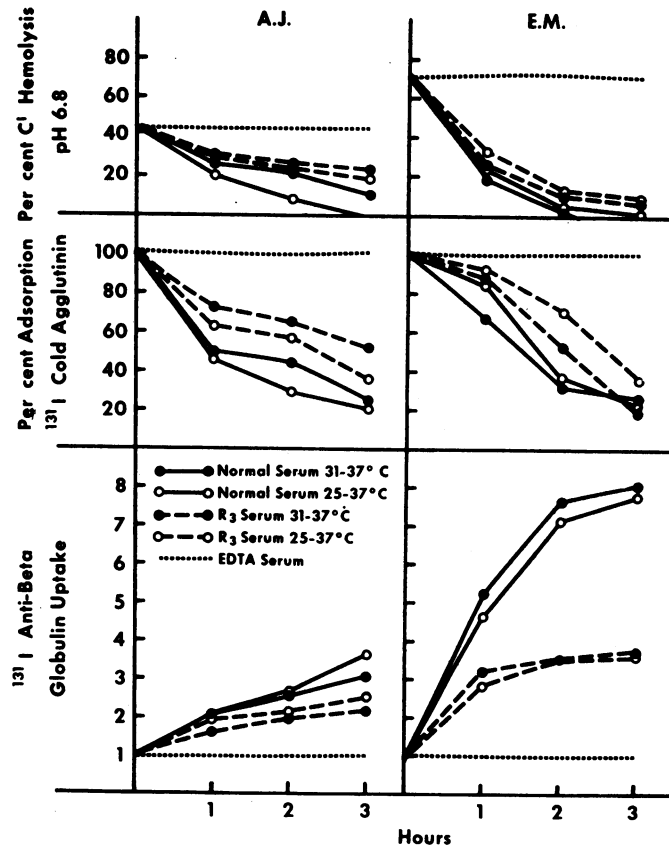


FIG. 2. THE DEVELOPMENT OF RESISTANCE TO C' HEMOLYSIS BY COLD AGGLUTININ DURING INCUBATION OF NORMAL RED CELLS WITH COLD AGGLUTININ AND EITHER R3 SERUM OR NORMAL SERUM. Each suspension was divided in half, one set being incubated at temperatures alternating between 25° and 37°C and the other at temperatures alternating between 31° and 37°C. Top frame shows the percentage of C' hemolysis by cold agglutinin at pH 6.8, middle frame the percentage reduction in ^{131}I -labeled cold agglutinin adsorption, and bottom frame the adsorption of ^{131}I -labeled anti- β -globulin serum recorded as the multiple of background adsorption by normal cells. A suspension with EDTA added to block the action of C' served as a control.

Consumption of complement during the development of resistance to C' hemolysis by cold agglutinin. Normal red cells were suspended in an equal volume of the serum of the patients and 4 volumes of normal serum. The suspensions were divided into two aliquots. One was incubated at 33°C while the second was alternated between 31° and 37°C. Group-O I-negative cord cells were suspended and incubated in the same manner in control observations (20). At the end of each hour of incubation samples of the cells were tested for resistance to C' hemolysis and the adsorption

of ^{131}I -labeled anti- β -globulin serum. In addition, levels of C' in the serum mixture used for incubation were determined (Fig. 3).

As demonstrated in Fig. 3, the i-positive cord cells, deficient in I antigen, showed no increase in the uptake of ^{131}I -labeled anti- β -globulin serum as a result of incubation with anti-I cold agglutinin and C' and continued to be nonagglutinable in anti- β -globulin serum. Decreases in levels of serum C' during incubation with these cells were slight and no greater than decreases noted when normal serum was kept at 37°C without red cells.

