PHYSICAL PROPERTIES OF THE RED CELL AGGLUTININS IN ACQUIRED HEMOLYTIC ANEMIA

By H. H. FUDENBERG, M.D., and H. G. KUNKEL, M.D.

(From The Rockefeller Institute for Medical Research and the Department of Medicine, The Mount Sinai Hospital, New York)

(Received for publication, July 17, 1957)

Previous observations (1, 2) have demonstrated that human γ-globulin can readily be divided into 7 S and 19 S ultracentrifugal components which differ in chemical (2, 3) and immunological (4, 5) properties. Evidence has accumulated that a number of antibodies are associated with the 19 S fraction in normal and pathological animal (6) and human (6, 4) sera. As part of a broad study of these antibodies the present report is concerned primarily with the cold agglutinins found in patients with the syndrome of cold hemagglutination, chronic hemolytic anemia, and paroxysmal cold hemoglobinuria. The possibility that these cold agglutinins are high molecular weight proteins was raised by Gordon (7), who studied a purified preparation of cold agglutinins obtained from the serum of a patient with the cold hemagglutination syndrome by adsorption upon, and elution from, washed red cell stromata. The eluted protein showed a sedimentation coefficient of 18 S. The present study deals with the results obtained in an analysis of the sera of 12 patients with acquired hemolytic anemia, (8 with high titer cold agglutinins and 4 with warm incomplete antibodies). Cold agglutinins in all instances were found to be high molecular weight proteins of the 19 S class, in contrast to the warm variety of hemolytic anemia antibodies which had sedimentation coefficients of approximately 7 S.

Methods and Materials

Serologic Methods.—Titrations of serum and serum fractions were performed at 4 and 37°C. against 2 per cent suspensions of washed, pooled ORh_0^+ erythrocytes. Parallel titrations were performed using saline suspensions of trypsin-treated and of untreated erythrocytes. Titrations employing the indirect antiglobulin (Coombs) method were performed at 37°C. only. Separate pipettes were used for each dilution to prevent carry-over.

Appropriate serologic tests, using a panel of red cells of varying antigenic composition,[1] were performed with each serum to exclude the possibility of specific isoagglutinins toward a given blood group antigen.

All titrations utilizing isolated serum fractions obtained by zone electrophoresis or zone ultracentrifugation of a given serum were performed in parallel.

[1] Containing the antigens Rh_0, rh′, rh″, V, f, K, k, Fy^a, Le^a, Le^b, Jk^a, Jk^b, M, N, S, s, and P in various combinations.

Patient Material and Serum Titers.—A list of the 12 patients with the established diagnosis of acquired hemolytic anemia whose sera were examined in this study is shown in Table I. The majority were hospitalized in the New York area. Patient S. Y. was studied in detail over a 3 month period. Most of the sera were obtained 1 to 3 days prior to testing. Whole blood was drawn into warm syringes, allowed to clot at 37°C. for 1 to 2 hours, and the serum removed and frozen until tested. The sera of patients O. L., E. B., and T. R. were obtained 3 to 4 days prior to testing and shipped from Sweden, Milwaukee, and Boston, respectively. Prior to shipping 0.1 per cent sodium azide was added to these sera as a preservative. The sera of patients S. P. and A. S. had been stored in the deep freeze for periods of 1 and 3 years, respectively.

TABLE I

List of Patients Employed in This Study and Titers of Their Sera at the Time of Investigation

Name	Sex	Age	Cold agglutination titer		Duration of disease
			Trypsin	Saline	
1. A. S. (8)	F	62	160,000	40,000	5 yrs.
2. S. Y. (9, 10)	M	77	32,000	8,000	16 yrs.
3. S. P. (11)	F	70	32,000	8,000	6 mos.
4. O. L.	M	—	8,000	4,000	—
5. S. D.	F	65	8,000	2,000	5 yrs.
6. R. S.	F	75	2,560	1,280	3 yrs.
7. E. B. (12)	F	67	2,000	256	1 yr.
8. T. R.	M	—	1,024	512	—
			Warm titer (Coombs)		
1. Eds	M	11	128		3 mos.
2. M. T.	M	38	128		—
3. Hill	M	—	64		—
4. L. R.	F	26	32		—

Titer of the "cold hemolytic anemia" sera at the time of testing here ranged from 1,000 to 160,000 by the trypsin method, as is indicated in Table I. Saline agglutinin titers averaged 2 tubes lower (range: 1 to 4 tubes lower) each tube representing a two-fold dilution.

The sera of 11 patients with severe "warm hemolytic anemia" were screened for serum antibody. Only patients with severe anemia and 4+ direct antiglobulin (Coombs) reactions were examined. Only 4 of the 11 patients screened had serum antiglobulin titers greater than 32.

Typical Case Report.—S. Y., a 78 year old Negro male, moved to New York City from Virginia at age 45. For the next 5 years he worked in an aluminum refinery where he was exposed to molten aluminum and intense heat. At the end of this time he began to note burning and numbness of his fingers and toes on a 10 to 15 minute exposure to cold weather with rapid subsidence on return to a warm room. Such episodes were often followed by sharp epigastric pain and later by passage of coffee-colored urine.

