Serologic Properties of a Cold Hemolysin and an Acid Hemolysin Occurring in a Case of Syphilitic Paroxysmal Cold Hemoglobinuria

By E. T. Peterson, A.B. and R. L. Walford, M.D.

The clinical entity of syphilitic paroxysmal cold hemoglobinuria has been adequately described by Rosensbach,1 Donath and Landsteiner,2 Mackenzie,3 Ross4 and others. In this disease exposure to cold induces a paroxysm characterized by a shaking chill, fever, flank pain and hemoglobinuria. This phenomenon is thought to be due to a cold hemolysin found in the patient’s serum. In vitro experiments have shown that this antibody will sensitize the patient’s erythrocytes, or those of another individual, upon exposure to cold. Subsequent incubation at 37 C. in the presence of complement will bring about lysis of the sensitized cells. The most recent and detailed work on the exact mechanism of this reaction (the Donath-Landsteiner reaction) has been done by Jordan, Pillemer and Dingle.5–6

The case herein discussed presents the classic features of syphilitic paroxysmal cold hemoglobinuria. However, in addition to the usual cold hemolysin, a thermodilatable hemolysin, active only under conditions of slight acidification, was consistently found in the patient’s serum. The presence of such an acid hemolysin in syphilitic PCH has been suggested by the findings of previous investigators.3–5 Adequate evidence has not, however, been presented heretofore to support the view that it represents an antibody entirely separate from the cold hemolysin of the classical Donath-Landsteiner reaction.

Clinical History

Mr. D. G. is a 47 year old obese, Caucasian male truckdriver. Since 1937 he has had one or two yearly attacks of flank pain followed by the passage of dark red or brown urine. These attacks have always been associated with exposure to cold. The only attack free years since 1937 were those of 1942 and 1943, when the patient worked as an airplane mechanic in the warm climate of Texas. His most recent attack occurred three days prior to the present admission. His automobile broke down one cold morning while he was driving from Idaho to California. While repairing the engine, he became chilled. The chilling was accompanied by flank pain. The next urine voided was dark brown in color.

On admission the patient was lethargic and mentally dull, although well-oriented. The Wasserman and Kahn tests on his serum were strongly positive. His spinal fluid contained 105 lymphocytes per cu. mm. and 346 mg. per cent of total protein. The spinal fluid Wassermann was positive, and there was a Zone I type of colloidal gold curve. Biopsy of a deep punched-out lesion on the posterior pharyngeal wall revealed a nonspecific ulcer-
<table>
<thead>
<tr>
<th>No.</th>
<th>Test</th>
<th>Results from 10-31 to 12-3</th>
<th>Results on 12-4</th>
<th>Results on 12-10</th>
<th>Results on 12-28 (following ACTH)</th>
<th>Results on 12-31 (Follow-up study)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rosenbach Test</td>
<td>11-8-51: Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>Donath-Landsteiner Reaction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b. Confirmatory</td>
<td>Strong pos. on numerous occasions.</td>
<td>Pos.: 10% hemolysis with 1 to 8 serum dilution.</td>
<td>Pos.: 10% hemolysis with 1 to 8 serum dilution.</td>
<td>Negative</td>
<td>Strong positive</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Ham's Test</td>
<td>Consistently pos.</td>
<td>Positive</td>
<td>Positive</td>
<td>Weak positive</td>
<td>Positive</td>
</tr>
<tr>
<td>a. Presumptive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>b. Confirmatory</td>
<td>Consistently neg.</td>
<td>Negative 50% with undiluted serum. 30% with 1 to 2 dilution.</td>
<td>Negative 20% with undiluted serum. None with 1 to 2 dilution.</td>
<td>5% with undiluted serum</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Acid-hemolysin test</td>
<td>Over 70% hemolysis with undiluted serum</td>
<td>Pos.: 1 to 8 dilution</td>
<td>Pos.: 1 to 16</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Coombs Test</td>
<td>4+ 2+ Pos. with undiluted serum</td>
<td>4+ 1+ Pos. 1 to 8 dilution</td>
<td>4+ Pos. 1 to 16</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Direct: Chilled 21C.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Indirect: Chilled 37C.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Complement titer of patient's serum</td>
<td>40% of controls</td>
<td>Essentially zero</td>
<td>40% of controls</td>
<td>100% of controls</td>
<td></td>
</tr>
</tbody>
</table>
tion with granulation tissue and an acute polymorphonuclear reaction. From October 15 to October 31, 1951, the patient received a total of 9 million units of penicillin. The pharyngeal lesion healed promptly under this therapy.