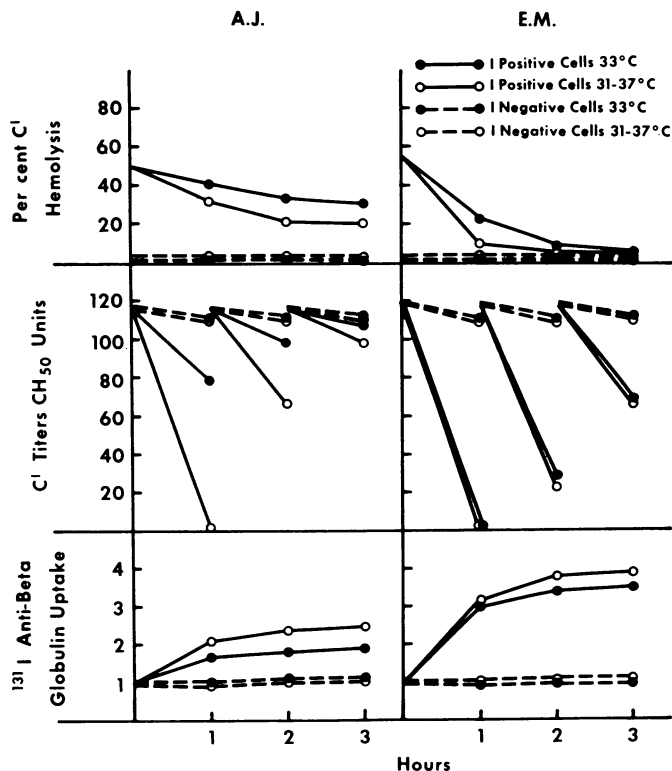


FIG. 3. THE CONSUMPTION OF C' FROM THE INCUBATING MEDIUM DURING THE INCUBATION OF NORMAL I-POSITIVE RED CELLS WITH COLD AGGLUTININ AND NORMAL SERUM COMPARED WITH I-NEGATIVE CORD RED CELLS. The development of resistance of the I-positive cells to C' hemolysis by cold agglutinin at pH 6.8 is shown in the top frame and the adsorption of anti-B-globulin activity in the bottom frame.

The depletion of C' from the serum in which I-positive cells were incubated was directly reflected in the increase in adsorption of ¹³¹I-labeled anti-β-globulin by these cells. The alternation in

temperature between 31° and 37°C was more effective in producing C' fixation than incubation at a constant temperature of 33°C. The adsorption of C' by the I-positive red cells is also re-

TABLE IV
Development of agglutination of I-positive cells in dilutions of anti-β-globulin serum during C' consumption studies depicted in Fig. 3*

Temperature	Time	Reciprocal of dilution of antisera													
		Serum of A.J.						Serum of E.M.							
		5	10	20	40	80	160	320	5	10	20	40	80	160	320
33°C	1 hr	1	1	0	0	0	0	0	4	3	2	1	1	0	0
	2	1	1	1	0	0	0	0	4	3	3	2	1	1	0
	3	1	2	1	1	1	0	0	4	3	3	2	1	1	0
31°-37°C	1	2	2	1	1	0	0	0	4	3	3	1	1	0	0
	2	3	2	2	1	1	0	0	4	3	3	2	1	1	0
	3	3	3	3	2	1	1	0	4	4	3	2	2	1	1

* The I-positive cord cells were not agglutinated by anti-β-globulin serum before or after incubation.

