The Mechanism of Hemolysis in Paroxysmal Cold Hemoglobinuria

III. Erythrophagocytosis and Leukopenia

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THE PHENOMENON of erythrophagocytosis, known for over a half century, is said to have been described first in the blood of a patient with paroxysmal cold hemoglobinuria (PCH). Shortly after the classical studies of Metchnikoff on bacterial phagocytosis, Ehrlich,1 in 1891, observed red cells and red cell fragments in mononuclear leukocytes obtained from a patient's ligatured finger following the production of local hemolysis by chilling.2 This observation was confirmed by Eason3 and others,4-6 and phagocytosis of erythrocytes was shown to occur in vitro during the Donath-Landsteiner reaction.7

There was close parallelism between the development of knowledge concerning the immunologic mechanisms of cold hemoglobinuria and an understanding of the general phenomena of hemolysis and phagocytosis. Savtchenko8 showed that a substance which enhanced phagocytosis appeared in the blood of animals injected with foreign erythrocytes. He mentioned the analogy, later emphasized by others,9-10 between bacteriolytic and hemolytic serums. Donath and Landsteiner7 demonstrated that the abnormal factor responsible for hemolysis in paroxysmal cold hemoglobinuria is in the patient's serum. When Eason observed erythrophagocytosis, he too, pointed to the Metchnikovian theory, observing that there is "nothing new to the idea that an immune bacteriolytic or hemolytic serum induces increased phagocytosis of the cell or micro-organism to which immunity exists."9

Subsequent investigators11,12 have followed in more detail the course of erythrophagocytosis during paroxysms of cold hemoglobinuria, but the manner in which syphilis induces a serum "immune" factor that hemolyzes the patient's own erythrocytes is not known. The hemolysin has been shown to be a heat-stable, water-soluble (pseudoglobulin) gamma-globulin, similar to most other human antibodies,13 which is activated in an unusual antigen-antibody-complement system.14-17 The antibody can be detected and measured because it acts as a hemolysin and causes erythrocytes to be agglutinable by antiglobulin serum. Erythrocytes adsorb antibody when chilled with complement in PCH serum,
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and such sensitized cells are then hemolyzed when warmed with complement (Donath-Landsteiner reaction) or agglutinated when incubated with antiglobulin serum (indirect Coombs test).16, 17

Phagocytosis of these sensitized cells was observed both in vivo and in vitro, and occasioned a review of the earlier studies. Both Uchida11 and Björn-Hansen12 followed a number of patients, and little can be added to their descriptions of the occurrence and course of erythrophagocytosis and leukopenia in vivo. But the in vivo changes may be newly interpreted in the light of recent studies on leukocytes18, 19 and the alarm reaction.20 In vitro studies with sera from 2 patients have provided further data on the mechanism of phagocytosis, data which may be applicable to the other hemolytic anemias in which erythrophagocytosis has been described.21–29

MATERIALS AND METHODS

The case histories of the 2 patients with paroxysmal cold hemoglobinuria have been summarized elsewhere.17 Case 1, a 30 year old white female, had a hemolysin titer of 64, and her attacks were precipitated by exercise in the cold. Case 2, a 27 year old colored male, had a hemolysin titer of 4, and his attacks were easily produced by exposure to cold.

The methods used for the following procedures were those previously described: (1) hemolysin titration, (2) direct Coombs test, (3) indirect Coombs tests (a) and (b), and (4) complement titration. Blood was drawn from Case 1 with saline rinsed syringes, from Case 2 with oil-lined syringes. In one instance (Case 2, third chill) it was felt that some degree of mechanical hemolysis may have raised the level of serum hemoglobin. Serum and urine hemoglobin were measured in a spectrophotometer.* Serum hemoglobin was determined by a modification of the cyanmethemoglobin method of Stadie,3 using large enough samples to provide sufficient color and the control specimen as a blank for color control. The urine specimens were diluted to the same minute volume. The control specimen was used as zero for reading the subsequent specimens, the results being calibrated by comparison with urine specimens to which known amounts of hemoglobin had been added. Leukocyte counts were performed in the usual manner; the differential formula was based on a count of 200 cells.

The methods used for the in vitro experiments are described in the protocols.

OBSERVATIONS IN VIVO

Case 1

An attack was induced in Case 1 by exposure in a cold room at 5 to 6 C. for 25 minutes during which time she exercised for two 5 minute periods by stepping up and down on a stool. Ten minutes after chilling the patient was put to bed and covered with blankets. At this time the serum hemoglobin was 62 mg./100 ml, and erythrophagocytosis was noted in a neutrophil and a monocyte. Although an insignificant drop of the leukocyte count had occurred (7,900 to 7,300), the number of neutrophils decreased from 75.5 per cent to 47.5 per cent of the total count, and the lymphocytes increased from 18 per cent to 45 per cent. Almost an hour after leaving the cold room, the patient had a 5 minute rigor followed by a 30 minute period of chilliness. Two hours after leaving the cold, and 75 minutes after the onset of the reaction, the leukocyte count had risen to 9,900 with 83.5 per cent neutrophils, and erythrophagocytosis was observed in a neutrophil.

