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Research Article

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Measurement of the Third Component of Complement Bound to Red Blood Cells in Patients with the Cold Agglutinin Syndrome

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ABSTRACT The amount of the third component of complement (C3) bound to red cells of patients with the cold agglutinin syndrome was determined by a quantitative assay, measuring the fixation of the first component of complement by anti-C3. Abrupt reduction in the serum concentration of cold agglutinin by plasmapheresis markedly decreased the hemolytic rate, but the amount of C3 bound to circulating cells did not change appreciably. When this patient was transfused with normal cells, C3 accumulated on the transfused cells within 48 h to the level present on his own cells, but selective destruction of the transfused cells did not occur. When patients were subjected to acute cold stress, cell-bound C3 rose abruptly and intravascular hemolysis occurred. These studies suggest that most of the C3 detected on the circulating red cells of cold agglutinin patients is in an inactive form, and that the rate of attachment of C3 to the membrane is important in determining hemolysis.

INTRODUCTION

Immune hemolytic anemia may be associated with complement fixation and direct intravascular hemolysis may result. If lysis does not occur, complement components may accumulate on the membranes of unlysed cells (1, 2). The fourth component (C4), the third component (C3), and the fifth component of complement (C5) have been shown to be attached in vivo to the red blood cells of some patients with immune hemolytic anemia (3). Of these components, C3 is attached in large amounts and its presence on the membrane is largely responsible for the positive "non-gamma globulin" direct antiglobulin reaction. Until recently, methods for the quantitation of C3 on the red cell membrane have not been available (4). In the present paper, we describe a method for measuring membrane-bound C3 which is based on the fixation of the first component of complement ($C\bar{1}$) by a rabbit anti-human C3 antibody reacting with membrane-bound C3. The amount of $C\bar{1}$ fixed to red cell-C3-anti-C3 complexes was measured by the method of Borsos and Rapp (5) and the relationship of the amount of $C\bar{1}$ fixed to the amount of C3 bound was established.

The cold agglutinin syndrome provides an opportunity to study the consequences of attachment of complement to the membrane. Large amounts of C3 are fixed to the red cells but the antibody is readily dissociated from the membrane by warming (6). Thus it is possible to examine the C3 attached to the membrane without interference by the presence of antibody.

Using the anti-C3-$C\bar{1}$ fixation and transfer technique, we have examined the binding of C3 to red cells in patients with the cold agglutinin syndrome, and the relationship of bound C3 to the destruction of the cells. It is apparent from these studies that most of the C3 which is detected on the membrane in the absence of cold stress is hemolytically ineffective. With cold stress, C3 is rapidly added in large amounts and increased hemolysis results. The rate of addition of C3 to the membrane appears to be important, since even larger amounts may be fixed slowly without hemolytic consequences.

METHODS

Red blood cells and serum. Blood was collected in EDTA from patients with cold agglutinin disease and the red cells were immediately separated and washed at 37°C. Serum from these patients was obtained by collecting blood and maintaining it at 37°C until the clot had retracted. The serum was then removed, incubated at 56°C for 30 min and frozen at − 20°C.

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Blood from normal donors was collected in an equal volume of Alsever's solution (7) and stored at 4°C until the time of use. "Standard" suspensions of red cells containing 2.2×10^8 cells/cm^3 were made as in reference 8. To obtain normal human serum as a source of complement, blood, drawn aseptically was immediately cooled and centrifuged at 0°C. The plasma was removed and was maintained at room temperature until a fibrin clot was formed, which was removed. The serum was then frozen at −90°C until use.

Buffers and complement reagents. Veronal-buffered saline (VBS),[1] isotonic sucrose buffer, 60% isotonic sucrose-40% VBS ("60% sucrose") buffer, and buffered 0.015 M ethylenediamine-tetraacetic acid disodium salt (EDTA) were prepared according to the methods outlined in reference 9. Sheep red cells, rabbit antibody to boiled sheep red cell stroma, guinea pig serum as a source of complement, the first (C1) and second (C2) components of complement, and sensitized sheep cells with the fourth component of complement (C4) on the surface (EAC4) were prepared as previously described (9, 10).