In January, 1942, he was exposed to a temperature of about 5°F. for 1 hour and the usual symptoms followed. However, on this occasion burning and pain persisted for several weeks, unimproved by environmental warmth. Dry gangrene of the toes and fingertips followed over the next 4 to 5 weeks, with slough of the gangrenous areas. Studies performed

during hospitalization at Harlem Hospital revealed a normal hemoglobin (14.5 gm. per cent) and a saline cold agglutinin titer of 3,000 to 30,000. Immersion of one arm in a 4°C. water bath for 20 minutes resulted in marked intravascular hemolysis, with plasma hemoglobin values rising to 500 mg. per cent in blood drawn from the chilled arm. No elevation of plasma hemoglobin was present in blood obtained from the contralateral arm, kept in a water bath at room temperature during the immersion period. A paroxysm of hemoglobinuria followed.

After 3 months' hospitalization, the patient was discharged with instructions to avoid exposure to cold. He subsequently remained indoors during winter months and remained well until 1945, when he first noted symptoms of mild congestive failure. In 1951 he was admitted to the Mt. Sinai Hospital for acute pulmonary edema, and responded well to digitalis and mercurials during a 10 day hospitalization. Hematologic studies other than routine blood count (hemoglobin 12.0 gm.) were not performed.

The patient was readmitted to the Mt. Sinai Hospital in July, 1956, because of refractory congestive failure. The patient responded slowly to the usual cardiac regime, with a weight loss of 45 pounds over a 3 month period. Despite loss of overt edema and return of circulation time and venous pressure to normal levels, the liver remained palpable (4 cm. below the right costal margin). Small cervical and axillary lymph nodes were also palpable, and a peripheral lymphocytosis was present. Erythrocyte sedimentation rate (Westergren) was 128 mm. in 1 hour. Hemoglobin was 9.7 gm. per cent and cold agglutinin titer was 8,000 with untreated cells and 32,000 with enzyme-treated cells. Peripheral blood films demonstrated 5 to 8 per cent reticulocytosis, and moderate aniso- and poikilocytosis and polychromatophilia. Stool guaiac determinations were persistently positive despite absence of demonstrable lesions on repeated gastrointestinal tract x-ray studies. Bone marrow aspirations revealed hypercellular erythroid marrows, with a marked increase in cells morphologically intermediate between plasma cells and lymphocytes. These cells, suggestive of those seen in the macroglobulinemia of Waldenström totalled 80 per cent of the marrow white cell forms.

In November, 1956, the patient was transferred to a hospital for chronic disease. He died suddenly 3 weeks later of a pulmonary infarct. Permission for postmortem examination was not obtained.

Summary.—A 78 year old Negro male with high titer cold hemagglutination, Raynaud's phenomenon, chronic hemolytic anemia, and paroxysmal hemoglobinuria for at least 15 years, developed refractory congestive failure, mucous membrane bleeding, and enlarged liver, greatly accelerated erythrocyte sedimentation rate, and a bone marrow picture resembling that seen in some cases of Waldenström's macroglobulinemia.

Elution of Cold Agglutinins.—Red cell stromata were prepared from 100 ml. of washed, packed erythrocytes, utilizing the method of Gordon (7). 6 cc. of packed stromata was obtained and divided into two equal portions. Each portion was incubated with a twofold volume of a 1 per cent solution of trypsin for 15 minutes at 37°C., then washed twice with normal saline.

0.6 ml. of serum with high titer cold antibodies (1/32,000) was diluted to 6.0 ml. with normal saline. The diluted serum was absorbed with 3 ml. of the stromata preparation for 6 hours at 4°C. The mixture was then centrifuged at 4°C., the supernatant removed, and again absorbed with the second aliquot of the stromata preparation, under the same conditions.

After centrifugation and removal of the supernatant from the second absorption, the two aliquots of stromata preparation were pooled. The stromata were then washed 4 times with cold normal saline, centrifugation and washing being carried out in the cold room. After 4 washings an equal volume of saline was added and the stromata-saline mixture incubated at 37°C. After 1 hour, the mixture was centrifuged and the supernatant eluate removed.