It was not possible to repeat this experiment and obtain adequate data. The hematologic changes noted served to introduce the problem and stimulated the observations in Case 2.

* We are indebted to Dr. J. W. Price for these determinations.
Case 2

Although this patient's hemolysin titer was low, an attack was easily induced by placing his hand in ice water. Three such attacks were studied and are represented graphically in figures 1 and 2. Since the results of the first two episodes of chilling were similar, the data from both are shown in figure 1.

In the first experiment, one hand was chilled for 18 minutes and one, simultaneously, for 10 minutes. In the second experiment, both hands were chilled for 10 minutes. Hemolysis occurred promptly, serum hemoglobin levels of 420 and 486 mg./100 ml. being reached 12 and 20 minutes, respectively, after exposure to cold. This hemoglobin was excreted rapidly, the maximum rate of excretion occurring during the interval between 45 and 60 minutes after exposure. Twenty minutes after termination of the first chilling, the patient had a brief rigor, unaccompanied by elevation of temperature, followed by a 15 minute period of chilliness. Although comparable blood changes occurred during the second experiment, the patient did not experience any systemic reaction. His hand, of course, felt cold, and two fingers became swollen.

In addition to hemoglobinemia and hemoglobinuria the most striking phenomena observed were leukopenia and erythrophagocytosis. The changes in the total and differential leukocyte counts are plotted in figure 1 as percentage increases or decreases above or below
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the initial absolute control levels (table 1). The total leukocyte counts fell 67 per cent and 69 per cent in the first and second experiments, respectively. The number of atypical mononuclear cells remained constant during the immediate reaction, but decreased by 37 per cent to 41 per cent in 2 to 6 hours. With the exception of these cells (similar to those seen in infectious mononucleosis), all leukocytes participated in the leukopenia.

The neutrophils, both segmented and unsegmented, the eosinophiles, and the monocytes were equally depressed, all decreasing by about 85 per cent. The lymphocytes decreased by approximately half this amount—48 per cent and 33 per cent in the first and second experiments, respectively. The neutrophils recovered first, there being a 69 per cent increase of the unsegmented forms within 2 hours. In 6 hours, there were no unsegmented cells, but a

![Graph showing changes in hematocrit, total leukocytes, segmented neutrophils, unsegmented neutrophils, eosinophiles, lymphocytes, monocytes, and urine hemoglobin over time.](attachment:image)

**Fig. 2.**—The course of hemoglobinemia and leukopenia during and after third episode of chilling—Case 2.

92 per cent increase in segmented neutrophils had occurred. Slight recovery of monocytes was evident in 2 hours, and they had increased by 116 per cent in 6 hours. Eosinophiles and lymphocytes had increased by only 13 per cent and 23 per cent, respectively, at the end of 6 hours.

Direct Coombs tests with erythrocytes obtained before both of these chilling experiments were positive. Prior to the first, phagocytosis of an erythrocyte by a single neutrophil was observed. Erythrophagocytosis by neutrophils was marked at the height of the hemolytic reaction, at which time phagocytosis by monocytes was observed (fig. 3; table 1). Some phagocytic cells contained whole, apparently undamaged erythrocytes. In others, all stages of erythrocyte destruction were evident, some showing only a clear vacuole the approximate size of an erythrocyte. Although the direct Coombs test remained positive for at least 4 hours, no erythrophagocytosis was present in the 2 hour blood films. The maximum fall in hematocrit had occurred in 2 hours, and neutrophil leukocytosis had begun. The occurrence of erythrophagocytosis, as observed in the peripheral blood, thus appeared to be correlated with the period of active intravascular erythrocyte destruction.
In an attempt to follow these changes more closely, the patient was chilled a third time and leukocyte counts done at 5 and then 15 minute intervals (fig. 2). Although the hematocrit fell, and the patient had a shaking chill 27 minutes after his hand was placed in ice water, hemoglobinemia was moderate and he did not have hemoglobinuria. The leukocyte changes were similar to those observed in the first two experiments. The leukopenia was evident within 5 minutes, was most marked in 10 minutes, and persisted for 135 minutes. Within 10 minutes the total count had decreased by 68 per cent, the neutrophils by 78 per cent, the monocytes by 83 per cent, and the lymphocytes by 46 per cent. This time, the atypical lymphocytes also participated in the drop. After 135 minutes, segmented and unsegmented neutrophils had increased by 11 per cent and 19 per cent, respectively, over the control levels, but the other cells remained depressed. After 5 hours, all but the eosinophiles and lymphocytes had recovered, there being a marked increase in unsegmented neutrophils. The number of erythrophagocytes observed during the reaction was but little more than present in the control smear. The erythrocytes behaved in the direct Coombs test as in the first two experiments.

