A male, 47, developed chronic hemolytic anemia in association with high titers of cold agglutinins in 1966. His symptoms of the cold agglutinin syndrome began in 1951 with cyanosis of the extremities and face after exposure to cold. In 1956, hemoglobinuria was first noted after chilling. In 1962, the cold agglutinin titer was 1:16,000, and his red cells were coated with complement globulins. In 1966, he developed chronic hemolytic anemia and a macroglobulin M component appeared in the serum protein electrophoresis. The titer of cold agglutinins was 1:64,000. The hemolytic anemia responded to chlorambucil with a decrease in cold agglutinin titer and the level of macroglubulin. Serum complement (C) values returned toward normal. Cessation of chlorambucil was followed by relapse. There was a second response to chlorambucil, but reactivation of the hemolytic anemia occurred following a respiratory infection, despite the reduced titer of cold agglutinin. Chlorambucil was discontinued and has not been administered since. There have been remissions and exacerbations of the hemolytic anemia and a return of the macroglubulin and cold agglutinins toward pretreatment levels. During the past 2 yr there has been spontaneous improvement in his symptoms, and the level of macroglubulin has decreased. Despite the appearance of a monoclonal macroglubulin having kappa light chain and with cold agglutinin activity, there is reason to classify his abnormal immunoglobulin production as benign.

CHRONIC HEMOLYTIC ANEMIA with acrocyanosis due to cold agglutinins is a manifestation of macroglubulinemia in which a relatively small increase in the abnormal protein can produce intravascular red cell agglutination leading to stasis and cyanosis in the skin and mucous membranes. Hemoglobinuria from acute red cell destruction may follow episodes of chilling although this is not a uniform occurrence. Finally, some patients develop a chronic hemolytic anemia that persists despite maintenance of a warm environment.

In this report, studies of a middle-aged male with a 20-yr history of symptoms of the cold agglutinin syndrome are presented. The slow progression of his disorder can be attributed to both a gradual increase in cold agglutinin titer and its thermal amplitude. Recent studies of the interaction of cold agglutinins and complement permit some tentative explanations of the course of his hemolytic disease, though obscurities of the mechanism remain. Response

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Submitted June 1, 1972; revised January 10, 1973; accepted February 9, 1973.

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to administration of chlorambucil was observed on two occasions, though exacerbations occurred while he was receiving maintenance therapy.

CASE REPORT

D.P., a 47-yr-old man, was seen twice during 1962 because of cyanosis of face, hands, and feet with exposure to cool air since 1951. On many occasions during the past 6 yr, his urine had been red or coffee colored after episodes of chilling. The acrocyanosis developed in cool atmosphere and cleared completely after warming.

The patient had always had good health. The family history was negative for lymphoma, anemia, and other blood disorders.

He was a robust male without notable physical abnormalities. The lymph nodes were not enlarged, and the liver and spleen were not palpable.

When first seen in 1962, the hematocrit was 43% and reticulocytes were 2%. The red cells were normal in morphology and hemoglobin content. The leukocytes and platelets were also normal. Two urinalyses were normal, and hemosiderin granules were not demonstrated.

The titer of cold agglutinins was 1:16,000 with group 0, 1 positive red cells at 5°C and 1:120 with group 0, 1 positive cord red cells. Serum complement (C) values were 50%, of normal. The patient’s red cells washed at 37°C were agglutinated by antisera with specificity for βE (C4) and βC1.C3 (C3): (1:5000–1:10,000).

Three years later, in January 1965, no change in his symptomatology or laboratory values had occurred, except for a further decrease in serum C levels. Serum electrophoresis was normal.

Fourteen months later, in March 1966, he reported that easy fatigue had been present for 3 mo. He was pale, and the spleen was now palpable 4 cm below the costal margin. The hematocrit was 29%, reticulocytes were 15%, and there was significant spherocytosis of the red cells. Hemolysis in hypotonic solution began in 0.65% of sodium chloride. Leukocytes and platelets were normal. There was normoblastic hyperplasia, but no evidence of lymphoid or plasma cell increase in bone marrow aspiration smears. Serum electrophoresis had an M-component band in the fast gamma region. The cold agglutinin titer was 1:64,000. Titration of serum C showed no CH50 units on repeated examinations. The patient’s serum was not anticomplementary.

He was given 6 mg of chlorambucil in a single dose daily. The titer of cold agglutinins, serum complement values, and the hematocrit and reticulocyte counts during the ensuing 30 mo are presented in Fig. 1. The hematocrit remained stationary for 2 wk and then rose to normal levels during the next 6 wk, while reticulocytes decreased to 2.5%. At the same time, there was a gradual fall in the titer of cold agglutinins. After 3 mo of chlorambucil medication, the hematocrit was 42%, but the patient’s red cells continued to be agglutinated by anti-C serum in high dilution. There was a decrease in acrocyanosis. The chlorambucil was discontinued, and during the next
6 mo there was a gradual fall in hematocrit to 28%, and a rise in reticulocytes to 8%. The cold agglutinin titer had increased, and serum C was again not measurable.