C3 and anti-C3. Human C3 was purified by a modification of the method of Nilsson and Müller-Eberhard (11). A euglobulin precipitate of fresh human serum was applied to a DEAE column equilibrated with 0.03 M pH 8.1 phosphate buffer and eluted with a gradient from 0.03 M phosphate buffer, pH 8.1 to 0.2 M NaH_2PO_4. The C3-containing fractions detected by immunoprecipitation were applied in a batch technique to hydroxyapatite which had been equilibrated with 8 m mho phosphate buffer pH 7.9, the hydroxyapatite was then washed with 12 and 13 m mho phosphate buffer pH 7.9 and the C3-containing material was eluted with 14.5 m mho phosphate buffer. The C3-containing supernatant fluid was then concentrated by the method of Alper and Rosen (12). The purified human C3 produced a single band on microimmunoelectrophoresis against anti-whole human serum.[2] A single protein band was present on

Several techniques were used to label this purified protein with ^{125}I. When C3 was iodinated by the technique of McConahey and Dixon (13), using chloramine T, it was converted to β_{1A} and α_{2D} fragments as shown by immunoelectrophoresis against anti-C3 (14). When the iodine monochloride method (15) was used, the C3 was not fragmented. When this C3 was incubated with washed sheep or human red cells, about 10% was attached in the absence of immune reactions. A large amount of non-specific attachment of ^{125}I-labeled C3 to red cells has been previously noted by others (16).

Anti-C3 antiserum was obtained by injecting rabbits with 0.75–2.0 mg of purified C3 emulsified in Freund's complete adjuvant. The rabbits were immunized three times over a 6 wk period and the antisera used for this study were harvested 2 wk after the third immunization. The antisera were absorbed with bis-diazotized benzidine (17) aggregated IgG and human red cells. When fresh human serum was examined by microimmunoelectrophoresis against these antisera, only a β_{1C} immunoprecipitin was obtained. When human serum, incubated at 4°C for 2 wk was similarly examined, β_{1A} and α_{2D} immunoprecipitins were produced. When partially aged serum was examined by this antiserum. β_{1C}, β_{1A}, and α_{2D} arcs were present, and spurring of the β_{1C} arc beyond the β_{1A} arc indicated the presence of antibodies to antigens present on β_{1C} which were not detectable on β_{1A}. 5 ml of the anti-C3 antiserum were passed through a Sephadex G-200 column and the content of anti-C3 was analyzed in the effluent fractions by the agglutination of C3-coated red cells and by the fixation of $\overline{C1}$ to such cells. Nearly all the anti-C3 activity was found in the IgG-containing fractions; only trace amounts were present in the exclusion volume.

In vitro C3 attachment. In order to coat human red cells in vitro with C3, serum containing cold agglutinin diluted in VBS was mixed with washed red cells in standard suspension and fresh compatible human serum as a source of complement. The mixture was incubated at 0°C for 15 min, then at 37°C for 45 min. The cells were sedimented by centrifugation at 37°C and the supernatant fluid was analyzed for the presence of hemoglobin by measuring optical density at 412 nm. The cells (EC3) were washed twice with 0.015 M EDTA at 37°C, then thrice with VBS at 37°C and resuspended to the standard cell concentration in 60% sucrose buffer.

C1 fixation and transfer. The amount of $\overline{C1}$ fixed by anti-C3 to C3-coated red cells was determined by a modification of the $\overline{C1}$ fixation and transfer test (18). Cells coated with C3 (EC3) in a suspension containing 2.2×10^7 in 0.1 ml, were mixed with 0.1 ml of anti-C3 and 0.2 ml functionally pure C1, containing at least 10^{12} hemolytically active molecules per ml (19). After 60 min, the excess C1 was removed by washing with 60% isotonic sucrose-40% VBS buffer. The amount of C1 fixed was determined by the method of Borsos and Rapp (5). When membrane-bound C3 was estimated repeatedly on the same sample of EC3 by this method, the amount of $\overline{C1}$ fixed varied from the mean by less than 12% in all instances.

In order to ascertain the concentration of anti-C3 which fixed the greatest amount of C1 to EC3 cells, two sets of EC3 cells, one of which had been reacted with cold agglutinin and a high concentration of serum as a source of complement ("high EC3") and the other with the same amount of cold agglutinin and a lower concentration of fresh serum ("low EC3"), were reacted with varying dilutions of anti-C3. The amount of C3 fixed to these EC3 cells by varying amounts of anti-C3 is shown in Fig. 1. For the cells heavily coated with C3, the same number of molecules of $\overline{C1}$ were fixed with dilutions of anti-C3 from 1:40 to 1:320. For "low EC3" cells, the maximum amount of $\overline{C1}$ was fixed at a 1:50 dilution of anti-C3. This dilution was used in all studies unless otherwise stated. The amount of C3 on the red cell membrane was varied by varying either the amount of fresh serum as a source of complement, or the amount of cold agglutinin antiserum used in the reaction mixture. The fraction of cells lysed during attachment of C3 was determined and the amount of $\overline{C1}$ fixed by anti-C3 was measured in duplicate. The results of such experiments are shown in Figs. 2 and 3. The amount of $\overline{C1}$ fixed, expressed as the mean of duplicate determinations, varied directly with the amount of fresh serum or cold agglutinin present in the initial reaction mixture.