**Table 1.—Total and Differential Leukocyte Counts after First Two Episodes of Chilling—Case 2**

<table>
<thead>
<tr>
<th>Leukocytes</th>
<th>Control counts</th>
<th>Time after beginning of chilling—minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st chilling</td>
<td>2nd chilling</td>
</tr>
<tr>
<td>Total/mm.(^3)</td>
<td>5,000</td>
<td>7,000</td>
</tr>
<tr>
<td>Differential count/mm.(^3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>2,830</td>
<td>3,990</td>
</tr>
<tr>
<td>Segmented</td>
<td>25</td>
<td>8</td>
</tr>
<tr>
<td>Unsegmented</td>
<td>7,000</td>
<td>7,000</td>
</tr>
<tr>
<td>Eosinophiles</td>
<td>150</td>
<td>140</td>
</tr>
<tr>
<td>Basophiles</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1,250</td>
<td>1,260</td>
</tr>
<tr>
<td>Monocytes</td>
<td>250</td>
<td>594</td>
</tr>
<tr>
<td>Atypical mononuclears</td>
<td>400</td>
<td>664</td>
</tr>
<tr>
<td>% of cells phagocytic</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>Neut.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mono.</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Comment

Hemoglobinemia was induced promptly in Case 2 on three occasions following exposure to cold. Twice the degree of hemolysis was equally marked, but a systemic reaction occurred only once. In the third experiment, the extent of hemolysis was less, but a systemic reaction, albeit milder than the first, was produced. Marked leukopenia developed each time, and erythrophagocytosis was greatest during the first experiment. These results suggest that neither the degree nor type of leukopenia was (1) influenced by the extent of erythrocyte destruction as reflected by serum hemoglobin levels of from 78 to 486 mg./100 ml. or (2) dependent on the occurrence of erythrophagocytosis. It was manifestly impossible to determine that leukocytes had been removed from the peripheral circulation because they were phagocytic, the net result being the observed leukopenia with few phagocytic cells. It was
possible that leukocytes as well as erythrocytes, had been destroyed by lysis. A solution to these and other problems was sought by a number of in vitro experiments.

**Fig. 3.—Erythrophagocytosis in cold hemoglobinuria; in vivo, top, and in vitro, bottom.**

(a and b) Phagocytic neutrophils in blood of Case 2 twenty minutes after beginning of first episode of chilling.

(c and d) Phagocytic neutrophil and monocyte observed after whole blood from Case 2 was chilled and warmed with extra complement (Tube 1, table 2).

**Observations in Vitro**

**Erythrophagocytosis by Whole Blood in the Presence of Additional Complement**

The first two experiments were performed to determine if erythrophagocytosis occurred when whole PCH blood, fortified by extra complement, was chilled and warmed. Blood from Case 2 was tested first; stored serum from Case 1 was then added to normal blood.

**Experiment 1**

Amounts of 1 ml. of fresh heparinized whole blood from Case 2 and from a normal control were added to tubes as indicated in table 2. Because extra complement was usually required to activate this patient's hemolysin, 0.25 ml. of a 1:2 dilution of freshly reconstituted "lyovac" guinea pig serum (approximately 20 units of hemolytic complement) was added to one tube each of patient and control blood. The tubes were chilled in an ice bath for 30
minutes and then warmed in a 37 C. water bath for 30 minutes. Blood films were made before and after chilling and after warming. Two hundred leukocytes on each film were examined to determine the differential counts and the degree of erythrophagocytosis.

There were no significant changes in the differential formulas, the ones after chilling and after warming closely resembling the control counts. The patient's direct Coombs test was positive before chilling, but erythrophagocytosis was not present then or immediately after chilling, although chilling in the presence of complement fixes PCH antibody to the erythrocytes. Phagocytosis in the patient's blood was evident after warming. In the tube with added complement, the supernatant was dark red, and phagocytosis by both neutrophils and monocytes was marked (Tube 1, table 2; fig. 3). There was no visible hemolysis in Tube 2, but a few leukocytes showed erythrophagocytosis.

Table 2.—The Occurrence of Erythrophagocytosis when Whole Blood Was Chilled and Warmed

<table>
<thead>
<tr>
<th>Reagents and results</th>
<th>Tube number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Patient's blood</td>
<td>1.0 ml.</td>
</tr>
<tr>
<td>Normal blood</td>
<td></td>
</tr>
<tr>
<td>Complement</td>
<td>0.25</td>
</tr>
<tr>
<td>Before chilling</td>
<td></td>
</tr>
<tr>
<td>Hemolysis</td>
<td>0</td>
</tr>
<tr>
<td>Phagocytosis</td>
<td>0</td>
</tr>
<tr>
<td>After chilling</td>
<td></td>
</tr>
<tr>
<td>Hemolysis</td>
<td>0</td>
</tr>
<tr>
<td>Phagocytosis</td>
<td>0</td>
</tr>
<tr>
<td>After warming</td>
<td></td>
</tr>
<tr>
<td>Hemolysis</td>
<td>+++</td>
</tr>
<tr>
<td>Phagocytosis</td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>67%</td>
</tr>
<tr>
<td>Monocytes</td>
<td>56%</td>
</tr>
</tbody>
</table>

Experiment 2

Serum from Case 1 that had been stored at -25 C. was tested for its effect on normal blood of the same type. The reagents were added as follows: 0.2 ml. serum, 0.2 ml. of 1:2 guinea pig serum and 0.2 ml. of fresh heparinized whole blood. This mixture was chilled and warmed, smears being made after each step. After warming, hemolysis occurred, and 5 per cent of the neutrophils and 25 per cent of the monocytes showed erythrophagocytosis. Again the differential count was not affected. Thus sera from both patients promoted both hemolysis and erythrophagocytosis.

