Chronic Acquired Hemolytic Anemia Associated with Hemoglobinuria and Raynaud's Phenomena

By J. A. Bonnin

The antibody patterns in certain types of acquired hemolytic anemia were described in detail by Dacie and de Gruchy in 1951. The patients they investigated were grouped into three clinicopathological syndromes: Group I, idiopathic acquired hemolytic anemia, Group II, hemolytic anemia following virus pneumonia and Group III, chronic hemolytic anemia with hemoglobinuria and Raynaud's phenomena. A detailed account of the clinical aspects of the three patients of Group III was published by Ferriman, Dacie, Keele and Fullerton who also made an analysis of nine similar cases reported in the literature. All these patients had certain features in common: all except one were over 50 years of age, all suffered from a chronic hemolytic anemia, and most of them from Raynaud's phenomena also. The hemolytic anemia was worse in winter time, and episodes of acute hemolysis with hemoglobinuria occurred upon exposure to cold. The sera of those patients who were adequately investigated were found to contain cold antibodies in high titres, capable of causing agglutination, hemolysis and sensitization to antilgobulin serum. Their Wassermann reactions were negative and the cause of their disease unknown.

The patient now to be described may be considered to belong to this third group. His serum was, however, unusual, for, although it contained an extremely high-titre cold agglutinin, its behaviour in respect to hemolysis resembled that of the Donath-Landsteiner type of hemolysin, rather than that of the 'acid hemolysins' which have previously been reported in this type of acquired hemolytic anemia. Studies on the ability of this antibody to produce erythrophagocytosis were also carried out, and in this respect, too, the behaviour differed from that of an 'acid hemolysin'.

Clinical History

The patient, aged 63 years, was a tally clerk working on the wharves at Port Adelaide where he was exposed to all weather conditions. During the previous six years he had suffered from several episodes of sudden collapse associated with abdominal pain, dizziness, severe anemia and the passage of reddish-brown urine. These episodes always followed exposure to cold; in addition, he had experienced many milder attacks without noticeable discoloration of the urine. The first episode occurred in 1946 following a severe streptococcal pharyngitis for which he received penicillin.

The patient stated that he had been told at the age of ten years, when examined because of severe abdominal pain, that his spleen was enlarged, but this could not be confirmed. He had been quite healthy until his present illness.

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The author is grateful to Dr. E. F. Gartrell, Honorary Physician, Royal Adelaide Hospital, for his permission to publish this case, and to Dr. G. C. de Gruchy, Melbourne, for his advice. The serological investigations were carried out in the Department of Haematology, Postgraduate Medical School of London, and the author would like to thank Dr. J. V. Dacie for his help and interest.
There was no family history of anemia, jaundice or splenomegaly; moreover, he had an identical twin brother whose blood has been examined and found to be normal.

He was admitted to hospital in May, 1952, eleven days after a severe episode. Physical examination then revealed the following abnormalities: there was a generalized pallor with an icteric tinge of the conjunctivae and marked Raynaud's phenomenon affecting the ears, hands and feet, without trophic changes in these structures. The liver was enlarged and palpable 5 cm. below the right costal margin and the spleen was palpable 2 cm. below the left costal margin.

With rest in bed and with warmth, his clinical condition and hematological picture improved rapidly and neither adrenal cortical hormones nor transfusion were necessary. The patient was discharged from hospital when the hemoglobin level had risen to 13 Gm. per 100 ml. He was advised to change his occupation to an indoor job and, although his hemolytic anemia and slight jaundice have persisted, he has had no further acute hemolytic episodes.