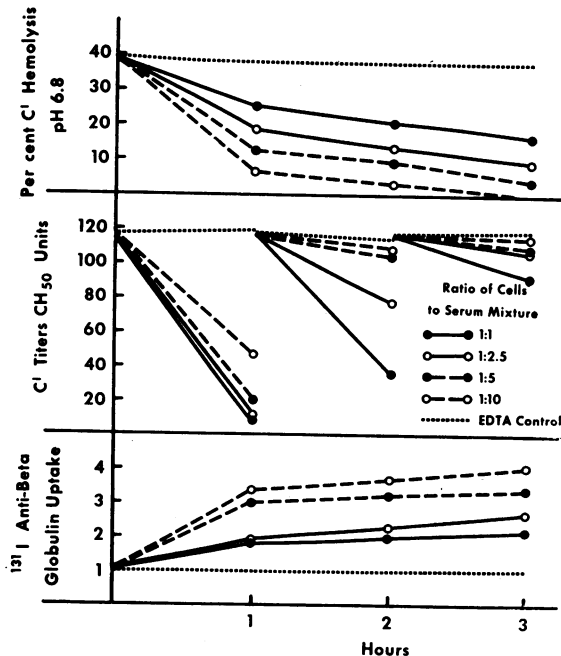


FIG. 4. THE EFFECT OF INCREASING THE RATIO OF A MIXTURE OF COLD AGGLUTININ AND NORMAL SERUM TO NORMAL RED CELLS UPON THE DEVELOPMENT OF RESISTANCE TO C' HEMOLYSIS BY COLD AGGLUTININ. Middle frame shows the consumption of C' during each hour of incubation; bottom frame indicates the adsorption of anti- β -globulin activity. A suspension with EDTA added to block the action of C' served as a control.

flected in the agglutination of the cells in dilutions of anti- β -globulin serum (Table IV).

The hemolysis of the cells during the 3 hr of incubation with the serum of A.J. was less than 1% at the constant temperature and approximately 7% when the temperature was alternated between 31° and 37°C. The serum of E.M. produced 15% hemolysis at both temperatures.

Development by normal red cells of resistance to C' hemolysis by cold agglutinin during incubation in varying ratios of cold agglutinin and normal serum. Normal red cells were suspended in 1, 2.5, 5, and 10 volumes of a mixture of one part serum of E.M. and four parts normal serum at pH 7.2. The uptake of ^{131}I -labeled anti- β -globulin serum and resistance to C' hemolysis by cold agglutinins was determined at the end of each hour. The C' level of each of the serum mixtures used for incubation was compared with the preincubation level and with the level of the control sample of EDTA serum. The results of these determina-

tions are presented in Fig. 4. The hemoglobin appearing in each supernatant serum sample during the hour of incubation was determined relative to 100% hemolysis (Table V).

The adsorption of ^{131}I -labeled anti- β -globulin serum, the agglutination in dilutions of anti- β -globulin serum, and the development of resistance to C' hemolysis were directly related to the volume of serum mixture to which the red cells were exposed. Larger volumes of serum produced greater hemolysis during the 1st hr of incubation but less hemolysis during the 2nd and 3rd hr. C' assays indicated that more complete adsorption of C' occurred from the smaller volumes of incubation medium though the total amount adsorbed on the cells as measured by adsorption of anti- β -globulin serum was less.

Effect of adsorbed C' globulins on the reaction with C' . Incubation of red cells with cold agglutinins and R3 serum produced a progressive decrease in the uptake of ^{131}I -labeled cold agglutinin. Experiments were designed to test whether inhibition of recombination with cold agglutinin accounted for all of the resistance to C' hemolysis or whether inhibition of C' action by C' complexes previously adsorbed by the cells might also be a factor. Cells were coated with C' globulin using R3 and cold agglutinin. Control cells were prepared in the same manner using EDTA in the serum to prevent C' coating. To observe the effect of concentration of cold agglutinin on C' hemolysis, we incubated both sets of cells at 25°C, pH 6.8, for 1 hr in dilutions of radioiodinated cold agglutinin plus a constant amount of human C' . Samples of the same suspensions were incubated with dilutions of the ^{131}I -labeled cold agglutinin, plus a constant amount of EDTA-treated

TABLE V
 C' hemolysis during incubation of I-positive cells with increasing volumes of E.M. serum and normal serum*

Time	Per cent hemolysis in a volume ratio of cells to serum of:			
	1:1	1:2.5	1:5	1:10
hr				
1	1.7	5.4	7.4	16.9
2	2.1	2.3	0.8	1.5
3	2.4	1.7	0	0

* The pH was adjusted to 7.2 and the incubation temperature alternated between 25° and 37°C.