Occurrence and Rate of Phagocytosis; Absence of Leukolysis

The next four experiments were designed to test the activity of cells and serum after separation and recombination, to determine the rate of occurrence of phagocytosis, to examine the behavior of homologous and heterologous erythrocytes and leukocytes, and to investigate the possibility of lysis of leukocytes.

Experiment 3

Leukocytes, erythrocytes and plasma from fresh heparinized blood from Case 2 were separated by centrifugation. The buffy coat from 8 ml of blood was placed in 1.5 ml of warm
### Table 3. The Occurrence of Erythrophagocytosis with Homologous and Heterologous Erythrocytes, Leucocytes and Plasma

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
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<tbody>
<tr>
<td><strong>Leukocytes:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Patient:</td>
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<td>0.5</td>
<td>0.5</td>
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<td>0.4</td>
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<tr>
<td><strong>Erythrocytes:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Patient:</td>
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<td>0.5</td>
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<td>0.5</td>
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<tr>
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<td>0.25</td>
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<tr>
<td><strong>Plasma:</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Patient:</td>
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<tr>
<td>Control:</td>
<td>0.25</td>
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<td>0.25</td>
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<td>0.25</td>
<td>0.25</td>
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Total leukocyte and differential counts (%) before chilling, B, and after warming, A

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>A</th>
<th>B</th>
<th>A</th>
<th>B</th>
<th>A</th>
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<th>B</th>
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<th>B</th>
<th>A</th>
<th>B</th>
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<tbody>
<tr>
<td>Leukocytes × 10³</td>
<td>2.35</td>
<td>3.25</td>
<td>3.95</td>
<td>3.70</td>
<td>3.00</td>
<td>3.90</td>
<td>3.75</td>
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<td>2.60</td>
<td>2.30</td>
<td>2.95</td>
<td>3.60</td>
<td>2.15</td>
<td>2.90</td>
<td>3.50</td>
<td>3.15</td>
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<tr>
<td>Neutrophils</td>
<td>56.0</td>
<td>42.5</td>
<td>57.5</td>
<td>45.5</td>
<td>60.5</td>
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<td>54.0</td>
<td>61.0</td>
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<td>47.5</td>
<td>49.0</td>
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<tr>
<td>Lymphocytes</td>
<td>30.5</td>
<td>27.0</td>
<td>40.5</td>
<td>21.0</td>
<td>32.5</td>
<td>39.5</td>
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<tr>
<td>Monocytes</td>
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<td>4.0</td>
<td>4.5</td>
<td>8.5</td>
<td>4.5</td>
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<td>6.5</td>
<td>4.5</td>
<td>3.5</td>
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<td>1.0</td>
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<tr>
<td>Phagocytic cells</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>% Neut.</td>
<td>0</td>
<td>31</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>77</td>
<td>0</td>
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<td>2</td>
<td>35</td>
<td>0</td>
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<td></td>
</tr>
<tr>
<td>% Mono.</td>
<td>20</td>
<td>16</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>80</td>
<td>8</td>
<td>0</td>
<td>33</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>50</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Hemolysis</td>
<td>+++</td>
<td>0</td>
<td>+++</td>
<td>0</td>
<td>++</td>
<td>+++</td>
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<td>+++</td>
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saline and held at 37 C. until used. Reagents were added to two tubes as follows: 0.2 ml. of packed erythrocytes, 0.5 ml. of plasma, and 0.25 ml. of 1:2 guinea pig serum. The tubes were chilled in an ice bath for 30 minutes and warmed at 37 C. One-half ml. of buffy coat suspension was added to one tube, warming continued for 30 minutes, and films made. Hemolysis occurred in both tubes, being equally marked in the tube to which no buffy coat had been added. Very few leukocytes were present in the film of this tube. In the smear from the tube containing leukocytes, 44 per cent of the neutrophils and 45 per cent of the monocytes showed erythrophagocytosis. The picture resembled that seen in vivo. The leukocytes contained one and often two or three erythrocytes appearing as vacuoles, fragments or undamaged cells. Control blood similarly treated showed no hemolysis or phagocytosis.

**Experiment 4**

Using a warm stage, it was possible to observe the process of erythrophagocytosis. Blood from Case 2 prepared as in the preceding experiment was used. A hanging-drop preparation was made consisting of one drop each of buffy coat, 1:2 guinea pig serum, and 5 per cent erythrocyte suspension, and two drops of plasma. The slide was chilled at 0 C. for 30 minutes, placed on a microscope stage warmed to 37 C., and observed under oil. Erythrophagocytic leukocytes were present as soon as the slide was brought into focus, the time elapsed since chilling being about 3 minutes. Initially, the leukocytes contained only single erythrocytes. As warming continued, some leukocytes were observed to ingest two and three erythrocytes, and others seemed to be surrounded by red cells.