Chlorambucil was again given in the same dose. The patient entered a second remission with a rise in hematocrit. Cold agglutinin titers decreased, and serum C again became detectable. The patient was again free of symptoms. Chlorambucil was reduced to 4 mg daily. During the ensuing 6 mo there was a decrease in hematocrit to 35%, and an increase in reticulocytes to 7%. During an acute upper respiratory tract infection in February 1968, there was rapid fall in the hematocrit to 25%. Reticulocytes were 10%, and the leukocyte and differential counts were normal. The chlorambucil was then discontinued at the patient’s request. The hematocrit rose to 35%, as the respiratory infection cleared. A second episode of respiratory infection with bronchitis developed 3 mo later and was again associated with a fall in hematocrit and a disappearance of detectable serum C.

Since March 1968, chlorambucil has not been reinstituted. Hematocrit levels have generally varied between 30% and 40%, but there have been three periods of several weeks in which the hematocrit has dropped to the level of 18%–21%. These episodes were associated with respiratory infections and were apparently due to increased destruction rather than to decreased formation of red cells, since reticulocyte counts have remained elevated. The cold agglutinin titer varied between 1:16,000 and 1:32,000, and the serum IgM increased gradually to 900 mg/100 ml 6 mo after discontinuing chlorambucil.

During the past 3 yr, the patient reports general improvement in symptoms with less acrocyanosis and greater endurance. A determination of macroglobulin in December 1970 was 300 mg/100 ml; the cold agglutinin titer was 1:4000, the hematocrit was 40%, and reticulocytes were 6%. The direct antiglobulin reaction was unchanged. Since then the patient has refused additional determination.

MATERIALS AND METHODS

The method of titration of the cold agglutinins and the serum complement levels has been described, as well as the methods used in preparation of the antiserums for C globulins, C4 (β1E) and C3 (βC-1A).2

The hemolytic and complement-fixing activity of the cold agglutinin with group 0, 1 positive red cells of a normal subject was determined as follows: One half milliliter of the patient's serum was mixed with 0.5 ml of normal serum. The pH was adjusted to 7.2 or 6.8 by the addition of 0.1 N HCl. After all reagents had been equilibrated to temperature of 37°C, 0.25 ml of a 20% suspension of normal red cells was added to 1.0 ml of the serum mixture and incubated for 30 min at the desired temperature. The red blood cells were sedimented at the temperature of incubation, and the hemoglobin concentration in the supernatant was compared with a control with 100% hemolysis. The residual cells were washed three times at 37°C and were tested for agglutination in the anti-C globulin serum. The susceptibility of the patient's red cells to hemolysis by cold agglutinin was compared to normal red cells. The remaining red cells were tested for coating by C components with anti-C serum.

Two milliliters of serum were fractionated on Sephadex G-200 using Tris-HCl buffer at pH 8. Fractions were tested for cold agglutinin activity with group 0, 1 positive red cells. Fractions were also tested by cellulose acetate electrophoresis, Ouchterlony analysis, and immunoelectrophoresis. The concentration of IgM was determined by radial diffusion (Hyland Laboratories). The method described by Lo Buglio et al. was used to demonstrate the formation of rosettes with the patient's washed red cells and leukocytes of normal subjects.3

The patient's red cells were tested with the complement-fixing antibody consumption test to demonstrate the presence or absence of low concentrations of gamma G globulin insufficient to produce agglutination by antigamma G antibodies.4 Typing of light chains of the cold agglutinin was carried out originally by Dr. Harboe.5 Cold agglutinins from later bleedings were typed for light chains in our laboratory.

For detection of Bence Jones proteinuria, 24-hr urine specimen was thoroughly dialyzed against distilled water and then lyophilized. The lyophilized urine was dissolved in water at a concentration of 100 mg/ml and then examined for Bence Jones protein by electrophoresis and immunoelectrophoresis. Serum hemolytic C determinations were done by a modification of the method of Kabat and Mayer,6 in which normal values ranged from 80 to 160 CH50 U/ml.
Cr tagging was used to observe the survival of autologous and homologous red cells in the patient and the survival of complement-coated red cells of the patient in a normal subject.

RESULTS

The cold agglutinin activity was found in the first peak of the Sephadex G-200 separation. This material produced a homogeneous spike on protein electrophoresis. Ouchterlony analysis of isolated cold agglutinin prepared by repeated adsorption and elution from red cells showed reaction with anti-\(\kappa\) antibody but not with anti-\(\lambda\). Bence Jones protein could not be demonstrated in urine that had been highly concentrated.