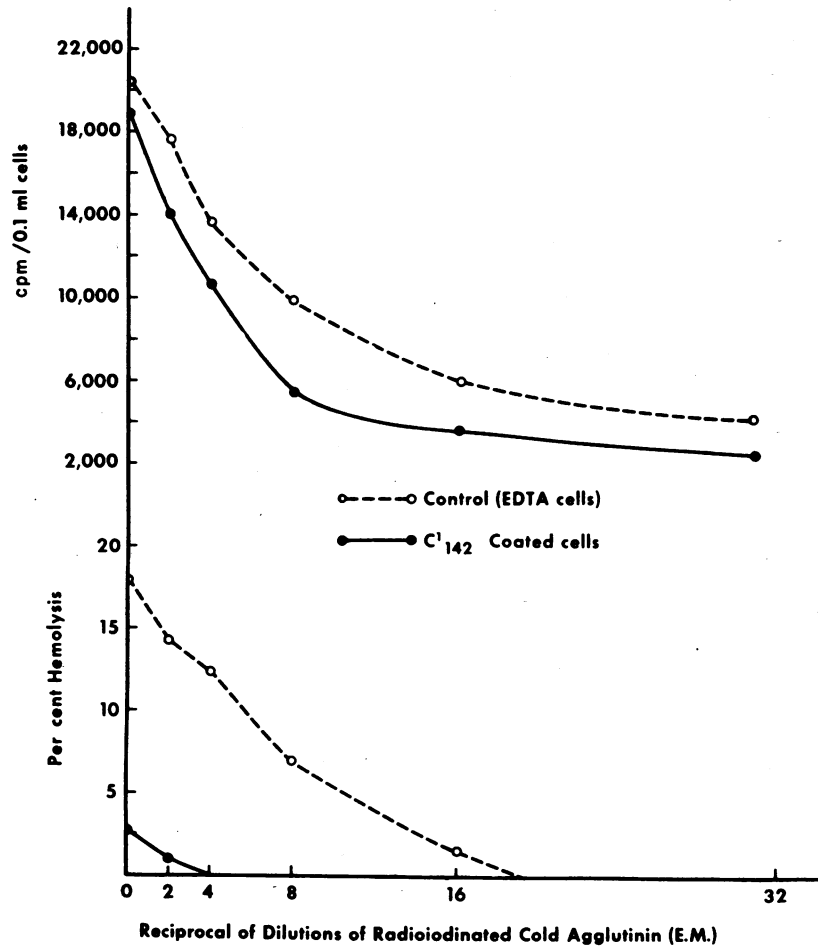


FIG. 5. ADSORPTION OF ^{131}I -LABELED COLD AGGLUTININ AT 25°C BY $\text{C}'142$ CELLS AND BY CONTROL CELLS DURING INCUBATION IN DILUTIONS OF ^{131}I -LABELED COLD AGGLUTININ (UPPER SCALE) AND THE PER CENT OF C' HEMOLYSIS OF SAMPLES OF THESE CELLS BY THE SAME CONCENTRATIONS OF THE ^{131}I -LABELED COLD AGGLUTININ (LOWER SCALE). Reactions were carried out at pH. 6.8.

serum, to measure the uptake of ^{131}I cold agglutinin activity in the absence of hemolysis (Fig. 5).

