In Vitro Erythrophagocytosis in Acquired Hemolytic Anemia

By William H. Zinkham, M.D.* and Louis K. Diamond, M.D.

In vitro erythrophagocytosis has been studied in 4 children with acquired hemolytic anemia. In 3 of these children the hemolytic anemia was of the so-called primary or idiopathic variety; in the remaining child it was associated with another disease.

Peripheral erythrophagocytosis has been described in a variety of hemolytic disorders (see table 1), and in these reports 2 types of erythrophages have been observed. The first is characterized by cells which are thought to originate from the reticulo-endothelial system, and to them has been given the name clasmatocyte, histiocyte or endothelial cell. They are regarded as cellular elements not normally occurring in the peripheral blood stream, and morphologically they can be differentiated from the circulating phagocytes. The second is characterized by normally occurring peripheral leukocytes (neutrophiles, eosinophiles and monocytes) which have ingested red cells. These are usually seen in disorders associated with episodes of acute hemolysis: potassium chlorate poisoning; transfusion reactions, erythroblastosis fetalis and paroxysmal cold hemoglobinuria.

This second type of erythrophage has also been found to develop in vitro. Huck, while studying wet preparations of peripheral blood from patients with sickle cell disease, observed the active ingestion of red cells by monocytes. Similar observations were made by Rowley in a case of subacute bacterial endocarditis. Other workers, using the hemolysin occurring in cases of paroxysmal cold hemoglobinuria, have been able to sensitize normal red cells in the presence of complement so that they are phagocytized by the white cells of normal blood. Hektoen, by reproducing in the test tube the conditions of a mismatched transfusion, has been able to show that marked erythrophagocytosis occurs when fresh human serum is added to an incompatible buffy coat. For example, if one adds fresh group O serum to a group A, B, or AB buffy coat, the red cells are altered by a thermostable serum factor in the presence of complement and are then ingested by the surrounding leukocytes. To this thermostable serum factor Hektoen has given the name hemopsonin.† This thermostable serum factor has also been found in human serum and in cases of erythroblastosis fetalis. These "hemopsonins" or thermostable serum factors are the same as the anti-A or anti-B antibodies (unpublished data).

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† Recent work by us has shown that these "hemopsonins" or thermostable serum factors are the same as the anti-A or anti-B antibodies (unpublished data).
phenomenon has also been observed in vivo in people who have received trans-
fusions of mismatched blood.\textsuperscript{11}

During the past year we have studied 4 children with idiopathic acquired
hemolytic anemia. Two of these children showed an occasional erythrophage
on direct smears of their peripheral blood, and this finding led to the study of all
the bloods of these patients for the occurrence of erythrophagocytosis by in
vitro methods. The purpose of this paper is to report the results of this study
and to suggest the possibility of using in vitro erythrophagocytosis as an aid
in demonstrating abnormalities of red cells in patients with acquired hemolytic
anemia.

\textbf{Table 1.—Hemolytic Disorders in Which Erythrophages Have Been Described in
Peripheral Blood}

I. Congenital Red Cell Defects
   A. Sickle cell disease\textsuperscript{1}

II. Acquired Red Cell Defects
   A. Secondary to chemicals:
      1. Potassium chlorate\textsuperscript{2}
      2. Naphthalene\textsuperscript{3}
   B. Secondary to infectious agents:
      1. Bacterial
         a. Sub-acute bacterial endocarditis\textsuperscript{4, 5}
         b. Typhoid fever\textsuperscript{6}
         c. Tuberculosis\textsuperscript{7, 8}
         d. Streptococcal septicemia\textsuperscript{9}
         e. Meningococcemia\textsuperscript{9}
      2. Protozoal\textsuperscript{10}
         a. Malaria
         b. Trypanosomiasis
         c. Ankylostomiasis
   C. Secondary to serum antibodies:
      1. Transfusions with incompatible blood\textsuperscript{11-12}
      2. Erythroblastosis foetalis\textsuperscript{14-17}
      3. Paroxysmal cold hemoglobinuria\textsuperscript{18-20}
   D. Symptomatic:
      1. Leukemia\textsuperscript{21}
   E. Idiopathic:
      1. Acute (Lederer’s type)\textsuperscript{22, 23}