Ultracentrifugation and Electrophoresis.—Zone ultracentrifugation in a sucrose density

gradient was performed in a Spinco model L centrifuge. The technique finally adopted was a modification of the procedure employed by Brakke (13) for virus work. A swinging bucket rotor was used and the plastic containers filled with 4 cc. of sucrose in isotonic saline, with the sucrose concentration ranging from 12 per cent at the top of the tube to 37 per cent at the bottom. (A gradient was formed of 3 sucrose layers by the combination of slight stirring with a platinum wire loop and diffusion for 24 hours.) A special effort was made to form the gradient in the same manner in each experiment. Some experiments were carried out in very homogeneous gradients made with a special mixing device. Similar results were obtained by the 2 procedures. Serum, either whole or diluted with an equal volume of saline, was layered in a volume of ½ cc. over the sucrose gradient. Droplet formation at the interphase (13) was minimized by working entirely at 4°C., by stirring this region with the platinum loop, and by starting the ultracentrifugation very rapidly after applying the serum. Centrifugation was usually carried out for approximately 15 hours at 35,000 R.P.M. Several bottom fractions were analyzed in the analytical ultracentrifuge to test the separatory capacity of the procedure. This was somewhat difficult because of the large amount of material necessary for an analytical pattern, but the samples examined showed more than 90 per cent material with sedimentation coefficients of 19 S or greater. A small amount of brom-phenol blue added to the serum served to localize the albumin. Samples were taken relative to the albumin position at different levels in the centrifuge tube. This was usually done with a mounted capillary tube although a tube slicer was used at times. Diluted serum gave better results than whole serum and was always used when antibody titers were high. However, some results could be obtained with whole serum.

Antiserum against 7 S γ-globulin was prepared as previously described (4). This reacts weakly with 19 S γ-globulin but this did not interfere because of the usual relatively low concentration of 19 S material. Antiserum to 19 S γ-globulin was absorbed with 7 S material and was specific for the 19 S fraction. Semiquantitative determinations of antigen were carried out by serial dilution of antigen followed by layering in capillary tubes with antiserum.

Analytical ultracentrifugation was carried out in the Spinco model E centrifuge. Zone electrophoresis was performed at pH 8.6 by the starch procedure previously described (1).

RESULTS

Electrophoretic Distribution.—

The cold hemagglutinins from the sera of different patients showed considerable variation in mobility. In most instances the peak of activity appeared in the fast migrating portion of the γ-globulin (Fig. 1 *a*). In two cases the mobility was even faster and the peak fell into the β-globulin region. The activity was found directly under the γ-globulin peak in only one instance. Repeated analyses of the same or of different samples of serum from a single patient showed that the characteristic mobility was a distinct property of the individual. In one instance an early specimen obtained 2 years previously and stored in the deep freeze was compared directly with a fresh serum and identical fast mobilities were obtained for the hemagglutinating activity.

In 4 of the 8 cases, the protein responsible for the biological activity was present in sufficient concentration to cause a visible abnormal peak in the serum protein pattern. Fig. 1 *b* illustrates the pattern of the serum of patient A. S. with the highest titer of cold agglutinins (1/160,000). All of the activity was found associated with a large protein peak in the γ-globulin region. Fig. 2 illustrates the pattern of the

serum of a second patient (S. Y.) where an abnormal peak was found in the β-globulin region. In this experiment approximately 90 per cent of the original activity was recovered from the pooled eluate of segments 10, 11, and 12.

Fig. 3 *a* shows the pattern obtained with another serum (S. P.) with a very high titer of cold agglutinins and an abnormal peak in the fast γ-region. Following repeated absorptions with red cell stromata in the cold, the abnormal peak disappeared

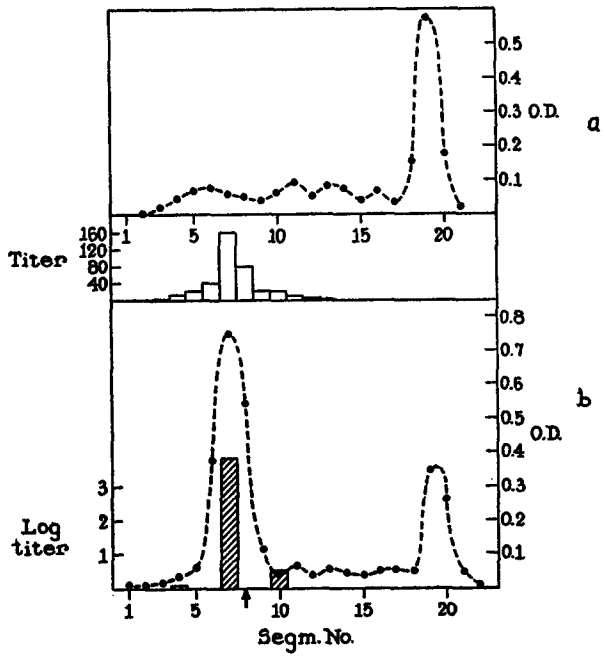

FIG. 1. Curves obtained by protein analyses and the cold agglutinin titer of the electrophoretic fractions of two sera of patients with increased cold agglutinins and hemolytic anemia. The top pattern of T. R. with relatively low titers shows a normal protein curve. The bottom (A. S.) shows a huge abnormal peak associated with high titer of cold agglutinins (trypsin method).

(Fig. 3 *b*). The position of the red hemoglobin added with the stromata is indicated. Fig. 3 *c* illustrates the pattern of the red cell eluate after dissociating the agglutinins from the red cell stromata preparation by raising the temperature to 37°C. The agglutinins were eluted into a volume of saline equal to that of the serum originally used, so that the three curves in Fig. 3 are strictly comparable on a quantitative basis. The amount of protein lost in the abnormal peak after absorption is close to that found in the similarly migrating peak of the eluted protein. The titer of the eluate corresponded closely to that lost from the serum by absorption.