For purposes of calculation, we assumed the radioactivity of the red cells after washing three times at 25°C to reflect the amount of cold agglutinin adsorbed to the cell, although we recognize some was eluted during washing. From the ^{131}I activity of the original preparation of tagged protein using 1.0×10^6 as the molecular weight of the antibody, we calculated that 40,000 molecules of cold agglutinin per cell were present when 2.5% hemolysis of the control cells occurred, whereas 112,000 molecules were present when 2.5% hemolysis of the cells previously coated with C' globulin occurred (2). These data indi-

cate that several times the concentration of cold agglutinin must be adsorbed to the surface of red cells coated with C' globulins compared to uncoated cells to produce equal hemolysis. This finding suggests that the adsorbed C' globulins inhibited C' action as well as the reaction with cold agglutinin.

I antigen content of red cells of patients with hemolytic disease due to cold agglutinins. As shown in the above experiments, resistance to C' hemolysis by cold agglutinin can be acquired by normal cells through accumulation of C' globulin. An alternative explanation for the resistance of patients' red cells to C' hemolysis by cold agglutinins might be lack of I antigen. Preparations of

TABLE VI
Susceptibility of group O I-positive red cells to C' hemolysis after separation by agglutination in decreasing cold agglutinin concentrations*

Dilutions of E.M. serum	Per cent of original cells		Per cent hemolysis
	Unagglutinated	Agglutinated	
1:20	3	—	9
1:80	14	—	17
1:320	32	—	26
1:1280	38	—	38
1:5120	11	—	43
1:5120	—	2	43
1:1280	—	19	42
1:320	—	55	42
1:80	—	11	29
1:20	—	2	21
1:20	11	—	17

* An aliquot of the same population was separated by susceptibility to agglutination in increasing concentration of cold agglutinin.

stroma from normal adult and cord red cells have been found to reflect the I content of the cells (2). Stroma prepared from measured quantities of whole cells of E.M. and A.J. were found to adsorb as much ¹³¹I-labeled cold agglutinin as similar preparations of normal adult I-positive cells.

Heterogeneity of normal red cells in susceptibility to agglutination and C' hemolysis by cold agglutinin. The resistance of the circulating cells of the patients to C' hemolysis by cold agglutinins might be due in part to heterogeneity of the red cells of the patient to reaction with cold agglutinins and survival of the most resistant population. To test this hypothesis, we separated I-positive cells of normal individuals into fractions on the basis of their susceptibility to agglutination in increasing or in decreasing concentrations of cold agglutinin. Cells from each population were tested for susceptibility to C' hemolysis by cold agglutinin. The

results of one of several experiments from normal I-positive donors are shown in Table VI.

It is evident that red cells of a normal I-positive subject can be separated by susceptibility to agglutination in both increasing and decreasing concentrations of cold agglutinins into populations that show some variation in susceptibility to C' hemolysis by cold agglutinin. In both methods of separation, the red cells that showed least susceptibility to agglutination were least susceptible to C' hemolysis. A small percentage (0.5–2.0%) of red cells of normal subjects was found to be completely resistant to both agglutination and C' hemolysis by cold agglutinin. Such cells agglutinated very weakly in anti- β -globulin serum after 1 hr of incubation with cold agglutinin and normal serum, indicating a decreased ability to acquire a coating of β_{1e} - and β_{1c} -globulins. Reticulocytes were not more numerous in the small population of resistant cells.

Resistance of reticulocytes to C' hemolysis. Susceptibility of red cells to C' hemolysis may be influenced by changes brought about by cell age. Red cells produced by the bone marrow under stress have been shown to have increased reactivity with cold agglutinins of anti-i specificity, although there is no evidence of an accompanying decrease in I factor (21). Young red cells produced in experimental hemolytic anemia have been found to be more resistant to heterospecific antibody than normal red cells (22). C' hemolysis of populations with increased reticulocytes was investigated to determine if young red cells were more or less susceptible to C' hemolysis by cold agglutinins than mature cells. Reticulocyte counts were performed by the same technique before and after the production of partial hemolysis by normal serum and cold agglutinin at pH 6.8 and 25°C

TABLE VII
Susceptibility to C' hemolysis by cold agglutinin after separating red cells of two patients with posthemorrhagic reticulocytosis into a reticulocyte-rich and a reticulocyte-poor layer