The concentration of C3 bound to sheep cells was varied by reacting sheep EAC4 with excess C1 and C2, then with dilutions of purified human C3. These cells were then

[1] *Abbreviations used in this paper:* C3bINA, a serum factor which inactivates membrane-bound C3; EAC4, sensitized sheep red cells coated with guinea pig C4; EAC1,4,2, sensitized sheep red cells coated with guinea pig C1, C2, and C4; EC3, human red cells coated with human C3; PNH, paroxysmal nocturnal hemoglobinuria; VBS, veronal-buffered saline.

[2] Meloy Laboratories, Inc., Springfield, Va.
polyacrylamide gel disc electrophoresis.

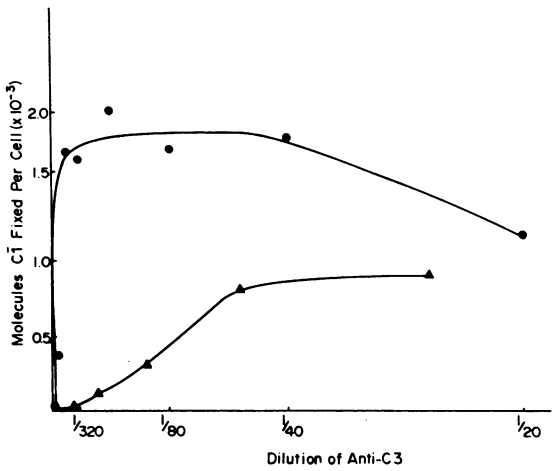

FIGURE 1 Determination of optimal anti-C3 concentration. The molecules of $C\overline{1}$ fixed per cell to human cells coated with C3 are plotted against the dilution of anti-C3 used (see text). The triangles indicate human cells coated with small amounts of C3, and the circles indicate human cells coated with larger amounts of C3. A 1 : 50 dilution of anti-C3 was chosen for subsequent experiments.

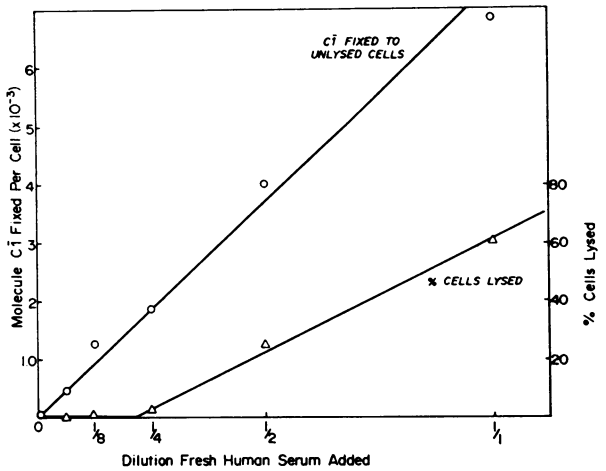

FIGURE 2 $C\overline{1}$ fixed by anti-C3 to cells which had been reacted with cold agglutinin antibody and varying amounts of fresh human serum. The percent of cells lysed by antibody and fresh serum and the $C\overline{1}$ fixed to the remaining washed, unlysed cells by anti-C3 are compared with dilution of fresh serum added.

washed twice in 0.015 M EDTA and twice in veronal-buffered saline. The washed cells were reacted with anti-C3 and the amount of $C\overline{1}$ fixed was assayed. The results of one such determination are shown in Fig. 4. There is a linear relationship between the amount of C3 added to the initial reaction mixture and the number of molecules of $C\overline{1}$ fixed by anti-C3.