Fig. 4 shows the pattern of the serum of S. D. another patient with this syndrome. Here an abnormal peak was observed which did not have cold agglutinin activity. This lack of association of the abnormal peak with the protein responsible for the

agglutinin activity was suspected because the titer of this serum was not sufficiently high to be associated with such a large component. The activity showed a clearly faster mobility and was associated with a minor peak. The nature of the elevated γ-globulin in this serum was not established. Ultracentrifugal analysis demonstrated that it had a sedimentation coefficient of 7 S and thus differed from the abnormal component of the other sera which showed a much higher s rate. This serum illustrates one of the pitfalls that may be encountered, if the protein fractions responsible for such abnormal peaks are not isolated. Examination of the fractions themselves rather than the whole serum is essential. An unusual electrophoretic component

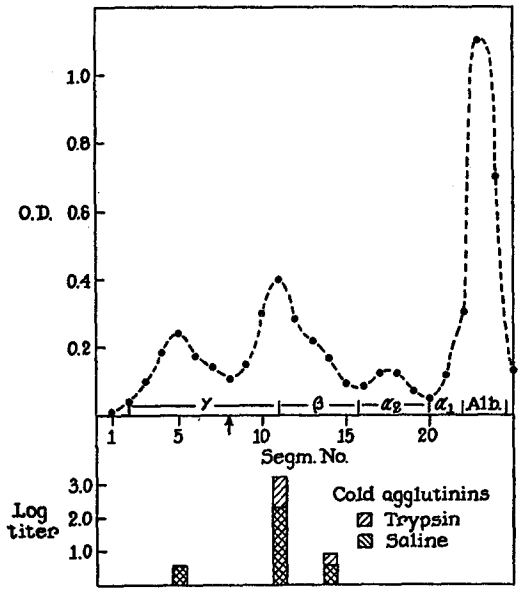

FIG. 2. Electrophoretic pattern of the serum of patient S. Y. showing an abnormal peak of relatively fast mobility associated with a high titer of cold agglutinins.

and a high antibody titer may be completely independent and the identification of a relationship between the two requires the accumulation of several lines of evidence.

The sera of 11 patients with severe "warm antibody" acquired hemolytic anemia and strongly positive (4+) direct antiglobulin (Coombs) reactions were screened for serum antibody. Of the 11 selected patients, only 4 had warm indirect antiglobulin titers of 32 or greater. No abnormal components related to the antibody were observed. The mobility of the biological activity was measured in 3 of these sera and in general corresponded with the central peak of the γ-globulin component. The fast mobility observed for many of the cold agglutinins was not encountered.

Ultracentrifugal Studies.—

All the 8 sera with high titer cold agglutinins were analyzed by density gradient ultracentrifugation in order to localize the biological activity in the 7 S fraction or in a higher molecular weight fraction corresponding to 19 S or greater. The 7 S γ-glob-

ulin usually sedimented approximately one-third of the way down the tube in these experiments and could be readily determined by means of an antiserum against 7 S γ-globulin. The 19 S material sedimented to the bottom of the tube and was localized by an antiserum specific for 19 S γ-globulin. It was also identified in some experiments by direct measurement of the sedimentation rate in the analytical ultracentrifuge. Specimens completely free of material lower than 19 S could be obtained.

Table II shows the results of one experiment on the serum of patient S. P. The large amounts of 19 S material in this serum caused a clear peak of protein in Fr. V. This fraction, reacting specifically with 19 S antiserum, contained most of the cold

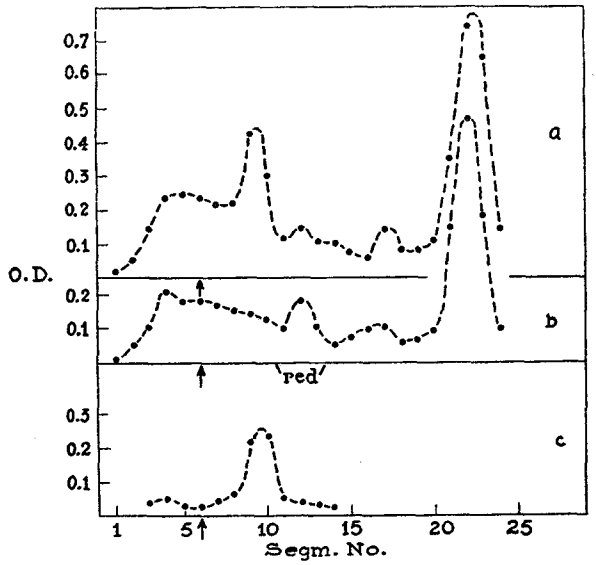

FIG. 3. Electrophoretic patterns of serum of patient S. P. before and after removal of the abnormal component by absorption with red cell stromata in the cold. The curve for the protein eluted from the stromata is shown at the bottom.