Patient	Test	Whole blood cells	Retic.-rich cells	Retic.-poor cells
M.C.	C' hemolysis with cold agglutinin	29	30	30
	Reticulocytes before C' hemolysis, %	4.2	8.8	2.0
	Reticulocytes after C' hemolysis, %	3.7	8.6	2.4
F.J.	C' hemolysis with cold agglutinin	45	42	42
	Reticulocytes before C' hemolysis, %	7.9	32.7	4.2
	Reticulocytes after C' hemolysis, %	8.2	32.4	3.9

and after incubation of samples of the same cells with cold agglutinin and heat-inactivated normal serum.

In two of 15 patients having increased blood formation and a variety of hematologic disorders, reticulocytes were somewhat more resistant to C' hemolysis than normal cells. Reticulocyte counts increased from 8 to 11% after 51% hemolysis of the whole cells of one patient with uremia and from 6 to 10% after 50% hemolysis of the red cells of a patient with chronic myelogenous leukemia. Reticulocyte counts of other patients remained unchanged after hemolysis by cold agglutinins.

The susceptibility of reticulocytes to C' hemolysis by cold agglutinins was also determined after separating whole blood from patients with a posthemorrhagic reticulocytosis into a reticulocyte-rich layer and a reticulocyte-poor layer by differential centrifugation (Table VII). Varying the number of reticulocytes did not significantly influence the susceptibility of the red cells to C' hemolysis by cold agglutinin.

Resistance of cells coated with C' globulins by action of cold agglutinin to C' hemolysis by anti-A. Normal group A, I-positive cells were made resistant to C' hemolysis by anti-I cold agglutinin and C'. Control suspensions were prepared by the addition of EDTA to the incubation medium to block the action of C'. Suspensions of the resistant and control cells were then tested for susceptibility to C' hemolysis in 20 volumes of anti-A human serum.

Such C'-coated cells exhibited slight resistance to C' hemolysis by anti-A as shown in Table VIII. In 10 other experiments, the C'1423 cells were 2-30% more resistant to C' hemolysis by anti-A than were the normal cells. At the same time, these cells were 60-77% more resistant to C' hemolysis by cold agglutinin.

Effect of coating normal red cells with incomplete anti-D antibody on susceptibility to C' hemolysis by cold agglutinin. Normal group O, R1 R2 I-positive cells were incubated with 10 volumes of incomplete anti-D antibody with a titer of 1:1000. The washed cells were shown to be agglutinated by antiglobulin serum and were presumed from previous experience with this serum to have nearly maximal coating of anti-D antibody (23). These anti-D coated cells were found

TABLE VIII
C' hemolysis of normal group A I-positive red cells by anti-A antibodies and by cold agglutinins of A.J. before and after coating with C'1423 complex

Red cells	Per cent hemolysis		
	Anti-A serum		Anti-I serum
	M.B. Group O	J.P. Group B	A.J. Group A
E.T. (A ₂)	20	34	46
E.T. (A ₂)C'1423	19	30	19
J.B. (A ₁)	73	84	50
J.B. (A ₁) C'1423	70	79	12
Patient A.J. (A ₁)	73	85	8

The susceptibility to hemolysis was determined by incubation of the cells in 20 volumes of serum for 1 hr at 25°C, pH 6.8. Normal serum was mixed with serum of A.J. to provide human C'.

to be as susceptible to C' hemolysis by cold agglutinins as samples of the same suspension before coating with anti-D antibody. Cells coated with anti-D antibody also adsorbed as much radioiodinated cold agglutinin as normal cells.