To determine what per cent of the patient’s serum IgM was participating as cold agglutinins, an aliquot of serum was absorbed repeatedly with stroma prepared from group 0, 1 positive red cells. The concentration of IgM was measured by radial diffusion, and the cold agglutinin titer was determined following each absorption. Seven absorptions were necessary for complete removal of cold agglutinin activity. Cold agglutinin activity represented 84% of the patient’s serum IgM at this particular bleeding (Table 1). Repeat studies using a control serum with 2 g of macroglobulin, but without cold agglutinin activity, showed less than 20% nonspecific absorption of macroglobulin.

The patient’s washed red cells were agglutinated by anticomplement serums throughout the period of study. His red cells were relatively resistant to C hemolysis by cold agglutinins when compared to normal red cells.2 There was less than 5% hemolysis of the patient’s red cells, as compared to 20%-50% hemolysis of normal red cells under the same conditions.

The red cells of the patient washed at 37°C formed rosettes with monocytes and polymorphonuclear leukocytes from a normal donor when incubated at 25°C. The adherence of red cells to leukocytes appeared to be due to C components, since the amount of \(\gamma\) G globulin detectable on the red cell surface was not increased above that found on normal red cells.4 Both polymorphonuclear neutrophils and mononuclear cells formed rosettes with the complement-coated red cells of the patient.

Survival of the patient’s \(^{51}\)Cr-tagged red cells in his own circulation was observed during treatment with chlorambucil. The \(t_{1/2}\) survival was 11 days at the beginning of chlorambucil treatment and 15 days when he was in partial remission 3 mo later. The \(t_{1/2}\) survival of normal group 0, 1 positive cells in the patient’s circulation was 13 days. The initial phase of rapid destruction of normal

<table>
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<th>No of Absorptions</th>
<th>(\gamma)M (mg/100 ml)</th>
<th>Reciprocal of Cold Agglutinin Titer</th>
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<tr>
<td>Before</td>
<td>650</td>
<td>25,800</td>
</tr>
<tr>
<td>1</td>
<td>510</td>
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<td>7</td>
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*Three milliliters of serum were absorbed with stroma from 2 ml of red blood cells.
cells seen in other patients with chronic hemolytic anemia due to cold agglutinins was not observed. The patient's red cells injected into a normal subject showed a biphasic curve with an initial period of rapid removal of 30% during the first 90 min. The remaining red cells were removed at a gradual rate with a \( t_\frac{1}{2} \) of 24 days.

**DISCUSSION**

The history of acrocyanosis beginning in 1951 indicates that abnormal levels of cold agglutinin had been present at least 15 yr prior to the onset of chronic hemolytic anemia. The titer of cold agglutinins had increased until detectable M component appeared in the serum protein electrophoresis, and his serum produced complement coating of normal red cells at 37°C. The patient's own red cells were heavily coated with C components and were relatively resistant to C hemolysis through the action of cold agglutinin. This abnormal resistance has been shown by in vitro studies to be due to the accumulation of complement components (primarily C3) on the red cells, a process that takes place at temperatures above that required for agglutination. Further studies demonstrated that the fixation of C globulins to circulating red cells is a continuing process due to repeated reactions between the cold agglutinin and red cells at body temperature.

Following the onset of chronic hemolytic anemia, the patient noted no further episodes of hemoglobinuria. This change may have been the result of depletion of serum C, as well as the acquired resistance of his circulating cells to complement hemolysis. He is unique in our group of patients with this syndrome in having an absence of measurable total serum hemolytic complement, rather than a partial depletion when the hemolytic disease was active. The lack of serum complement is the probable explanation for the absence of an initial period of rapid destruction when normal red cells susceptible to C hemolysis in vitro were introduced into his circulation. It is reasonable to postulate that complement was consumed as it was released by a steady deposition on the surface of circulating red cells. Transfused red cells, as the patient's own red cells, acquired C components gradually, and the acute form of membrane damage that results in rapid removal from the circulation did not occur.

The significance of the coating of red cells with the C components by cold agglutinin as a cause of red cell destruction has been enhanced by observations that 30% of \( ^{51}\text{Cr} \)-tagged red cells of this patient were removed rapidly from the circulation of a normal subject. Furthermore, it was shown that his red cells formed rosettes with both macrophages and polymorphonuclear leukocytes similar to those seen in patients whose red cells are coated with IgG. The existence of receptor sites for C3 and C4 on human monocytes and polymorphonuclear leukocytes has been reported. Others have failed to confirm the existence of such sites.