agglutinins with some appearing in Fr. IV. However, Fr. III which contained most of the 7 S γ-globulin was completely devoid of agglutinin activity. Table III shows similar results with two other sera containing increased cold agglutinins. The reaction with 7 S and 19 S antisera was carried out on the fractions of serum of E. B. Since almost identical results were obtained for the sera of patients S. Y. and Eds. these are included in the same table. The cold agglutinin activity resided primarily in the bottom fractions. The warm incomplete Coombs type antibody in the serum of patient Eds. was found in the upper fractions with the peak of 7 S γ-globulin and no activity was present in Fr. V. Thus the two types of agglutinins fell into completely different fractions. The use of the two types in individual experiments repeatedly brought out their contrasting sedimentation rates. Similar results were obtained with the other 5 sera containing cold agglutinins and the other 3 sera containing the warm type antibodies listed in Table I.

The serum of 1 patient with atypical pneumonia who had an elevated cold agglutinin titer without hemolytic anemia was separated in a similar fashion. Here again the agglutinin activity was localized clearly in the bottom or 19 S fraction.

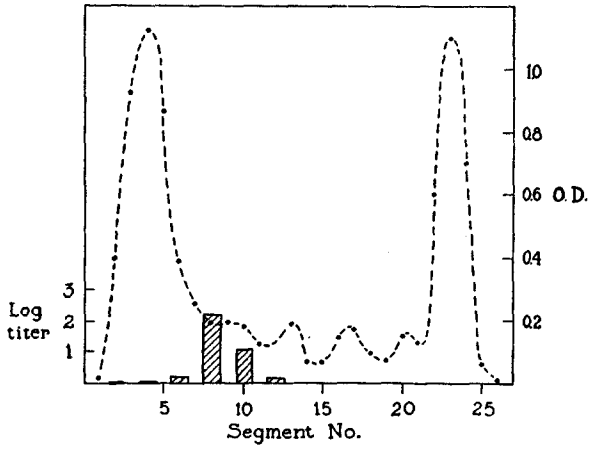

FIG. 4. Electrophoretic pattern of serum of S. D. showing a huge abnormal peak not associated with cold agglutinin activity.

TABLE II

Distribution of Total Protein, 7 S and 19 S γ-Globulin, and Cold Agglutinins in Various Fractions of Serum S.P. Separated by Density Gradient Zone Centrifugation

Fraction	Protein concentration	Reaction with 7 S As.	Reaction with 19 S As.	Cold agglutinin titer	
				Saline	Trypsin
	mg./cc.				
Top I	0.5	Tr.	0	0	0
Albumin II	6.1	2+	0	0	0
III	6.2	4+	0	0	0
IV	0.5	+	+	1/100	1/400
Bottom V	1.5	+	4+	1/1000	1/2000

Analytical ultracentrifugation was also carried out on the electrophoretic fractions which showed a high titer of cold agglutinins. Fig. 5 A illustrates the pattern of tube 7 of the fractionatior of the serum of A. S. shown in Fig. 1. Fig. 5 B shows the pattern of the pool of tubes 10, 11, and 12 of the fractionation of serum S. Y. shown in Fig. 2. In each case the primary component showed a sedimentation coefficient of approximately 19 S.[2] Heavier substances were also observed as is usually the case when a large 19 S fraction is present (14). No 7 S material could be observed in the peak

[2] The observed s rates for the 3 components shown in Fig. 5 B were 6.19, 16.5, and 24.5 respectively. Correction for concentration dependence and for the electrolyte used (1) gave $s^0_{20,w}$ = 18.7 S for the major middle component.

TABLE III

Comparison of Distribution of Cold Agglutinins from Sera of Patients E. B. and S. Y. with Warm Incomplete Antibodies from Patient E. L. in Various Fractions Separated by Density Gradient Zone Centrifugation

Fraction	Reaction with 7 S As.	Reaction with 19 S As.	Cold agglutination titer Patient E.B.			Cold agglutination titer Patient S.Y.			Warm incomplete antibody titer Patient Eds.		
			1/10	1/100	1/1000	1/10	1/80	1/160	1/1	1/5	1/10
Albumin II	2+	0	0	0	0	0	0	0	+	0	0
III	4+	0	0	0	0	1+	0	0	3+	2+	1+
IV	1+	Tr.	1+	0	0	2+	1+	0	2+	0	0
Bottom V	1+	3+	4+	3+	1+	4+	3+	2+	0	0	0