Discussion

The reaction between cold agglutinins and red cells is only transient at normal body temperature. Above 31°C most cold agglutinins do not produce agglutination and are sufficiently dissociated to be completely removed from the cell suspension by repeated washings at 37°C. Despite this, there is evidence of transient reaction between cold agglutinins and the red cell surface at 37°C, since the cold agglutinins of some patients are capable of producing partial C' hemolysis at 37°C. The red cells which do not undergo hemolysis are coated with C'1423 complex. The cold agglutinins of other patients deposit C'1423 complex at 37°C without producing hemolysis.

Evidence is lacking that the fixation of C' binds the cold agglutinin to the red cell and interferes with dissociation at body temperature. Since cold agglutinin dissociates readily, it probably reacts repeatedly with the same or other antigen sites and fixes C'1423 complex with each reaction. Accumulation of C' complexes on the red cell surface with time decreases the reactivity of the red cell with both cold agglutinin and C' globulins.

The adsorption of ^{131}I activity from anti- β -globulin serum and the agglutination in dilutions of the same antisera provide the best methods available for demonstrating the accumulation of C' globulins. The anti- β -globulin serum used in this study had not been purified by adsorption and elution from its antigen because of difficulties in obtaining pure β_{1c} - and β_{1e} -globulin. Consequently, the radioiodinated antisera had a relatively high nonspecific activity due to the adherence of radioiodinated globulin or lipoprotein to the red cells in nonimmune reaction (24). It has been demonstrated repeatedly that the nonspecific adsorption of such protein does not result in a positive agglutination test with any antiglobulin antibody (23, 25). The red cells of normal subjects do not vary significantly in the adsorption of the labeled non-antibody protein. There is evidence that adsorption of radioactivity above the base line for normal red cells in the experiments described above was due to the anti- β_{1c} - and anti- β_{1e} -antibodies. Only red cells known to have adsorbed C' globulins were agglutinated by the antisera or adsorbed radioactivity above the base line. The adsorption of ^{131}I -labeled anti- β -globulin was directly related to the time of incubation with cold agglutinin and C' and to the amount of C' provided by the incubation medium. Concomitantly, the degree of agglutination of the incubated cells in dilutions of anti- β_{1c} - and anti- β_{1e} -antibody increased. These observations suggest that the reaction between anti- β_{1c} - and anti- β_{1e} -antibodies and their respective antigens will prove to be stoichiometric, as has been shown to be true of anti- γG and incomplete γG antibodies adsorbed to red cells (26, 27).

The development of resistance to C' hemolysis by cold agglutinin which followed prolonged incubation of normal red cells with cold agglutinins and R3 serum or with cold agglutinins and whole serum is presumed to be due to steric hindrance by the accumulated C' globulins. Resistance to hemolysis was directly related to the quantity of C' globulin complex adherent to the cell, as measured by radioiodinated anti- β -globulin serum. Adsorption of ^{131}I -labeled cold agglutinin was reduced as C' globulins accumulated. In addition, there was evidence that C'-coated red cells must adsorb more cold agglutinin than normal red cells to undergo similar degrees of hemolysis. This suggests that the residual C' complex also inter-

fered with reinitiation of C' hemolysis even though antibody attachment had occurred.

Some degree of specificity of the steric hindrance produced by deposition of C' complexes is suggested by the failure of anti-D antibody coating of red cells to inhibit either C' hemolysis or the reattachment of ^{131}I -labeled cold agglutinin. It must be added that the concentration of globulin in the two systems was probably not comparable, since it has been computed that there are approximately 500,000 antigen sites for the cold agglutinin and less than one-tenth that number for anti-D antibody (2, 27). Probably many more molecules of C' globulin can be attached to red cells by cold agglutinin than the number of γG molecules represented by maximum concentration of D antibody. Furthermore, some fractions of C' are macromolecular and may be more effective in producing steric hindrance than the anti-D antibody. The C' globulins deposited on the red cell surface by the cold agglutinin inhibited hemolysis of red cells by anti-A antibody to a lesser degree than the inhibition of C' hemolysis by cold agglutinin. This suggests the possibility of proximity of A and I antigen sites, as proposed by previous work (28).