**Experiment 5**

Plasma, erythrocytes and buffy coat were separated from 20 ml. of fresh heparinized blood from Case 2. The buffy coat was washed twice in warm saline, centrifuged in hematocrit tubes, carefully pipetted from the few remaining erythrocytes and placed in a final volume of 4 ml. of warm saline. The erythrocytes were washed once with saline and 0.4 ml. placed in a tube containing 0.5 ml. of plasma and 0.25 ml. of 1:2 guinea pig serum. The tube was chilled for 30 minutes in an ice bath and then warmed at 37 C. for 30 minutes. An amount of 0.5 ml. of the leukocyte suspension was added, the tube returned to the 37 C. water bath, and films made immediately, after 2 minutes, after 10 minutes and after 15 minutes.

Only a few leukocytes were present in the erythrocyte-plasma-complement mixture, and no phagocytosis of the few erythrocytes still present in the leukocyte suspension had occurred. On addition of the leukocytes, phagocytosis occurred promptly, 16 per cent of neutrophils showing erythrophagocytosis after 2 minutes. Within 10 minutes, 56 per cent of the neutrophils and 45 per cent of the monocytes were phagocytic. The same degree of phagocytosis (53 per cent neutrophils and 39 per cent monocytes) was present after 15 minutes. The amount of hemolysis was similar to that obtained in Experiment 1. There was a slight decrease in the number of neutrophils and an increase in the number of lymphocytes. That this shift was not due to destruction of neutrophils is indicated by the next experiment.

**Experiment 6**

Blood from Case 2 and control blood of the same type was processed as described in Experiment 5 and added to tubes as shown in table 3. The contents were mixed and leukocyte and differential counts done on each tube. The tubes were then chilled in an ice bath for 30 minutes, incubated at 37 C. for 30 minutes, inverted several times, and leukocyte and differential counts again made. The tubes were then centrifuged and read for hemolysis.

As expected, hemolysis occurred in all tubes containing the patient's plasma (table 3). Erythrophagocytosis occurred in these same tubes regardless of the source of erythrocytes or leukocytes, being most marked in the tube containing patient's leukocytes and control erythrocytes. In three of the films made before warming, 1 to 2 per cent of the neutrophils were phagocytic; in 3, 8 to 20 per cent of the monocytes were phagocytic. In the two of these instances which involved control plasma, phagocytosis was not present after warming. Destruction of leukocytes did not occur, for the total and differential leukocyte counts
appear to reflect only sampling variation. It was concluded that the PCH antibody is the essential factor which, in the presence of complement, hemolyzes erythrocytes and initiates erythrophagocytosis by leukocytes.

Relation of Phagocytosis to Hemolysis

The next two experiments were designed to determine whether phagocytosis would occur if hemolysis were prevented by omitting either the cold or warm phase of the Donath-Landsteiner reaction.

Experiment 7

Since chilling is necessary for the fixation of PCH antibody and subsequent hemolysis, erythrophagocytosis may be affected by omission of the cold phase. Serum from Case 2, complement and compatible fresh heparinized whole blood were added to a tube as in Experiment 2. After incubation at 37 C. for 30 minutes a blood film was made, and the tube centrifuged and examined for hemolysis. No hemolysis and no erythrophagocytosis occurred.

Experiment 8

In previous experiments, erythrophagocytosis had not occurred after chilling alone, but this may have been due to suppression of leukocyte activity in the cold. Accordingly, erythrocytes coated with PCH antibody, but not hemolyzed by the addition of complement, were warmed with leukocytes. Reagents were added in amounts comparable to those used for hemolysin titration and the indirect Coombs test (a): 0.2 ml. of serum from Case 2, 0.2 ml. of 1:2 guinea pig serum, and 0.4 ml. of a 5 per cent suspension of normal, group O erythrocytes. This mixture was chilled in an ice bath for 30 minutes, the erythrocytes washed three times with cold saline, and 0.2 ml. of a leukocyte suspension added. A blood film was made, the cells warmed at 37 C. for 30 minutes and another film made. There was a faint trace of hemolysis, and it is possible that some complement was added with the buffy coat. Erythrophagocytosis did not occur although agglutination of the erythrocytes by antiglobulin serum indicated the presence of antibody on these cells. The experiment was repeated using inactivated plasma as the washing and suspending medium. No hemolysis or phagocytosis occurred.

Phagocytosis in Two Other Erythrocyte-Antibody Systems

In the next experiment blood group antibodies were employed to examine the capacity of another human hemolysin to promote erythrophagocytosis.

Experiment 9

One ml. amounts of fresh heparinized group A and O bloods were centrifuged, and the plasma exchanged. Extra complement was added to each tube as 0.25 ml. of 1:2 guinea pig serum. The tubes were incubated at 37 C. for 30 minutes. Agglutination, but no hemolysis, occurred in the tube containing A cells and O plasma. No erythrophagocytosis was observed. The amount of plasma was then increased to 2 ml. and the amount of complement to 0.5 ml. in another preparation. Both hemolysis and agglutination of the A cells occurred, and erythrophagocytosis was marked. All of the neutrophils and 64 per cent of the monocytes showed phagocytosis, most of the neutrophils containing more than one red cell.