General laboratory workup included a blood urea nitrogen of 13 mg. per cent, creatinine 1.3 mg. per cent and total protein 7.9 Gm. per cent with an A/G ratio of 1.9. The urine showed a normal Addis count and occasionally a one or two plus reaction for albumin. Throughout hospitalization the patient's red blood cell count, hemoglobin, white cell count and differential count remained within normal limits. The shape of the red blood cells on stained smears was normal on numerous occasions. A bone marrow aspiration revealed only a mild normoblastic and pronormoblastic hyperplasia. The reticulocyte count was 1.2 per cent.

After the basic immunologic responses of the patient had been determined, he was placed on 80 mg. daily of ACTH for two weeks. The drug was then abruptly withdrawn and the tests repeated at appropriate time intervals. (Table 1.)

### Table 2—Rosenbach Test

<table>
<thead>
<tr>
<th>Date</th>
<th>Serum hemoglobin, mg %</th>
<th>Serum bilirubin, mg %</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>before chilling</td>
<td>15 min. after chilling</td>
<td>30 min. after chilling</td>
</tr>
<tr>
<td>11/8</td>
<td>4.5</td>
<td>41.5</td>
<td>—</td>
</tr>
<tr>
<td>12/10</td>
<td>0</td>
<td>60</td>
<td>55</td>
</tr>
<tr>
<td>12/28*</td>
<td>0</td>
<td>—</td>
<td>48</td>
</tr>
<tr>
<td>12/31</td>
<td>24.9†</td>
<td>82.5</td>
<td>—</td>
</tr>
</tbody>
</table>

* The last day of ACTH therapy.
† This figure is the only elevated serum hemoglobin ever obtained except following a Rosenbach test, and doubtless represents a technical error. On two occasions in the study of the case, blood specimens were examined for serum hemoglobin at 4 hour intervals over a 24 hour period. All values were normal.

### METHODS

Defibrinated blood was employed as a source of erythrocytes and of plasma except where otherwise designated. As soon as possible, all cells were washed three times with about 10 times their volume of normal saline and stored either as packed cells or in a saline suspension. Serum was used for all comparative work. Whole blood specimens were never refrigerated. The great majority of the work was completed on the day that the specimens were drawn.

All pH determinations and adjustments were made with a Beckman pH meter. Fresh, guinea pig serum was used as a source of complement. For the serologic tests of in vitro hemolysin activity, percentage-of-hemolysis standards were prepared from a stock hemoglobin solution. This was obtained by taking a volume of packed red blood cells appropriate to the specific test with distilled water, and then bringing to isotonicity by adding a buffered salt solution. The required amounts of cell suspension, hemoglobin solution and saline were added to tubes in preparing the percentage-of-hemolysis standards for the specific tests.

#### Rosenbach Test

The patient's feet were chilled in ice water for 15 minutes, after which he was covered with warm blankets for one hour. Blood and urine specimens were obtained as indicated in table 2. Serum hemoglobin was determined by a modification of the method of Bing and Baker,10 serum bilirubin by the method of Watson and Ducci.11
Donath-Landsteiner Reaction

Three ml. of the patient’s serum, 3 ml. of a 10 per cent suspension of erythrocytes and 0.3 ml. of undiluted complement were chilled in an ice water bath for 30 minutes, incubated at 37 C. for 30 minutes and centrifuged. The percentage of hemolysis was determined by comparing the supernatant fluid with appropriate percentage-of-hemolysis standards. The titer of these reactions was estimated by employing serial, saline dilutions of the patient’s serum. The “presumptive” test refers to the action of the patient’s serum against his own erythrocytes; the “confirmatory” test, against control erythrocytes.

Acid Hemolysin

Unmodified Ham’s presumptive and confirmatory tests were used.18 The tubes were covered by oil as soon as the pH adjustments had been made, and prior to the incubation. To determine the effect of oxalate on the acid hemolysin system, tubes containing oxalate were prepared as described by Kolmer and Boerner,13 and appropriate volumes (up to 5 ml.) of blood or serum added.

Complement Titrations

Serum complement titrations were performed within a few hours after the specimens had been taken. A 1 to 100 dilution of the patient’s serum was made and quantities from 0.5 to 2.0 ml., with increases of 0.1 ml., were prepared. Saline was added to bring the volume in each tube to 2 ml. Five-tenths ml. of previously “sensitized” sheep cells14 were then added. The tubes were incubated for 30 minutes at 37 C. and the hemolysis compared with an appropriate 50 per cent hemolysis color standard. Quantities ranging from 0.6 to 0.7 ml. of a 1 to 100 dilution of normal control sera gave 50 per cent hemolysis with this system.

RESULTS

Rosenbach Test

The pathognomonic sign of a positive Rosenbach test is a marked rise in serum hemoglobin as compared with pre-test levels. In addition, a rise in serum bilirubin and hemoglobinuria without hematuria may be observed. As indicated in table 2, this test was positive on all occasions. ACTH had at best only a moderate effect upon the patient’s response to this test.