The composition of the C' globulins remaining adherent to the red cells after incubation with cold agglutinin and R3 is uncertain. The R3 serum produced by reacting whole serum with zymosan at 37°C was devoid of hemolytic activity due to the inactivation of some of the fractions of C'3, including the conversion of some but not all β_{1c} - to β_{1a} -globulin (14). Red cells incubated with cold agglutinin and R3 serum were agglutinated in antiserum believed to contain only anti- β_{1e} -antibodies and to a lesser degree in antisera with only anti- β_{1c} -antibodies. The amount of β_{1c} -globulin present and the extent to which it and other fractions of the third component of C' stabilized the C'142 complex on the red cells is not known (15). Incubation of the C'-coated cells at 37°C for 1 hr to prepare EC14 cells did not result in return of susceptibility to C' hemolysis by cold agglutinin. Similarly, incubation with EDTA for 3 hrs at 0°C to elute C'1a did not affect the resistance to hemolysis (29).

The red cells of normal subjects vary considerably in susceptibility to agglutination and to C' hemolysis by cold agglutinins of anti-I specificity (30, 31). Moreover, the red cell population of a

single individual showed significant heterogeneity with regard to susceptibility to agglutination. Resistance to agglutination was reflected in greater resistance to C' hemolysis. How much the heterogeneity of the red cells of one individual contributes to the resistance of the red cells of patients to complement hemolysis by cold agglutinin is uncertain at present. Red cells of the patient which are most resistant to the action of cold agglutinin probably do survive better in the circulation but it is unlikely that survival of such cells accounts for more than a fraction of the observed resistance to C' hemolysis. The small percentage of red cells of normal individuals which are resistant to C' hemolysis are also resistant to C' coating by action of cold agglutinin. In contrast, the patients' cells which are resistant to C' hemolysis are coated with C' globulin complexes, as shown by the high uptake of ¹³¹I-labeled anti- β -globulin serum and by intense agglutination in anti- β -globulin serum. Reticulocytes of a minority of patients with increased blood formation were somewhat resistant to C' hemolysis when compared to red cells beyond the reticulocyte stage. The contribution of the young red cells in cold agglutinin hemolytic anemia to the resistance of the circulating cells of the patient to C' hemolysis by cold agglutinin appears to be a minor factor.

Others have noted the inhibition of antigen antibody reactions by adsorption of C' globulins to red cells. Laurell observed that sheep cells which had adsorbed C' complex from the action of human R3 and rabbit anti-sheep cell antibody were more resistant to agglutination by rheumatoid factor than control cells which had not adsorbed C' complex (32). Möller has studied the resistance of mouse cells to C' hemolysis by isoantibodies which developed through prior exposure of the cells to the antibody and normal serum factors presumed to be C' (33). The development of resistance to antibody action may occur in a variety of systems in which fixation of C' fractions is followed by dissociation of the antibody.

It is probable that C' globulins accumulate on the red cells in vivo as the result of mechanisms similar to those used in the in vitro studies. Alterations in temperature of the blood flowing from skin vessels to viscera was postulated to be a factor in the adsorption and release of cold agglutinin by the red cells. In vitro experiments in

which the temperature was kept constant at 33°C as shown in Fig. 3 or at 31° or 37°C in other experiments resulted in a somewhat slower accumulation of C' globulins, compared with those in which the temperature was alternated between 31° and 37°C. These observations suggest that the normal variations of temperature of the circulation between viscera and surface vessels were not essential for the association and dissociation of the cold agglutinin in vivo and the consequent accumulation of C' globulins on the red cell surface. Although the development of resistance to C' hemolysis by accumulation of C' globulin was accompanied by a decreased susceptibility to agglutination by dilutions of cold agglutinins, this did not prevent the development of acrocyanosis due to intravascular agglutination when the temperature of the skin was sufficiently reduced.

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