\textbf{Methods}

Five milliliters of venous blood were placed in a dry centrifuge tube containing either
0.6 cc. of 0.1 M. sodium oxalate or 1 mg. of sodium heparin (Roche)\textsuperscript{*}. The sample was al-
lowed to stand at room temperature (about 23 C.) for 30 minutes and the blood was cen-
trifuged at 2,000 r.p.m. for 10 minutes. The buffy coat was drawn off with a capillary pipet
and placed in test tubes (\(\frac{1}{2}\) " x 3") which were then incubated at 37 C. for a period of two
hours. Where the peripheral white count was over 10,000, whole blood was used instead of
the buffy coat. Three cover slip smears were made: the first after whole blood or buffy
coat had stood at room temperature for 30 minutes; the second after incubation of

\* The type of anticoagulant used apparently had no effect on the mechanism of in
vitro erythrophagocytosis, for similar erythrophagocytic indexes were obtained with ox-
lated, heparinized, or defibrinated blood. Blood collected in a siliconized system also gave
similar results.
whole blood or buffy coat for one hour; the third after incubation for two hours. These
smears were stained with Wright's stain. If erythrophagocytosis occurred, it was usually
observed on the first smear and had become more prominent on the second smear, and still
more marked on the third. In most instances the second and third smears were not neces-
sary for the rapid detection of erythrophages.

To determine a numerical value for the degree of phagocytic activity, 250 consecutive
phagocytic leukocytes (neutrophiles, eosinophiles and monocytes) were counted on each

TABLE 2.—A Summary of the 4 Children with Acquired Hemolytic Anemia Studied for
In Vitro Erythrophagocytosis, together with Tests to Demonstrate Abnormal Antibodies
in the Serum and on the Red Cells of These Patients. The Results Listed were Obtained on
the First Day of Observation of Each Patient

<table>
<thead>
<tr>
<th>Patient, Diagnosis, and Period of Observation</th>
<th>Erythrophagocytic Index on Direct Smer of Peripheral Blood</th>
<th>Erythrophagocytic Index of Buffy Coat Incubated 1 hr. at 37 C.</th>
<th>Mechanic-al Frailty of Incubated Red Cells</th>
<th>Acid Agglutinin and Hemolysin Tests</th>
<th>Titer Direct Coombs Test</th>
<th>Indirect Coombs Test</th>
<th>Trypsinized Red Cell Test</th>
<th>Course</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. H.—acute idiopathic aq. hemolytic anemia (8 months)</td>
<td>&lt;0.1%</td>
<td>62.9%</td>
<td>25.6%</td>
<td>0</td>
<td>1:256</td>
<td>2+</td>
<td>0</td>
<td>Spontaneous recovery over a period of 2 months</td>
</tr>
<tr>
<td>R. T.—chronic idiopathic aq. hemolytic anemia (7 months)</td>
<td>0.1%</td>
<td>27.2%</td>
<td>40.4%</td>
<td>0</td>
<td>1:2048</td>
<td>1+</td>
<td>4+</td>
<td>Relapsed after 2 courses of cortisone therapy and splenectomy. Now being maintained on oral cortisone</td>
</tr>
<tr>
<td>N. K.—acute idiopathic aq. hemolytic anemia (6 months)</td>
<td>0</td>
<td>0</td>
<td>26.8%</td>
<td>0</td>
<td>1:1024</td>
<td>0</td>
<td>4+</td>
<td>Relapsed after 1 course of cortisone therapy. Splenectomy done during 3rd month observ. Well since</td>
</tr>
<tr>
<td>L. P.—rheumatoid arthritis with aq. hemolytic anemia (3 weeks)</td>
<td>0</td>
<td>4.6%</td>
<td>28.2%</td>
<td>0</td>
<td>1:64</td>
<td>0</td>
<td>2+</td>
<td>Anemia temporarily improved after course of ACTH</td>
</tr>
</tbody>
</table>

of two paired cover slips, a total of 500 cells in all. The number of phagocytic cells con-
taining red cells was divided by 500, and this value was called the erythrophagocytic index.