Two other experiments were done using different group A and group O donors. Plasma and cells were separated from fresh heparinized blood and added to tubes according to the protocol shown in table 4. The tubes were incubated at 37 C. for 30 minutes, blood films were made and the tubes read for hemolysis and agglutination.

Both anti-A antibodies were lytic (table 4), but more hemolysis occurred with one set of bloods than with the other. The degree of phagocytosis, particularly by neutrophils,
corresponded to the degree of hemolysis. Inactivation of complement by heat prevented hemolysis and decreased, but did not prevent, phagocytosis. The plasma which had been the more lytic when unheated also was more active in promoting phagocytosis when heated.

Thus, in another human hemolytic system, the degree of erythrophagocytosis paralleled the degree of hemolysis. In this instance, antibodies which acted directly as agglutinins also rendered erythrocytes susceptible to phagocytosis. Although the degree of agglutination was comparable, one anti-A agglutinin was more active than another in promoting phagocytosis.

Erythrophagocytosis was sought in one animal hemolytic system.

Experiment 10

On three occasions, anti-sheep cell rabbit serum was added to fresh whole sheep blood and the mixture incubated at 37 C. for 30 minutes. Both agglutination and hemolysis occurred; no phagocytosis was observed.

Table 4.—The Occurrence of Erythrophagocytosis when Anti-A Antibodies Were Used as Hemolysins and Agglutinins

<table>
<thead>
<tr>
<th>Tube Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
</tr>
<tr>
<td>O plasma</td>
</tr>
<tr>
<td>Inactivated O plasma</td>
</tr>
<tr>
<td>Complement</td>
</tr>
<tr>
<td>Inactivated complement</td>
</tr>
</tbody>
</table>

Results with 2 sets of group A and O bloods (Leukocytes and erythrocytes from 1 ml. of group A blood)

| Agglutination | ++ | ++ | ++ | + | ++ | ++ | + | ++ | ++ | ++ | 0 |
| Hemolysis | +++ | +++ | +++ | + | ++ | ++ | ++ | + | ++ | ++ | 0 |
| % of cells phagocytic | 98 | 4.5 | 83 | 1.7 | 0 | 0 | 39 | 0 | 25 | 0 | 0 |
| Neutrophils | 100 | 25 | 100 | 90 | 0 | 0 | 33 | 0 | 0 | 6 | 0 |
| Monocytes | 100 | 25 | 100 | 90 | 0 | 0 | 33 | 0 | 0 | 6 | 0 |

Absence of Phagocytosis following Nonspecific Damage to Erythrocytes

Experiment 11

One ml. of fresh whole blood was adjusted to pH 5.0, another 1 ml. to pH 6.1, and the two tubes incubated at 37 C. for 30 minutes. At the more acid pH, hemolysis produced a dark red supernatant; slight hemolysis occurred in the other tube. No erythrophagocytosis was observed in either tube.

Experiment 12

Hypotonic saline was used to damage erythrocytes by adding 0.1 ml. of red cells to 0.5 ml. 0.40 per cent NaCl, shaking for 10 minutes and centrifuging. Hemolysis resulted. The supernatant was removed; 0.2 ml. of a leukocyte suspension in 0.9 per cent NaCl solution was added to the remaining red cells and incubated at 37 C. for 30 minutes. Additional hemolysis resulted, but no erythrophagocytosis was observed.
Discussion

The ease with which a hemolytic reaction can be induced in most patients with paroxysmal cold hemoglobinuria provides a unique opportunity for observing the hematologic changes that accompany the intravascular hemolysis characteristic of this disease. That leukopenia and erythrophagocytosis occur simultaneously with hemoglobinemia has long been known, but there has been no agreement as to the mechanisms involved. Erythrophagocytosis and leukopenia may be considered separately as simultaneous, but unrelated events, or collectively, as cause and effect phenomena. The theories regarding leukopenia will be considered first.

The most extensive in vivo studies have been reported by Uchida. He observed a slight leukocytosis and neutrophilia, similar to that found in 6 controls, immediately on exposure to cold. The leukocyte changes which promptly followed he divided into two phases: (1) leukopenia, neutropenia and relative lymphocytosis and (2) leukocytosis, neutrophilia and relative lymphopenia. The most intense leukopenia developed within 5 to 20 minutes, the percentage drop ranging from 25 to 72. In 3 cases the absolute number of neutrophils decreased to 152, 253 and 347 per cu. mm., the lowest levels in the group. The duration of leukopenia usually varied from 10 to 40 minutes, but in 3 cases, it persisted for 2 hours. About 2 hours after the beginning of hemolysis, leukocytosis was evident. Uchida stated that the leukocyte changes were not observed until hemoglobinemia appeared. Since he found no evidence of leukolysis in vitro, and had observed erythrophagocytosis, he surmised that leukopenia resulted because leukocytes, loaded with products of hemolysis, lodged in such organs as lungs, liver and spleen.