Donath-Landsteiner Reaction

Reference is made to table 1. The presumptive test was strongly positive on all examinations up to and including December 15, 1951 (not charted), at which time the patient was put on ACTH. Four days later (not charted), the reaction was negative. It was negative on the day that ACTH was discontinued. Three days later it was again positive. The presumptive test was consistently stronger than the confirmatory test. The titer of the presumptive test was unaffected by the Rosenbach test. Serum dilutions of 1 to 8 showed 10 per cent hemolysis of the patient’s erythrocytes with specimens drawn both before and 45 minutes after the completion of the Rosenbach test.

Inactivated serum (56 C. for 30 minutes), to which complement had not been added, failed to produce hemolysis in the Donath-Landsteiner reaction. However, when complement was added to this system, hemolysis occurred. The cold hemolysin was stable when stored in the refrigerator, or in a frozen state.

Acid Hemolysin

As shown in table 1, Ham’s presumptive test was consistently positive except during the follow-up period. The confirmatory test was always negative. This
led to the discovery of a hemolysin in the patient’s serum which was active against either the patient’s or control human red blood cells under slight acidification. (Table 3.) The hemolysin was slightly more active against patient than control cells, possibly due to some in vivo sensitization. It was extremely labile, being completely destroyed by incubation at 56 C. for only 5 minutes, or by storage overnight in the refrigerator. The reaction was inhibited by oxalate, roughly in proportion to the amount used. Whether this effect was the result of the action of the oxalate upon the calcium and magnesium ions—and thus indirectly upon complement—was not determined. In any event, the activity was not restored by the addition of excess complement to the oxalate-inhibited system. The role of intact complement (but not the fractions thereof) was investigated using serum drawn on December 4, 1951. On that date the patient’s intact complement titer was essentially zero. (Experiments that were simultaneously performed showed that this negative titer was not due to any anti-complementary activity of the serum.) The relative lack of intact human complement, as compared with a great excess of added guinea pig complement, had no significant effect upon the activity of the acid hemolysin.

**Table 3—pH Range of the Acid Hemolysin**

<table>
<thead>
<tr>
<th>pH</th>
<th>7.5</th>
<th>7.3</th>
<th>7.0</th>
<th>6.8</th>
<th>6.5</th>
<th>6.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>per cent of hemolysis</td>
<td>0</td>
<td>trace</td>
<td>5%</td>
<td>70%</td>
<td>over 70%</td>
<td>over 70%</td>
</tr>
</tbody>
</table>

A Coombs test using the patient’s serum with his own cells was performed with specimens of defibrinated blood which had been chilled for 30 minutes, and with specimens left at room temperature for 30 minutes. Results are referred to in table 1 as the direct Coombs test to distinguish it from the test using the patient’s serum with control erythrocytes, which is referred to as the indirect test. When oxalated instead of defibrinated blood was employed, the reaction was much weaker in both chilled and unchilled specimens. Recent workers have stressed the necessity of complement in the Coombs test in relation to the syphilitic PCH cold hemolysin. The addition of one-tenth the volume of undiluted complement to the oxalated specimens yielded a macroscopically visible $2+$ reaction in the chilled specimen. It had no effect upon the specimen left at room temperature. These results are shown in table 4.

The indirect Coombs test was performed with specimens which had been chilled for 30 minutes and with specimens incubated at 37 C. for 30 minutes during the “sensitization” period. A slight rise in titer was demonstrated in the chilled specimens. On the other hand, a marked drop in titer was observed with the specimens sensitized at 37 C. (Table 1.) The role of intact complement in the indirect Coombs test is illustrated in table 4.
Complement Titer of the Patient's Serum

When first examined, the serum contained complement with an activity about 40 per cent of that of 3 random controls. Seven days later, complement was absent, using a test system sensitive to less than 6 per cent of complement found in the controls. Thereafter, the titer rose, and two weeks following ACTH therapy it was 100 per cent of normal. Fluctuations in complement titer have been found in similar cases.4

Miscellaneous Tests

The patient's erythrocytes were more fragile than those of the control in hypotonic saline, and showed no abnormal response to mechanical agitation. A cold hemagglutinin titer of 1 to 20 was observed on several occasions.