Methods employed for doing peripheral counts, for direct Coombs test, indirect Coombs test,
trypsinized red cell test, acid agglutinin test and acid hemolysin test have been de-
scribed elsewhere. The direct Coombs method and the Coombs sera used in these studies
were the same as those used by Gardner. In doing the indirect Coombs test and trypsinized
red cell test, the patient's undiluted serum was used, and no attempt was made to find the
titer of serum at which these tests became negative.
RESULTS

In Vitro Erythrophagocytosis in Normal Blood

The bloods of 95 healthy adults were studied for in vitro erythrophagocytosis. Most of these individuals were selected at random from blood bank donors, and all had hemoglobin values of 12 Gm. or above.

Sixty-five of these bloods were collected in sodium oxalate, while the remaining 30 were collected in sodium heparin. Smears of the buffy coats were made after they had been incubated at 37 C. for one hour. Approximately two to three thousand phagocytic leukocytes were viewed with the high dry lens (a total of about two hundred thousand cells), and out of this entire number only six definite erythrophages were seen. The cells phagocytizing were monocytes, and a neutrophilic erythrophage was never seen in the incubated buffy coats of normal blood. Occasionally a monocyte containing a large vacuole was observed, but even if these were counted as erythrophages, the erythrophagocytic index of each preparation was always less than 0.1 per cent, i.e., less than one erythrophage was seen per one thousand phagocytic leukocytes.

In Vitro Erythrophagocytosis in Diseases Other than Acquired Hemolytic Anemia

In vitro erythrophagocytosis was looked for in the blood from 32 children who had clinical conditions not associated with an acquired hemolytic anemia. These patients were selected at random from the Hematology Clinic, and among them were cases of iron deficiency anemia, idiopathic thrombocytopenic purpura, infectious mononucleosis, hypoplastic anemia, megaloblastic anemia dis-
ease, and infectious hepatitis. Many of them had moderate to severe anemia, but none had signs of increased breakdown of red cells, and none had a positive direct Coombs test.

The number of erythrophages seen in the buffy coats of the blood from these patients was no greater than the number seen in the buffy coats of blood from normal adults. The erythrophagocytic index of each preparation was found to be less than 0.1 per cent.

**In Vitro Erythrophagocytosis in Cases of Acquired Hemolytic Anemia**

Four cases of acquired hemolytic anemia were studied for in vitro erythrophagocytosis. The results of this study, together with tests to demonstrate

<table>
<thead>
<tr>
<th>Titer Direct Coombs Test</th>
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<td>1:2048</td>
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<table>
<thead>
<tr>
<th>Indirect Coombs</th>
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<table>
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<tr>
<th>E-P Index Buffy layer incubated 2 hr.</th>
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<tr>
<td>50</td>
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<table>
<thead>
<tr>
<th>E-P Index Direct Smear</th>
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<tr>
<td>0.1%</td>
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<table>
<thead>
<tr>
<th>R.B.C. millions</th>
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<td>800</td>
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<table>
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<tr>
<th>Abs Retic count</th>
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<td>400</td>
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<tr>
<th>Transfusions 200 packed R.B.C.</th>
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<td>200</td>
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<table>
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<tr>
<th>ACTH 250 Cortisone 100</th>
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<tr>
<td>100</td>
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**Chart 2.—Effect of cortisone therapy and splenectomy on the erythrophagocytic index** (E-P index) of the incubated buffy coat in Case R. T.

abnormal antibodies in the serum and on the red cells of these patients, are presented in table 2. Two of the 4 children had an occasional erythrophage in the peripheral blood, while 3 of the 4 showed moderate to marked erythrophagocytosis when their buffy coats were incubated at 37°C for one hour (figs. 1 and 2). One of the four children, Case N. K., whose hemolytic studies were similar to those of Case L. P., never showed erythrophages, even when the buffy coat was incubated in vitro.