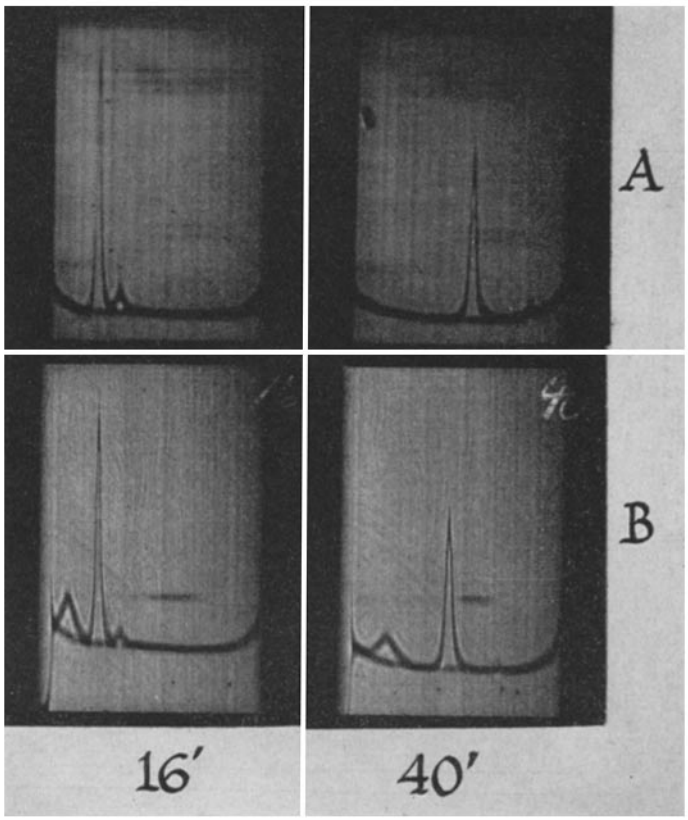

FIG. 5. Ultracentrifuge patterns of the active electrophoretic fractions of the sera of patients A. S. and S. Y. Photographs taken at 16 and 40 minutes. The major peak is of the 19 S type in each case.

tube of the serum of patient A. S., and the electrophoretic peak represented almost entirely high molecular weight materials. The fraction shown in Fig. 5 B shows 26 per cent 7 S material. This was expected because this serum showed a relatively small abnormal peak by electrophoresis and the latter must have contained some normal 7 S γ-globulin. Patterns were also obtained on the peak tubes of 3 other sera including those of patient S. P. (Fig. 3). In each case in which an abnormal electrophoretic component associated with cold agglutinin activity was observed, it was accounted for by an elevation of the 19 S class of proteins. A distinct correlation was found between the titer of activity, the height of the electrophoretic component, and amount of 19 S material in the peak tubes.

Dissociation of the Cold Agglutinins.—

An effort was made to dissociate the abnormal 19 S components of 2 of the sera containing high titer cold agglutinins. No dissociation was obtained with 6 M urea, acid buffer (pH 3.0), 15 per cent NaCl, or parachloromercurybenzoate. However, ready dissociation was obtained with the sulfhydryl reagents mercaptoethanol and cysteine. This was followed in the analytical ultracentrifuge and resulted in the appearance of proteins of low molecular weight. The details of these experiments will be published separately. With the physical dissociation there was a concomitant disappearance or marked loss in cold agglutinin activity. This was demonstrated both for the whole serum as well as for the isolated fractions. A search for incomplete antibodies by means of various antisera in a modified Coombs test failed to reveal any protein with affinity for the red cells after the dissociation. Control experiments with incomplete "warm" antibodies of the anti-Rh_0 type showed no loss of activity with addition of the sulfhydryl compounds.

DISCUSSION

The procedure of density gradient zone ultracentrifugation demonstrated that the cold agglutinins, in contrast to the warm type of incomplete antibodies found in acquired hemolytic anemia, are of high molecular weight. This method offers a simple means of grouping γ-globulins and antibodies into the 7 S or 19 S class. However, under the conditions used here it does not distinguish the 19 S materials from others of higher molecular weight. (Minor fractions with sedimentation coefficients of approximately 26 S and 33 S are usually associated with high concentrations of 19 S components.) However, the role of the 19 S fraction in cold agglutinin activity was clearly apparent from analyses in the analytical ultracentrifuge of the active electrophoretic components, particularly in the experiments in which loss of the abnormal peaks occurred after absorption with red cell stromata. Almost all of the protein eluted from the stromata was of the 19 S type.

Although all of the first group of patients whose sera were analyzed in the present study suffered from a similar disease with the syndrome of cold hemagglutination, chronic hemolytic anemia, and paroxysmal cold hemoglobinuria, they showed wide variation in cold agglutinin titers. The amount of 19 S

material in the active electrophoretic fractions showed a similar variation. In some instances no detectable increase above the normal 19 S concentration could be found while in others huge 19 S peaks could be seen in the ordinary electrophoretic pattern. The latter group might well be classified as macroglobulinemias of the Waldenström type and indeed the literature on this subject contains at least one case report (15) in which high titer cold agglutinins were encountered. In addition, one of the patients (S. Y.) included in this study displayed clinical features suggestive of Waldenström's macroglobulinemia. An abstract of his clinical history was given. However, this is a purely artificial classification because the same syndrome occurs in the absence of any distinguishable macroglobulinemia. The critical finding is the cold agglutinin activity and the very high titers represent simply one extreme of this condition.