Varying amounts of C3 labeled with ^{125}I (2.2×10^8 cpm/mg protein) was reacted with sheep EAC1,4,2 and EAC4 cells and the amount of C1 fixed in the presence of anti-C3 determined. The radioactivity bound to EAC4 was subtracted from that bound to EAC1,4,2 cells to determine the amount of labeled C3 attached immunologically. The results of such an experiment are shown in Fig. 5. Although immunologically attached C3 could be detected by this method, the large amount of labeled C3 attached to the EAC4 (60-80% of that bound to EAC142) makes interpretation of such data difficult.

Detection of C3 added in vivo. The relative amount of C3 on the cells of patients with cold agglutinin hemolytic anemia was measured, using anti-C3 and $C\overline{1}$ fixation and

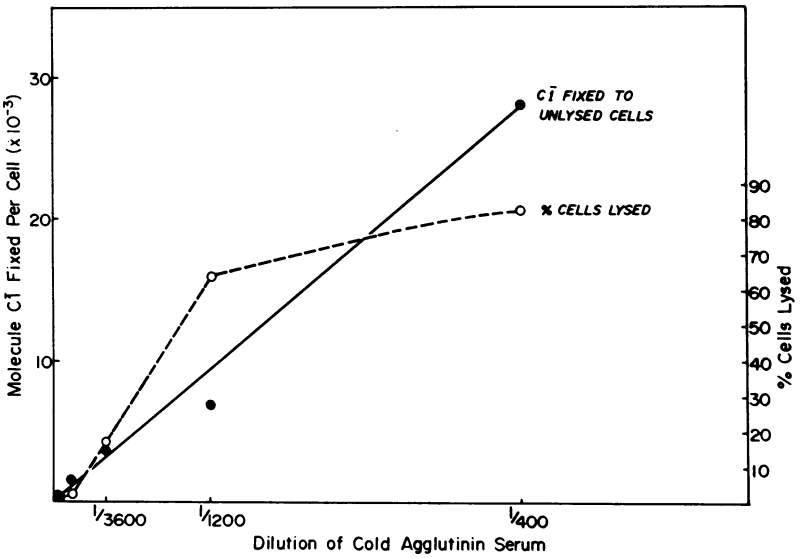

FIGURE 3 $C\overline{1}$ fixed by anti-C3 to cells which had been reacted with fresh human serum and varying amounts of cold agglutinin antibody. The percent of cells lysed by antibody and fresh serum and the $C\overline{1}$ fixed to the remaining washed, unlysed cells by anti-C3 are compared with dilution of cold agglutinin antibody added.

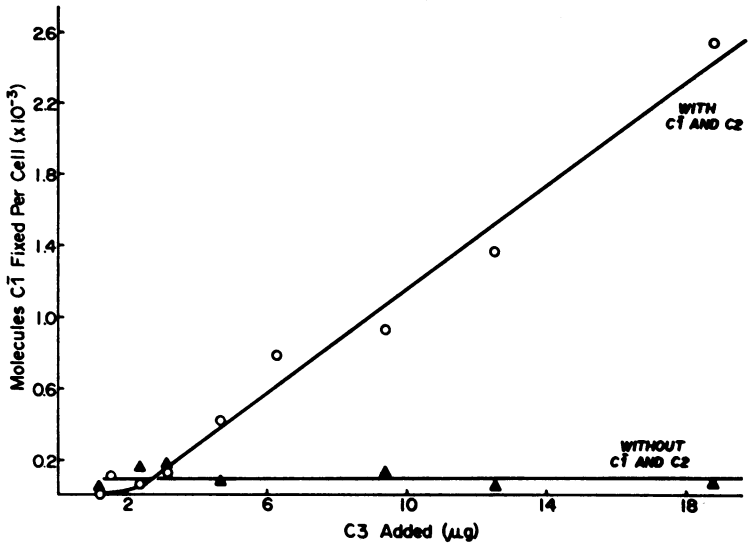

FIGURE 4 $C\bar{1}$ fixed by anti-C3 to sheep EAC1,4,2 cells which had been reacted with varying amounts of pure human C3 (see text). Sheep EAC4 cells treated with buffer rather than $C\bar{1}$ and C2 serve as controls and the molecules of $C\bar{1}$ fixed by anti-C3 to these cells are shown on the lower line. The C3 is expressed in micrograms of protein added to the initial reaction mixture.