<table>
<thead>
<tr>
<th>Sensitization periods*</th>
<th>Direct Coombs test</th>
<th>Indirect Coombs test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Defibrinated blood</td>
<td>Oxalated blood</td>
</tr>
<tr>
<td>Ice water</td>
<td>4+</td>
<td>±</td>
</tr>
<tr>
<td>Room temp.</td>
<td>2+</td>
<td>±</td>
</tr>
<tr>
<td>37 C</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Thirty minute sensitization periods, in ice water, room temperature, or 37C., prior to the washing of the erythrocytes and the addition of the Coombs serum.
† Inactivated at 56C. for 30 minutes.
‡ Two-tenths ml. of undiluted complement were added to 1 ml. of serum and 1 ml. of a 10 per cent suspension of erythrocytes, and the system sensitized at the indicated temperature.

Discussion

The consistently positive presumptive Ham's test associated with a consistently negative confirmatory Ham's test led to the discovery of a hemolysin present in the patient's serum that was capable of hemolyzing both patient's and control red blood cells under conditions of slight acidification. The finding of this acid hemolysin raised these questions: (1) Is it frequently associated with syphilitic PCH? (2) Is it distinct from the classical Donath-Landsteiner cold hemolysin antibody?

In 1909 Hijmans van den Bergh7 found that defibrinated syphilitic PCH blood could be hemolyzed by exposure to CO2. Normal red blood cells showed a similar reaction when mixed with the PCH serum and exposed to CO2. Inactivated PCH serum, to which complement had been added, failed to hemolyze normal erythrocytes under these conditions. These results were confirmed by Hannema and Rytma8 in 1922. Commenting on these reports in his review, Mackenzie said, "It is not clear from these experiments whether CO2 acts simply as an activating factor for the Donath-Landsteiner mechanism or whether it represents a distinct and separate hemolytic mechanism—the evidence points more to the latter assumption." We have found no further reference in the literature regarding this hemolytic mechanism until the report by Wagley, Zinkman and Siebens9
in 1947. These investigators found that when red blood cells were suspended in PCH serum and CO₂ added to a pH of 6.4, lysis rapidly occurred at 27 C.

Dacie[17] has reported an acid hemolysin occurring in 7 cases of non-syphilitic PCH, associated with titers of cold hemagglutinins ranging from 1 to 512, to 1 to 64,000. In our case the cold hemagglutinin titer was only 1 to 20. Elsewhere he has reported[18] an acid hemolysin in a case of severe acquired hemolytic anemia. In both instances these acid hemolysins were heat stable. In our case the hemolysin was exceedingly labile. Acid hemolysis in several cases of acquired hemolytic anemia have also been briefly reported by Gardner and Harris.[19] In their cases the "direct" type of hemolysis could be demonstrated only during the acute phase of the disease.

The acid hemolysin and the cold hemolysin in our case differed from one another in the following respects. The acid hemolysin was extremely labile, being destroyed by inactivation at 56 C. for 5 minutes, or by storage overnight in the refrigerator. The cold hemolysin, on the other hand, withstood heat-inactivation for a 30 minute period. The addition of complement to inactivated serum resulted in hemolysis in the Donath-Landsteiner test, whereas no hemolysis occurred in the acid hemolysin test under similar conditions. There was no correlation between the varying titers of the cold hemolysin and the acid hemolysin. This was particularly evident during the follow-up study, when the cold hemolysin test was strongly positive, while the acid hemolysin had disappeared.

Furthermore, the diverging titers obtained with the indirect Coombs test indicate that two separate antibodies could be demonstrated simply by varying the temperature conditions of the sensitizing period. Jordan, Pillemer and Dingle,5-6 and Siebens, Zinkman and Wagley20 have reported that complement is necessary during the chilling "sensitization" period, in order to obtain a positive indirect Coombs test with PCH serum. We agree with this finding. Heat-inactivated serum in our case gave a negative indirect test unless complement was added before chilling. On the other hand, when the erythrocytes were sensitized at 37 C., the addition of complement to heat-inactivated serum did not restore the positivity of the test. (Heat-inactivation destroys the acid hemolysin.) It seems probable that the indirect Coombs test as performed at 37 C. measured the acid hemolysin, and as performed in the cold measured the cold hemolysin.

In short, we believe that Mackenzie's original speculation was correct, and that a hemolytic mechanism, distinct and separate from that involving the classical Donath-Landsteiner cold hemolysin, may sometimes exist in this disease.

**SUMMARY**

1. Two apparently separate hemolysins observed in a case of syphilitic paroxysmal cold hemoglobinuria are described.
   a. One hemolysin (cold hemolysin) is that responsible for the Donath-Landsteiner reaction.
   b. The other (acid hemolysin) is a thermolabile antibody requiring a lowered pH for the production of hemolysis.

2. Review of the literature indicates that a similar acid hemolysin may have been encountered in other reported cases of this disease.
REFERENCES


Serologic Properties of a Cold Hemolysin and an Acid Hemolysin Occurring in a Case of Syphilitic Paroxysmal Cold Hemoglobinuria

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