LABORATORY INVESTIGATIONS ON ADMITTANCE TO HOSPITAL

Shortly after his sudden collapse a hematological examination revealed the following:—

Hemoglobin 6 Gm. per 100 ml. (39 per cent); volume of packed cells 20 per cent; mean corpuscular volume 87 cu. micra; mean corpuscular hemoglobin concentration 30 per cent; reticulocytes 7.2 per cent; leucocyte count 8,300 per cu. mm.; differential count: myelocytes 16 per cent, metamyelocytes 11 per cent, neutrophils 63 per cent, eosinophils 1 per cent, monocytes 2 per cent, lymphocytes 7 per cent. There was marked anisocytosis and polychromasia, and 9 normoblasts per 100 leukocytes. Obvious cold autoagglutination was present and the blood was grossly hemolyzed. It was estimated that he had suddenly lost some 50 per cent of his erythrocytes, accounting for the pronounced leuko-erythroblastic blood picture. The immature granulocytes were present in the peripheral blood for over eleven days when 2 per cent metamyelocytes were reported, and normoblasts persisted for over two weeks. Subsequent examinations have revealed none of these cells. The osmotic fragility of the red cells was very slightly increased beyond normal. The Wassermann reaction was negative. The serum complement was reduced to 8 units compared with 64 and 128 units in two normal control sera. His blood group was O Rh positive. The direct Coombs' test was strongly positive. Bone marrow aspiration biopsy showed an intense normoblastic hyperplasia. No hemoglobin or its derivatives could be found in the urine at this stage and there was no increase in urinary urobilinogen, although a pathological increase was later found.

SEROLOGICAL INVESTIGATIONS

Materials and Methods

Unhemolyzed serum was readily obtained when blood was allowed to clot undisturbed at 37 C. The titration of cold agglutinins was carried out according to the method described by Dacie and de Gruchy,1 and the combined titration of hemolysins and erythrophagocytosis by the technique of Bonnin and Schwartz.*

* The combined titration of hemolysis and erythrophagocytosis. A concentrated suspension of actively phagocytic leukocytes was prepared from freshly drawn heparinized group O venous blood. 10 ml. of this blood was centrifuged for 10 minutes at 2500-3000 R.P.M. The upper half of the supernatant plasma was withdrawn with a Pasteur pipet. The tip of the pipet was then placed at the bottom of the tube and three quarters of the packed red cells were removed, rendering the blood artificially anemic. After thorough resuspension, the contents of the tube were allowed to sediment under gravity for 30 minutes at 37 C. in a tube of narrow diameter. The resulting supernatant leukocyte-rich plasma was found to contain some 40,000 leucocytes per cu. mm. and was relatively free from contaminating erythrocytes.

30 per cent cell suspensions of normal, trypsinised and P.N.H. erythrocytes were pre-
Three types of Group O red cells were used as indicators of agglutination, hemolysis and erythrophagocytosis; fresh corpuscles obtained from defibrinated normal venous blood; normal corpuscles freshly trypsinized according to a modification of the method of Morton and Pickles,\textsuperscript{6} and red cells from a patient with paroxysmal nocturnal hemoglobinuria (P.-N.H. red cells) which had been stored for short periods at 4°C in acid-citrate-dextrose solution. The corpuscles were washed three times in normal saline before use.

Variation of the pH of the serum was effected by the addition of one-tenth volume of various concentrations of hydrochloric acid and sodium hydroxide solutions as described by Dacie.\textsuperscript{3} pH was measured by a glass electrode using a ‘Cambridge’ pH meter.

Fresh normal group A human serum was used as a source of complement. Complement was titred by the method of Mayer, Eaton and Heidelberger.\textsuperscript{7}

Antiglobulin reactions were carried out according to the techniques of Dacie.\textsuperscript{8}

**Results**

The most significant observations are summarized in tables 1, 2 and 3. The temperatures up to which the patient's antibody caused agglutination, hemolysis, and sensitization to antiglobulin serum are shown in table 1. Normal erythrocytes were used, and the serum was unacidified; it was diluted 1 in 10 in fresh normal serum. In this experiment the antibody was active up to 33.5°C at least.

Agglutination titrations with normal red cells were also carried out in saline dilutions of the patient's serum. The titres between 2°C and 37°C were as follows: at 37°C: 0; 33.5°C: 16; 30°C: 64; 20°C: 16,000; 2°C: 500,000.*

The effect of varying the pH on hemolysis and sensitization to antiglobulin serum is shown in table 2. Normal erythrocytes were used and the experiment was carried out at 20°C. The optimum pH was found to be between 7.6 and 8.3.

pared in physiological saline. Samples of the patient's serum and normal compatible serum were acidified by the addition of one-tenth volumes of N/4 HCl.