The question of whether these cold agglutinins are true antibodies is not clear. Other work to be published separately confirms older observations (6) that a number of classical antibodies do fall into the 19 S fraction. This is true of at least some of the isoagglutinins in the human being and of certain pneumococcal antibodies in the horse and rabbit. The non-specificity of the red cell type required to show agglutination in the cold (16) might be cited as evidence against the antibody hypothesis. However, the reaction might well be caused by a broadly distributed determinant group on the red cell. It seems possible that the red cell is not the original antigen but rather possesses groups similar to some other cell type. It is difficult to explain the agglutination simply on the basis of the size and solubility properties of these proteins. The great majority of macroglobulins, indistinguishable in the ultracentrifuge or by electrophoresis from those encountered in this study, do not have the property of cold agglutination. Some of the cold agglutinins are cryoglobulins and precipitate in the cold (17). However, this was not the case with most of those encountered in the present analysis. (Only the serum of patient O. L. showed significant cryoglobulin.) In addition, other macroglobulins which precipitate in the cold do not agglutinate red cells. A final answer on the question of whether these proteins are antibodies is not as yet available.

In contrast to the saline cold agglutinins, the "autoantibodies" of acquired hemolytic anemia of the warm variety display both species and antigen specificity. Although the cold hemagglutinins non-specifically agglutinate the erythrocytes of all human beings and of various mammalian species the antibodies of warm acquired hemolytic anemia react only with human red cells (18, 19). In addition, the use of panels of red cells of varying antigenic composition has demonstrated that eluates obtained from the red cells of patients with warm hemolytic anemia often possess preferential specificity for one or another of the blood group antigens present in the red cells of the donor patients (20, 21).

Furthermore, in cases of acquired hemolytic anemia of the warm variety, antibody is first fixed to the red cell surface; not until the red cell is almost

fully saturated does "free" antibody "spill over" into the plasma. Consequently, 4+ direct antiglobulin reactions, and serum antibody titers greater than 100 are extremely rare. Conversely, in the instances of "cold" hemolytic anemia, serum antibody titers are rarely below 1,000 and often reach levels of 10,000 to 20,000 with little or no coating of the patients' erythrocytes *in vivo*.

These distinctions along with the variation in the physical and chemical properties of the active proteins suggest a fundamental difference in the etiology and pathogenesis of the warm and cold varieties of acquired hemolytic anemia. The data supporting the concept of a true erythrocyte autoantibody appear much stronger in the instance of the warm antibody.

The dissociation of the cold agglutinins by sulfhydryl compounds which are known to split disulfide bonds is of particular interest. In this respect these proteins resemble other pathological macroglobulins without demonstrable biological activity which are known to be broken down with similar reagents (22). It would appear that they represent polymeric types made up of subunits resembling 7 S γ-globulin linked together through disulfide bridges. The subunits are not identical with ordinary 7 S γ-globulin because they are particularly rich in carbohydrate (3) and possess specific determinant groups immunologically (4, 5).

Further data concerning the nature of the cold agglutinins in acquired hemolytic anemia have recently been obtained by Christenson and Dacie (17). In this work, which was reported after the present study was completed, distinguishable electrophoretic components were observed in sera with high titers of cold agglutinins. Evidence for classification of these proteins as belonging in the 19 S group has also been obtained by these workers (23, 24). Thus, on the basis of the two independent investigations employing different methods and case material there appears little doubt that the cold agglutinins should be added to the expanding list of antibodies and biologically active proteins that are of high molecular weight and usually migrate as fast γ-globulins.

SUMMARY

The sera of 8 patients with acquired hemolytic anemia associated with elevated levels of cold agglutinins were studied by various procedures of zone electrophoresis. The agglutinating activity was found associated with proteins of variable mobility in the different cases. The majority represented "fast" γ-globulins. The 4 sera with the highest titers of cold agglutinins showed distinguishable abnormal electrophoretic components. The titers correlated with the height of the abnormal components.

Ultracentrifugal analysis of the electrophoretic fractions indicated that the cold agglutinins were associated with proteins having a sedimentation coefficient of approximately 19 S. The abnormal component from the serum with the highest biological activity showed almost no contamination with lower

molecular weight proteins. The amount of 19 S material found correlated with the titer of agglutinating activity.

The high molecular weight character of the cold agglutinins was confirmed by procedures of density gradient zone centrifugation. The biological activity sedimented with proteins of the 19 S class in all the sera including those of relatively low titer with which no abnormal electrophoretic components were observed.

Dissociation of the abnormal high molecular weight components was possible by means of certain sulfhydryl compounds. This resulted in disappearance of cold agglutinin activity.

Some of the cases could be classified as macroglobulinemias because of the very large content of high molecular weight components. However, the same disease picture occurred without recognizable elevation of these components.

The sera of 3 patients with severe acquired hemolytic anemia of the warm type associated with warm incomplete antibodies failed to show similar abnormal electrophoretic components and the antibody activity sedimented with proteins of the 7 S class.