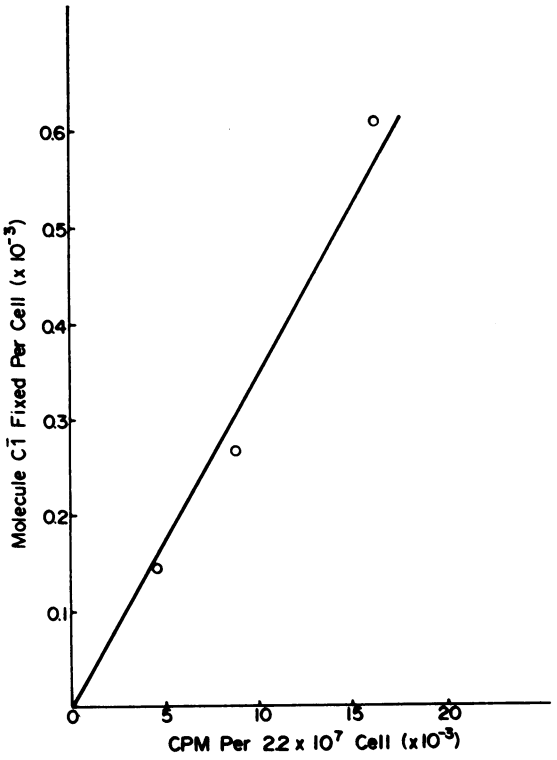

FIGURE 5 The molecules of $C\bar{1}$ fixed per cell to sheep EAC142 cells with anti-C3 is compared with the counts per minute of ^{125}I-labeled C3 attached to the cells. The values on the abscissa were obtained by subtracting the counts per minute attached to EAC4 cells from the counts per minute attached to EAC142 cells at each concentration of ^{125}I-labeled C3 added.

transfer. Cells from patients were drawn and maintained at 37°C, and were washed twice in 0.015 M EDTA and in VBS three times at 37°C. $\frac{1}{10}$ ml of a standard suspension of cells were reacted with 0.1 ml of anti-C3 (1/50 dilution) and 0.2 ml of $C\bar{1}$. The amount of $C\bar{1}$ fixed was determined. Analysis was performed on the same day as the blood was collected. Eight determinations on a single red cell sample produced a standard deviation of 6.1% of the mean.

RESULTS

Patients. Patient J. C., with the cold agglutinin syndrome and severe hemolytic anemia, underwent large volume plasmapheresis to reduce the concentration of cold agglutinin. The clinical and laboratory data of this patient are given in Tables I and II. Experiments to define the effect of sudden cold stress were performed on patient J. C. and on two other patients with the cold agglutinin syndrome, C. T. and C. P. Plasmapheresis and transfusion were not therapeutically necessary for patients C. T. and C. P.; these procedures could not be justified in these patients since the risks involved are too great.

The effect of changes in cold agglutinin titer. Rapid reduction in serum levels of cold agglutinin in patient J. C. was effected on two occasions by large volume plasmapheresis with a blood cell separator[3] in a room heated to 37°C. Venous whole blood was continuously removed from one arm and centrifuged. The plasma was removed and the cells, diluted in albumin-containing saline, were continuously returned to the other

[3] Aminco Celltrifuge, American Instrument Co., Silver Spring, Md.

TABLE I

Clinical and Hematological States of Patients with Cold Agglutinin Syndrome

Patient	Age	Sex	Clinical presentation, associated disease	Hemoglobin (g/100 ml), reticulocyte count (%)	Red blood cell life span by endogenous CO production	Serum complement
					days	*CH_{50} units/ml**
J. C.	50	M	Severe hemolytic anemia	8.5	12	24
(H9 0 082)			Carcinoma of the lung	13.0		
C. T.	82	M	Mild acrocyanosis	8.0	40	31
(1 1 164)			Hemolytic anemia	9.5		
			Carcinoma of bladder			
C. P.	51	M	Severe acrocyanosis	16.0	65	50
(H6 3 016)			No associated disease	4.3		

* Normal, 32–40 CH_{50} units/ml.

arm. 3,000 ml of plasma were removed in 4 h with a 70% fall in cold agglutinin level, as measured by ability of the cold agglutinin and fresh serum to lyse the red cells of patients with paroxysmal nocturnal hemoglobinuria (PNH) (20, 21). Within 1 week the antibody titer, as measured by the hemolytic assay, returned to the levels seen before plasmapheresis. The rate of endogenous CO production (22) fell following plasmapheresis, indicating that the hemolytic rate decreased; CO production increased as the plasma antibody concentration subsequently rose. The amount of cell-bound C3 detected by anti-C3 and the $C\bar{1}$ fixation and the transfer technique did not change appreciably after plasmapheresis or during the following week (Fig. 6).