The changes in the 12 patients chilled by Uchida were essentially duplicated in the 4 patients chilled by Björn-Hansen, but he proposed a different explanation for the leukopenia. Björn-Hansen noted that leukopenia occurred whether or not the patient had a generalized reaction, hemoglobinuria, or hemoglobinemia, a finding confirmed by MacKenzie and Totterman, and partially substantiated by the third episode of chilling of Case 2. Further, he interpreted as disintegration the changes seen in leukocytes on films made during an Ehrlich test. He pointed out that the shift to the left in the differential count indicated the formation of new leukocytes, but that the leukocyte count remained low. He concluded, therefore, that the leukopenia was the result of leukolysis.

In this respect, Björn-Hansen also differed from the Widal school, although he accepted the concept that the generalized reaction exhibited many of the stigmata of anaphylaxis. Widal, Abrami and Brissaud stated that the clinical picture was not only analogous but identical to that sometimes produced by a second injection of therapeutic serum. Montagnani later championed the anaphylactic nature of the "hemoclastic crisis," listing, in addition to leukopenia, hypotension, prolonged clotting time, decreased clot retraction and reduction of complement as features common to both. This concept is intriguing because an antigen-antibody-complement system is involved, but the analogy often breaks down. Hypertension, rather than hypotension, occurs; the changes in the coagulability of the blood are inconstant; eosinopenia, rather than eosino-
philiala, is the rule. In a sense, these patients do have “a specific reaction to an antigenic substance,” their own erythrocytes, but that reaction differs considerably from classical anaphylaxis. Constitutional symptoms, leukopenia and eosinopenia are also induced by the injection of bacterial pyrogens, but these changes in cold hemoglobinuria can no more logically be attributed to a pyrogenic reaction than to anaphylaxis.

The blood also shows characteristic changes during the alarm reaction, but the activation of pituitary-adrenal cortex mechanisms by exposure to cold does not account for the leukopenia and subsequent leukocytosis. Controls exposed to comparable degrees of cold showed only a slight leukocytosis. Men immersed in cold water developed leukocytosis and eosinopenia, both moderate. The occurrence of rapid erythrocyte destruction with the consequent release of hemoglobin would, of course, be a more “alerting” stimulus. Indeed, leukopenia is seen in certain acquired hemolytic anemias and paroxysmal nocturnal hemoglobinuria. Early leukocyte changes in association with hemolytic transfusion reactions in humans have not been stressed, leukocytosis at 5 hours apparently being the earliest change noted. During such reactions in dogs transient leukopenia and slight erythrophagocytosis were observed during the first few minutes along with the prompt development of hemoglobinemia. Hemoglobinemia, however, is said not to be essential for the production of leukopenia in cold hemoglobinuria, although Uchida always found hemoglobinemia prior to the leukocyte drop. Dissolution of leukocytes as a result of such pituitary-adrenal stimulation, if it occurred at all, would produce primarily a lymphopenia in addition to eosinopenia.

The second phenomenon to be considered, erythrophagocytosis, cannot be attributed to either anaphylaxis or the alarm reaction. A variety of cells distributed widely throughout the body may appear phagocytic. Since interest in phagocytosis has been related largely to bacterial invasion, most effort has been directed toward understanding bacterial phagocytosis in animal tissues. Many of the early observations of phagocytosis of erythrocytes were made in patients with cold hemoglobinuria, although erythrophagocytosis was noted at about the same time in patients with pneumococcal sepsis, bacterial endocarditis, and malaria. Erythrophagocytosis has now been observed in a wide variety of conditions including typhoid fever, cholera, erysipelas, leukemia, pernicious anemia, mushroom and potassium chlorate poisoning, dermatomyositis and scleroderma. In addition to producing L. E. cells, plasma from a patient with lupus erythematosus has been shown to stimulate myeloid leukocytes to phagocytize erythrocytes.

It was early postulated that phagocytosis played an important part in the removal of degenerated erythrocytes from the circulation, for phagocytosis was recognized as a physiologic function of the cell apart from the disposal of invading parasites. This concept visualized the chief application of phagocytic activity to be “the removal of a variety of particulate matter which, emanating from an endogenous or exogenous source, consists of substances which are useless or actually harmful to the normal physiologic processes of the body.” Although injury to erythrocytes cannot be demonstrated in all diseases in which erythro-
phagocytosis is found, such injury is probably the common factor responsible for the phagocytosis. Erythrophagocytosis has been frequently observed in certain hemolytic anemias,\textsuperscript{21-29} diseases in which damage to red cells is apparent, and particularly in hemolytic disease of the newborn,\textsuperscript{22, 24, 25, 27} a disease in which antibodies are demonstrable on the surface of erythrocytes.\textsuperscript{55} In none of these conditions is erythrophagocytosis as marked as in cold hemoglobinuria. In PCH, phagocytosis occurs, both in vivo and in vitro, when hemolysis occurs. In the human isoantibody system, when erythrocytes are agglutinated in vitro, phagocytosis occurs; phagocytosis increases when these sensitized cells are lysed in the presence of complement. These observations conform to the current tenets regarding the opsonic power of serum: opsonization depends on the cooperation of two components, thermostable antibody and thermolabile complement. The antibody by itself can opsonize, but its effect is enhanced by the presence of complement, which, alone, is incapable of inducing phagocytosis.\textsuperscript{25-56} It would appear that in cold hemoglobinuria and other diseases associated with hemolysis or other red cell injury, phagocytosis is one mechanism which operates to rid the blood of damaged and useless erythrocytes.