Four parallel titrations of hemolysis and erythrophagocytosis were carried out as follows: three sets of serial fourfold dilutions of the patient's serum were made in normal serum, and one of acidified patient's serum in acidified normal serum. 0.2 ml volumes were used. A one-tenth volume of the suspension of normal erythrocytes was added to each tube of one set of the unacidified serum dilutions and to each dilution of the acidified sera. Trypsinized and P.N.H. erythrocytes were added to each tube of the second and third sets of unacidified serum dilutions, respectively. Ten minutes at 20°C were allowed for sensitization of the erythrocytes, during which time one drop was removed from each tube and transferred to a duplicate tube. One drop of the leukocyte suspension was then added to each duplicate tube and a further ten minutes allowed at 37°C for erythrophagocytosis to take place. Smears were made from the serum-red cell-leukocyte mixtures, stained and examined for erythrophagocytosis. Hemolysis was recorded in the original tubes after one hour at 20°C and a second hour at 37°C. Appropriate control tests with normal serum were included. The titre of hemolysis was recorded as a reciprocal of the highest dilution at which definite hemolysis was observed macroscopically, and that of erythrophagocytosis as the reciprocal of the highest dilution showing phagocytosis greater than 1 per cent in the stained smears. At high dilutions of antibody, erythrophagocytosis was confined to monocytes.\textsuperscript{9}

\* Dacie and de Gruchy\textsuperscript{1} reported higher titres of cold agglutinins in the clinico-pathological syndrome to which this patient belongs than in either of the other groups. Wiener et al.\textsuperscript{10} drew attention to the possibility of carry-over in the preparation of serum dilutions when discussing reports of excessively high titres of anti-A agglutinins. While this possibility must be born in mind in the present case, the enormous titre of cold agglutinins occurring in this serum at 2°C has been kindly verified by both Dr. Dacie and Dr. de Gruchy on separate specimens of serum collected at different times.
TABLE 1.—The Effect of Temperature on Agglutination, Hemolysis and Sensitization to Antiglobulin Serum

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Agglutination</th>
<th>Hemolysis</th>
<th>Indirect antiglobulin reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>33.5°C</td>
<td>Trace</td>
<td>—</td>
<td>±</td>
</tr>
<tr>
<td>30°C</td>
<td>±</td>
<td>17%</td>
<td>+</td>
</tr>
<tr>
<td>20°C</td>
<td>±</td>
<td>72%</td>
<td>+++</td>
</tr>
</tbody>
</table>

TABLE 2.—The Effect of pH on Hemolysis and Sensitization to Antiglobulin Serum

<table>
<thead>
<tr>
<th>Concentration of alkali or acid added to patient’s serum*</th>
<th>Hemolysis</th>
<th>Indirect antiglobulin reaction</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/5 NaOH</td>
<td>5%</td>
<td>+++</td>
<td>8.6</td>
</tr>
<tr>
<td>N/10 NaOH</td>
<td>62%</td>
<td>+++</td>
<td>8.3</td>
</tr>
<tr>
<td>O</td>
<td>67%</td>
<td>+++</td>
<td>8.0</td>
</tr>
<tr>
<td>N/10 HCl</td>
<td>63%</td>
<td>+</td>
<td>7.6</td>
</tr>
<tr>
<td>N/5 HCl</td>
<td>50%</td>
<td>+±</td>
<td>7.0</td>
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<td>+±</td>
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</tr>
<tr>
<td>N/3 HCl</td>
<td>13%</td>
<td>+±</td>
<td>6.3</td>
</tr>
<tr>
<td>Normal serum control</td>
<td>—</td>
<td>—</td>
<td>8.0</td>
</tr>
</tbody>
</table>

* One volume of the patient’s serum was diluted with 9 volumes of normal serum and then 1 volume of alkali or acid added. Finally, 1 volume of a 30% suspension of normal red cells added.