The effect of addition of C3 to transfused normal cells. To determine the effect of addition of C3 to normal cells in the absence of cold stress, patient J. C., whose red cells were type B, was transfused with 2 U of type O packed cells which were warmed at 37°C prior to transfusion. A portion of the cells used in the transfusion was labeled with chromium-51 and was injected at the end of the transfusion. The survival of these labeled cells was followed by serial samples over the next 48 h (23). The amount of C3 bound to the transfused cells was serially determined after removal of the patient's own type B cells by agglutinating a 10% suspension of washed red cells with equal volumes of anti-B antisera. Separation by differential centrifugation was considered complete when further addition of anti-B antiserum produced no further microscopic agglutination of the cells. The transfused type O cells were then washed and resuspended to a concentration of 2.2×10^8 cells/ml and assayed for cell-bound C3 with anti-C3 and $C\bar{1}$ fixation and transfer.

The results of this study are shown on Fig. 7. 24 h after transfusion, the amount of C3 bound to the transfused cells was nearly the same as that on the patient's own cells. During the 48 h period after transfusion, the rate of destruction of the ^{51}Cr-labeled, transfused cells was not greater than the rate of destruction of the patient's cells as determined by endogenous CO production. Plasma hemoglobin was not higher on the day following transfusion than on the day preceding it. The patient's serum hemolytic complement level did not change significantly when measured on the day prior to transfusion, 24 h after transfusion and 1 wk following transfusion (44–46 CH_{50}/ml, normal range 32 to 42 CH_{50} units/ml).

Transfusion with type O packed red cells was repeated 3 months later. Survival of ^{51}Cr-labeled, transfused cells and red cell-bound C3 were measured in a manner identical to that of the first transfusion study. Again C3 accumulated on the transfused cells during

TABLE II

Characteristics of Cold Agglutinin Antibodies

Patient	Light-chain type	Cold agglutinin titer				
		Adult cells				Fetal cells
		0°	15°	22°	37°	0°
J. C.	Kappa	1/8,000	1/640	1/10	0	1/2,000
C. T.	Kappa	1/128,000	1/5,120	1/20	0	1/16,000
C. P.	Kappa	1/64,000	1/2,560	1/40	0	1/16,000

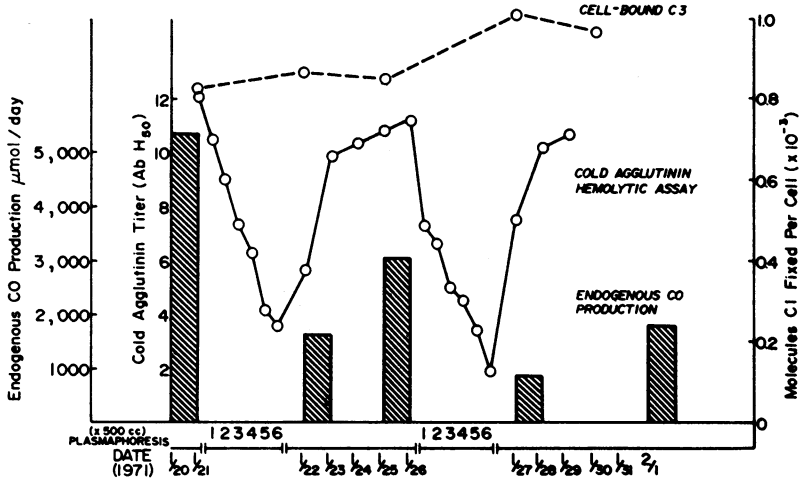

FIGURE 6 Cell-bound C3 and endogenous carbon monoxide production following the rapid reduction in cold agglutinin antibody by large volume plasmapheresis. Cell-bound C3, cold agglutinin hemolytic assay expressed as ability of the antibody to lyse cells from a patient with PNH, and endogenous carbon monoxide production as micromoles CO per day were measured as patient J. C. underwent plasmapheresis of 3,000 ml on two occasions (see text).

the first 24 h without accelerated destruction of the transfused cells.

The effect of acute changes in cell-bound C3 produced by cold. To determine the effect of cold stress on the amount of cell-bound C3 and its relationship to hemolysis, one arm of patient J. C. was immersed in water at 8°C for 1.5 min. At intervals of time, samples were drawn from this arm and from the uncooled arm, and the amount of red cell-bound C3 and the plasma hemoglobin (24) were determined (see Fig. 8). After removing the arm from cold water, both red cell-bound C3 and plasma hemoglobin rapidly increased; these measures did not change in the uncooled arm. After 20 min, the red cell-bound C3 and the plasma hemoglobin in both arms was the same as control values. The effect of cold stress on patient C. T. was tested in the same manner, and qualitatively identical results were obtained (see Table III).