Could the trapping of phagocytic leukocytes in organ capillaries account for the leukopenia? The leukopenia of both anaphylaxis\textsuperscript{57} and pyrogenic reactions\textsuperscript{36} has been attributed to storage of leukocytes in the lungs. Ichida's idea that phagocytic leukocytes are trapped in the capillaries of various organs merits consideration in the light of more recent work on the capacity of organs, particularly the lung, to filter leukocytes.\textsuperscript{18-19} If transfused leukocytes lodge in the lungs and cause normal leukocytes to be similarly stuck,\textsuperscript{55, 10} it is conceivable that distended phagocytes likewise become trapped. In cold hemoglobinuria neutrophils and monocytes are phagocytic, and various observers have emphasized the neutropenia and relative lymphocytosis. Actually, all leukocytes participate in the leukopenia, but the lymphocytes are least affected. Possibly the sticking and disintegration of the phagocytic leukocytes initiates a chain reaction which traps the nonphagocytic cells.

The in vivo observations in Case 2 do not fully support this hypothesis, for the degree of leukopenia was not related directly to either the extent of intravascular hemolysis or the amount of erythrophagocytosis. Yet maximum phagocytosis did occur at the time of maximum erythrocyte destruction, the phagocytic leukocytes disappearing from the peripheral blood after two hours. The in vitro observations do support the hypothesis, for the amount of erythrophagocytosis was directly related to the degree of hemolysis. Phagocytosis occurred rapidly and reached its maximum at a time consistent with the development of intense leukopenia in vivo, that is, within 5 to 20 minutes. The absence of leukolysis in vitro makes in vivo destruction of leukocytes by the PCH antibody a less attractive hypothesis.

It is likely that some mechanism common to such states as anaphylaxis, pyrogenic and alarm reactions contributes to the leukopenia of paroxysmal cold hemoglobinuria. In the light of present knowledge, it may be stated that leukopenia occurs in persons with cold hemoglobinuria as a part of the paroxysm of hemolysis, and that erythrophagocytosis probably plays an important role by inducing the trapping of leukocytes in organ capillaries.
SUMMARY

Attacks of hemoglobinemia were induced in 2 patients with paroxysmal cold hemoglobinuria (PCH). The occurrence of slight neutropenia and erythropagocytosis in the first patient occasioned more detailed observations of these phenomena in the second. The in vivo changes observed in Case 2 following three episodes of chilling were similar to those described by earlier investigators. Marked hemoglobinemia and hemoglobinuria occurred twice; mild hemoglobinemia once. The maximum number of phagocytes was observed at the time of maximum hemolysis. Leukopenia developed promptly during each attack, but the degree of leukopenia was not correlated with either the intensity of erythrocyte destruction or with the extent of phagocytosis.

Neutrophils and monocytes—the phagocytic cells—and eosinophiles were equally depressed, all decreasing by from 75 per cent to 80 per cent. Lymphocytes decreased by 33 per cent to 48 per cent. The neutrophils recovered within 2 hours, but the eosinophiles and lymphocytes remained depressed for 5 hours.

When PCH serum was chilled and warmed with complement and homologous or heterologous erythrocytes and leukocytes, erythropagocytosis occurred, reaching its maximum at a time consistent with the development of leukopenia in vivo. Although PCH antibody was fixed to erythrocytes in the cold phase of the Donath-Landsteiner reaction, phagocytosis did not occur if complement, with the attending hemolysis, was absent in the warm phase.

Erythropagocytosis was also produced in vitro when human isoaotibodies acted as either agglutinins or hemolysins. Phagocytosis was most marked when the sensitized cells were lysed, the degree of phagocytosis corresponding to the degree of hemolysis.

The in vivo and in vitro observations suggest that leukopenia occurs in persons with cold hemoglobinuria as part of the paroxysm of hemolysis, and that erythropagocytosis operates to remove damaged erythrocytes and probably promotes the trapping of leukocytes in organ capillaries.

REFERENCES

HEMOLYSIS IN PAROXYSMAL COLD HEMOGLOBINURIA

15 —: Paroxysmal hemoglobinuria; a review. Medicine 8: 159, 1929.
31 WIDAL, F., ABRAMI, P. AND BRISSAUD, E.: L'autoanaphylaxie. Son rôle dans l'hémo
globinurie paroxystique. Traitement anti-anaphylactique de l'hémo
36 BENNETT, I. L. AND BEeson, P. B.: The properties and biologic effects of bacterial pyro
54 Oxan, C. A. and Sabin, F. R.: Normal and pathological fragmentation of red blood cells, the phagocytosis of these fragments by desquamated endothelial cells of the blood stream; the correlation of the peroxidase reaction with phagocytosis in monocells. J. Exper. Med. 45: 839, 1926.
The Mechanism of Hemolysis in Paroxysmal Cold Hemoglobinuria: III. Erythrophagocytosis and Leukopenia

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