The concept that the C components on the surface of the circulating red cells in chronic hemolytic anemia due to cold agglutinins are \( \beta_{1E} \) and \( \beta_{1C} \) globulins (C4 and C3) has been challenged by Engelfriet et al. They presented evidence that \( \alpha_{2D} \) globulin (C3d), an enzymatic cleavage product of C3, is the only C fragment that remains on the red cells in vivo. Regardless of the nature of the
adherent complement fraction or fractions, the destruction of red cells in the chronic form of the disease is usually accompanied by some degree of spherocytosis, suggesting loss of membrane as a result of surface interaction with phagocytic cells as is postulated to occur with IgG antibodies. The formation of rosettes by this patient's red cells and leukocytes is interpreted as evidence for biologically active C components, probably C3b, on the surface of the circulating red cells.

Chronic hemolytic anemia due to cold agglutinins is an uncommon disorder, and so far, there are few descriptions of treatment by measures other than maintaining a warm environment. Unlike hemolytic anemia due to warm-reacting autoantibodies of the IgG variety, hemolytic anemia due to cold agglutinins appears to be resistant to steroids. Splenectomy may reduce the hemolytic activity in some patients, but its value as a general measure has not been established. Penicillamine and other mercaptanes inactivate cold agglutinin in vitro by splitting disulfide bonds of the IgM molecule. The value of these agents in the treatment of chronic hemolytic anemia due to cold agglutinins has not been established during the decade since the initial report of favorable response in two patients.

Several reports attesting value of long-term treatment of macroglobulinemia with alkylating agents, particularly chlorambucil, have appeared. Administration of these agents has caused a decrease in the M component and an improvement in anemia along with a decrease in the number of lymphoid cells in the marrow. Olesen has reported treatment of the cold agglutinin syndrome with chlorambucil. The concentration of M component was reduced, and the titer of cold agglutinins was decreased. Dacie and Worlledge have noted satisfactory improvement in three of nine patients given chlorambucil therapy, while three others had some benefit. Our attempts with alkylating agents in two other patients were not successful, since bone marrow depression occurred prior to any evidence of improvement in the hemolytic process.

Administration of chlorambucil to the patient reported above was followed by significant reduction in levels of IgM and in the titer of cold agglutinins. The hematocrit rose, and reticulocytes decreased. Total serum complement rose from undetectable levels to approximately 50% of normal during each remission. Reticulocytes were always elevated to 2%–3%, even when the hematocrit was within the range of normal. The 51Cr survival of 15 days during the partial remission was further evidence that the rate of red cell destruction had not returned to normal. The hemolytic anemia recurred 3 mo after chlorambucil was stopped, as the cold agglutinin titer returned to pretreatment levels. An exacerbation of equal severity occurred while chlorambucil was being administered in February 1968, following a respiratory infection when the cold agglutinin titer was at a relatively low level (1:2000). Reactivation of the hemolytic anemia occurred again 4 mo later in June 1968, following another respiratory infection when cold agglutinin titers were still relatively low. Such exacerbations may be related to an increase in complement formation and activation, as well as greater activity by phagocytic cells. Both factors are probably dependent on the thermal amplitude of the cold agglutinin, including complement deposition on red cells at normal body temperature. It is signifi-
cant that, despite the depression of the cold agglutinin level by chlorambucil, complement components were still bound to normal red cells at 31 and 37°C. Furthermore, the patient’s red cells had not lost resistance to hemolysis by sera with high titer of cold agglutinin as compared to normal cells.

The cold agglutinin isolated from our patient was a homogeneous immunoglobulin IgM with kappa light chains only. The M component observed on cellulose acetate electrophoresis appears to have been nearly all cold agglutinin, since it disappeared with repeated absorption of the serum with red cell stroma in the cold. Measurement of the patient’s serum IgM before and after removal of all cold agglutinin activity showed an 84% reduction of IgM. The identity of the M component on electrophoresis with cold agglutinin activity has been well documented by others.19 Similarly, antibody activity has been observed with other monoclonal immunoglobulins, including anti-γ G globulin (rheumatoid factor), antistreptolysin 0, and antilipoprotein activities.20

While the cold agglutinin activity of our patient’s M component led to symptoms, his monoclonal gammopathy can be classed as benign. Serum concentrations of IgG and IgA have remained normal, and bone marrow function has not been depressed. The failure to detect Bence Jones protein in a concentrated 24-hr urine sample has also been considered evidence for benign rather than malignant disease.21 The nature of the stimulus responsible for the production of the abnormal protein and the homeostatic mechanism limiting its production are unknown at present but pose important questions for future investigation. Finally, there is evidence that the M component decreased spontaneously during the 2 yr that followed the cessation of the cytotoxic drug.

REFERENCES

Chronic Hemolytic Anemia Due to Cold Agglutinins: A 20-Year History of Benign Gammopathy With Response to Chlorambucil

Robert S. Evans, Elizabeth Baxter and Bruce C. Gilliland