The authors wish to thank Dr. A. S. Wiener, Dr. W. Dameshek, Dr. C.-B. Laurell, Dr. A. V. Pisciotta, Dr. H. Ranney, Dr. R. E. Rosenfield, and Dr. R. Rosenthal for furnishing sera and clinical and serological data on their patients, and Dr. A. B. Gutman for making patient S. Y. available for study during his hospitalization.

BIBLIOGRAPHY

1. Wallenius, G., Trautman, R., Kunkel, H. G., and Franklin, E. C., Ultracentrifugal studies of major non-lipide electrophoretic components of normal human serum, *J. Biol. Chem.*, 1957, **225**, 253.
2. Müller-Eberhard, H. J., and Kunkel, H. G., The carbohydrate of γ-globulin and myeloma proteins, *J. Exp. Med.*, 1956, **104**, 253.
3. Müller-Eberhard, H. J., Kunkel, H. G., and Franklin, E. C., Two types of γ-globulin differing in carbohydrate content, *Proc. Soc. Exp. Biol. and Med.*, 1956, **93**, 146.
4. Franklin, E. C., and Kunkel, H. G., Immunologic differences between the 19 S and 7 S components of normal human γ-globulin, *J. Immunol.*, 1957, **78**, 11.
5. Korngold, L., Antigenic relationship of gamma globulins, cryoglobulins and macro-cryoglobulin, *Fed. Proc.*, 1956, **14**, 597.
6. Kabat, E. A., Blood Group Substances: Their Chemistry and Immunochemistry, New York, Academic Press, Inc., 1956.
7. Gordon, R. S., The preparation and properties of cold hemagglutinin, *J. Immunol.*, 1953, **71**, 220.
8. Wiener, A. S., Unger, L. J., Cohen, L., and Feldman, J., Type-specific cold auto-antibodies as a cause of acquired hemolytic transfusion reactions: biologic test with bovine red cells, *Ann. Int. Med.*, 1956, **44**, 221.
9. Stats, D., and Bullows, J. G. M., Cold hemagglutination with symmetric gangrene of the tips of the extremities, *Arch. Int. Med.*, 1943, **72**, 506.
10. Stats, D., Perlman, E., Bullows, J. G. M., and Goodkind, R., Electrophoresis

and antibody nitrogen determinations of a cold hemagglutinin, *Proc. Soc. Exp. Biol. and Med.*, 1943, **53**, 188.

11. Wiener, A. S., Gorow, E. B., and Gallup, B., Studies on auto-antibodies in human sera, *J. Immunol.*, 1953, **71**, 220.
12. Pisciotta, A. V., Cold hemagglutination in acute and chronic hemolytic syndromes, *Blood*, 1955, **10**, 295.
13. Brakke, M. K., Zone electrophoresis of dyes, proteins and viruses in density-gradient columns of sucrose solutions, *Arch. Biochem. and Biophysic.*, 1955, **55**, 175.
14. MacKay, I. R., Ericksen, W., Motulsky, A. G., and Volwiler, W., Cryo- and macroglobulinemia. Electrophoretic, ultracentrifugal and clinical studies, *Am. J. Med.*, 1956, **20**, 564.
15. Wilde, H., and Hitzelberger, A. L., Macroglobulinemia. Clinical features and differential diagnosis, *Blood*, 1954, **9**, 875.
16. Dacie, J. V., The Haemolytic Anaemias; Congenital and Acquired, London, J. and A. Churchill, Ltd., 1954.
17. Christenson, W. N., and Dacie, J. V., Serum proteins in acquired haemolytic anaemia (auto-antibody type), *Brit. J. Hematol.*, 1957, **3**, 153.
18. Komnios, Z. D., and Rosenthal, M. C., Studies on antibodies eluted from the red cells in auto-immune hemolytic anemia, *J. Lab. and Clin. Med.*, 1953, **41**, 887.
19. Weiner, W., Battey, D. A., Cleghorn, T. E., Marson, F. G. W., and Meynell, M. J., Serologic findings in a case of hemolytic anemia, *Brit. Med. J.*, 1953, **2**, 125.
20. Dacie, J. V., and Cutbush, M., Specificity of auto-antibodies in acquired hemolytic anemia, *J. Clin. Path.*, 1954, **7**, 18.
21. Crowley, L. V., and Bouroncle, B. S., Studies of the specificity of auto-antibodies in acquired hemolytic anemia, *Blood*, 1956, **11**, 700.
22. Deutsch, H. F., and Morton, J. I., Dissociation of human serum macroglobulins, *Science*, 1957, **125**, 600.
23. Christenson, W. N., and Dacie, J. V., Electrophoresis of acquired hemolytic anemia serum abnormal gamma peak composed of cold antibody protein (abstract), *Clin. Research Proc.*, 1957, **5**, 145.
24. Christenson, W. N., Dacie, J. V., Croucher, B. E. E., and Charlwood, P. A., Electrophoretic studies on sera containing high-titer cold haemagglutinins: identification of the antibody as the cause of an abnormal γ_1 peak, *Brit. J. Haematol.*, 1957, **3**, 262.