Patient C. P. volunteered to enter a walk-in refrigerator at 8°C for 25 min. During this time, moderate acrocyanosis was noted. Samples of blood from the fore-

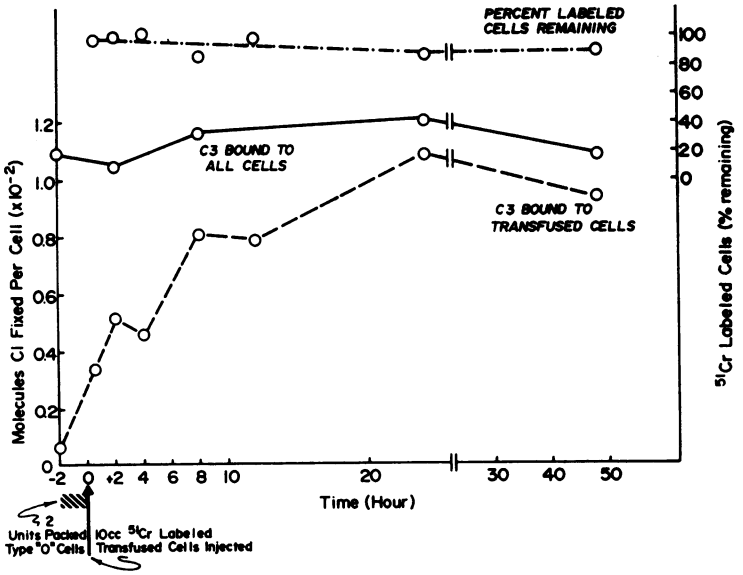

FIGURE 7 Addition of C3 to red cells transfused into a patient with the cold agglutinin syndrome and the survival of the transfused cells. The technique for separating transfused cells is described in the text.

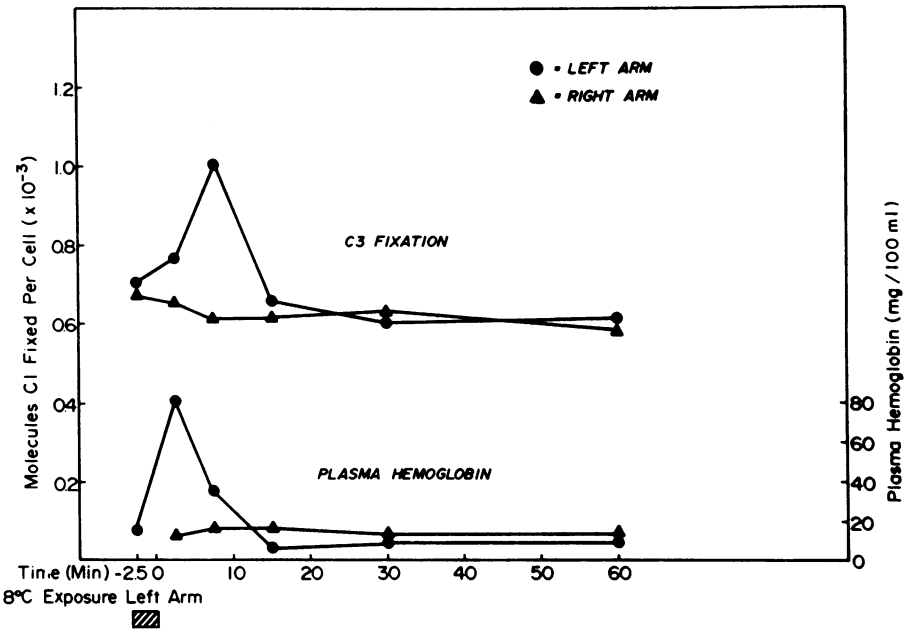

FIGURE 8 The effect of acute cold stress on the cell-bound C3 and plasma hemoglobin in the blood from the arm of a patient with the cold agglutinin syndrome. The patient's left arm was immersed in an 8°C water bath for 2.5 min and venous blood samples obtained from both the right and the left arm were analyzed (see text).

arm were withdrawn at intervals, after rewarming. The rate of hemolysis was estimated by the endogenous CO method (22) immediately after the patient re-entered a warm room. This was compared with the rate of endogenous CO production prior to cold exposure. Cell-bound C3, plasma hemoglobin, and endogenous CO production rose, as shown in Table III, indicating hemolysis associated with rapid accumulation of membrane-bound C3.