TABLE 3.—The Combined Titration of Hemolysis and Erythrophagocytosis

<table>
<thead>
<tr>
<th>Type of red cells</th>
<th>pH</th>
<th>Titre of hemolysis</th>
<th>Titre of erythrophagocytosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>8.0</td>
<td>8,000</td>
<td>8,000</td>
</tr>
<tr>
<td>Normal</td>
<td>6.7</td>
<td>256</td>
<td>2,000</td>
</tr>
<tr>
<td>Trypsinised normal</td>
<td>8.0</td>
<td>8,000</td>
<td>16,000</td>
</tr>
<tr>
<td>P.N.H.</td>
<td>8.0</td>
<td>400,000</td>
<td>32,000</td>
</tr>
</tbody>
</table>

The relative antibody titres for hemolysis and erythrophagocytosis using normal, trypsinsized normal and P.N.H. erythrocytes are summarized in table 3.

DISCUSSION

The serological observations now briefly reported resemble in most respects those previously recorded by Dacie and de Gruchy,1 and Ferriman et al.2 The effect of pH on hemolysis, however, is distinctly different. Whereas hemolysis of normal red cells in vitro can, in most instances, be demonstrated only if the patient’s serum is suitably acidified to the physiological level or below, the effect of acidification on this patient’s serum was to diminish its hemolytic activity rather than to increase it. In this respect the behaviour resembles that of the Donath-Landsteiner hemolysin. Sensitization by the antibody of normal corpuscles to agglutination by antiglobulin serum was similarly diminished by acidification, which is the opposite of what is usually found.8 The optimal conditions for erythrophagocytosis in vitro closely paralleled those for hemolysis.
The mechanisms of hemolysis in vivo in patients developing cold antibodies to very high titres are still not exactly understood. The high thermal amplitude of the antibodies present in the serum of the patient now described can probably be associated with the presence of a continuous hemolytic anemia. Whether the acute hemolytic episodes are due to the increased sensitivity of agglutinated corpuscles to mechanical trauma as suggested by Stats, or due to the direct hemolytic action of antibody and complement, is difficult to determine. Possibly both mechanisms are important. It is certainly tempting to correlate the repeated attacks of hemoglobinuria, from which the present patient suffered, with the remarkable ease with which hemolysis took place in vitro at the physiological pH.

**Summary**

The clinical history and serological findings in a patient suffering from chronic hemolytic anemia with Raynaud's phenomena and hemoglobinuria are briefly reported. The patient's serum contained a cold antibody in very high concentration, which was active up to a temperature of at least 33.5 C. Its activity in vitro differed from that of the sera of previously reported cases in that the hemolysis of normal red cells was diminished rather than increased by lowering the pH. It is thought probable that the patient's frequent attacks of hemoglobinuria may be correlated with the ease with which hemolysis took place in vitro at the physiological pH.

**Summario in Interlingua**

Es presentate un breve reporto del historia clinic e del constatationes serologic in un patiente de chronic anemia hemolytic associate con hemoglobinuria e phenomeno de Raynaud. Le sero del patiente contineva un anticorpore frigide in un altissime concentration. Illo se monstrava active usque a 33,5 C. o plus. Su activitate in vitro differeva de casos previemente reportate in tanto que le hemolyse de erythrocytos normal esseva diminuite e non augmentate per un reduction del pH. Nos considera como probable que le frequente attaccos de hemoglobinuria suffrite per le patiente es correlatate con le prompte occurringia de hemolyse in vitro con pH physiologic.

**Addendum**

Since this report was written, an almost identical patient has been referred to Dr. J. V. Dacie. Cold agglutinins were present to a titre of 500,000 and were associated with a Donath-Landsteiner type of hemolysin which reacted in the same way to acidification.

The patient described by Sweetnam, Murphy and Woodcock (Brit. Med. J., March 1st, No. 4756: 465, 1952) was also possibly of this type. Cold hemolysins were present to a titre of 4 and cold agglutinins to 256. The effect of acidification on the activity of the hemolysins was, however, not determined.

**References**


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