DISCUSSION

The role of membrane-bound C3 in the production of hemolytic anemia of patients with the cold agglutinin syndrome is controversial (3, 25). C3 is known to exist on the membrane in hemolytically active and inactive forms. There is some evidence that the antigenic determinants present in the inactive form are different than those present in the active form. This assay measures total membrane-bound C3, including both hemolytically active and inactive forms.

In the absence of cold stress, most of the membrane-bound C3 is apparently hemolytically inactive. The amount of cell-bound C3 detected was unchanged while large variations in cold agglutinin concentration and in hemolytic rate were produced by plasmapheresis (patient J. C.). Since the hemolytic rate was more closely related to the level of antibody than to the amount of cell-bound C3 on surviving cells, the amount of hemolytically active C3 present on the cells is largely determined by the concentration of antibody, but most of the cell-bound C3 is hemolytically inactive.

In the studies measuring the rate of C3 binding to transfused cells, large amounts of C3 accumulated on transfused cells without selective destruction of these cells. This again suggests that most, if not all, of the C3 which accumulated on these cells is in the inactive form. Evans, Turner, and Bingham noted increased hemolysis of transfused cells in patients with the cold agglutinin syndrome (26, 27). Their observations may differ from ours because of variations in the reactions of different cold agglutinins. Such variations will be the subject of a subsequent report.

TABLE III
Effects of Cold Stress

Patient	Red cell-bound C3 (molecules C1/cell)		Plasma hemoglobin		Endogenous CO production	
	Before	After	Before	After	Before	After
			mg/100 ml		*μmol/h*	
J. C.	700	1,020	5.0	82.0	—	—
C. T.	610	940	2.0	44.0	—	—
C. P.	530	1,100	—	—	24.4	107.1

On the other hand, acute cold stress causes rapid increase in detectable membrane-bound C3 and immediate hemolysis. The rate of increase of cell-bound C3 was much greater in the cold-arm experiment than in the transfusion studies, although the total amount of C3 added per red cell was less. The relationship between the rapid accumulation of membrane-bound C3 and immediate hemolysis was also confirmed in the cold stress experiments with patients C. T. and C. P. Red cell destruction correlated with the rate of accumulation of C3 rather than the absolute amount of C3 added in vivo in these studies. Thus, some of the C3 is in a hemolytically active form when added rapidly to the membrane, while that membrane-bound C3 which is detectable without cold stress is almost entirely inactive.

The presence in serum of an inactivator of membrane-bound C3 may account for the dependence of hemolytic activity upon the rate of attachment of C3 to the red cell (28, 29) and for the accumulation of large amounts of "inactive" membrane-bound C3. This inactivator (C3bINA) causes the loss of hemolytic activity and of immune adherence reactivity of membrane-bound C3. C3bINA presumably cleaves the activated C3 molecule to smaller fragments which may have different antigenic determinants (3). These C3 fragments cannot propagate the complement sequence, but are detectable by anti-C3.

The methods used to quantitate red cell destruction in these studies could not differentiate between complement-mediated hemolysis and red cell destruction produced by direct agglutination. Lewis, Dacie, and Szur have suggested that cold agglutinin-induced aggregation does not cause red cell damage which persists after the antibody is disassociated from the cell (6). The thermal amplitude of the antibody might be important in determining red cell destruction, either by aggregation or by complement fixation. The cold agglutinin of patient J. C. appears to have a thermal amplitude similar to that of the cold agglutinins of other patients.

C3 accumulation on red cells in vivo in patients with the cold agglutinin syndrome appears to be the result of a complex series of interactions. Human red cells are resistant to direct lysis by the completion of complement sequences but a large amount of C3 molecules are attached to the membrane (16). This C3 is probably inactivated by C3bINA, resulting in large amounts of "inactive" C3 fragments on circulating cells. In the absence of cold stress, inactivation of membrane-bound C3 occurs before sufficient active C3 accumulates to propagate the complement sequence to a hemolytic conclusion. With acute cold stress, C3 is attached to the membrane rapidly and its inactivation by C3bINA does not proceed quickly enough to prevent propagation of the complement sequence to completion, resulting in hemolysis.

ACKNOWLEDGMENTS

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