In Cases E. H. and R. T. the relationship between the erythrophagocytic index and the presence of abnormal antibodies in the sera and on the red cells was followed for a period of three months. These findings in Case E. H. who was followed throughout the entire course of his hemolytic anemia are listed on chart 1. It can be seen that as the positive direct Coombs and indirect Coombs
tests disappeared spontaneously without therapy the erythrophagocytic index of the incubated buffy coat became zero. Also it can be seen that in vitro erythrophagocytosis persisted for a period of two months after erythrophages were no longer observed on direct smears of peripheral blood.

The effect of cortisone therapy and splenectomy on in vitro erythrophagocytosis was observed in Case R. T. (chart 2). When enough cortisone was given to control the hemolytic process, the erythrophagocytic index was moderately reduced. During this period there was a slight decrease in titer of the direct Coombs test and the indirect Coombs test became negative. When cortisone was discontinued and activity of the hemolytic process increased, the erythrophagocytic index rose to 30 per cent, a value obtained before the institution of transfusion therapy. At this time there was a slight increase in the titer of direct Coombs test and the indirect Coombs test again became positive. Following a second course of cortisone begun just before and continued after splenectomy, the erythrophagocytic index was moderately reduced, and the red count rose to a normal level. About three weeks after splenectomy the activity of the hemolytic process increased, the erythrophagocytic index rose from 5 to 30 per cent, and the red count dropped to three million. These latter observations were made during a period in which the patient received no blood transfusions, and therefore the increased erythrophagocytic index could not have been secondary to blood group incompatibilities introduced by transfusions.* In fact, in no patient could blood transfusions, if compatible, be related to subsequent erythrophagocytic activity.

A Description of the Process of In Vitro Erythrophagocytosis

Wet preparations of defibrinated blood from Case E. H. were studied in a sealed cover slip preparation with the oil immersion lens at room temperature (about 23 C.), and the following stages in the process of erythrophagocytosis were observed. In the first stage one or more red cells became adherent to the surface of the white cell (figs. 2, 3, 6), and at times this adhesiveness of the red cells to the white cells was so marked that almost all of the red cells in the preparation appeared agglutinated.† This clumping of the red cells occurred only around neutrophiles and monocytes and was never seen around lymphocytes. In the second stage of erythrophagocytosis the white cell projected a thin stream of cytoplasm, a pseudopod, around the red cell that was to be ingested (fig. 4), and during the third stage this red cell was swept into the cytoplasm of the white cell (fig. 5). Stages 2 and 3 were repeated by the same white cell until its cytoplasm contained 2 to 8 red cells (figs. 6, 8, 9). In the fourth stage the ingested red cells lost their structure and faded into the cytoplasm of the white cell (figs. 10, 11). In the final stage the phagocytizing white cells became immobile, and on stained smears only nuclear fragments of these cells were seen (fig. 12).

* This patient received only transfusions of washed packed Group 0, Rh-negative red cells during the period of observation.
† This clumping of red cells around white cells in patients with hemolytic anemia showing in vitro erythrophagocytosis may produce false positive tests for direct Coombs and serum auto-agglutinins if the white cells are not completely removed from the red cells.
Fig. 1.—Direct smear of peripheral blood of case E. H. on day of admission to hospital. 500X

Fig. 2.—Smear of defibrinated venous blood after standing at 37 C. for one hour. From Case E. H. and done on same day as smear in figure 1. Note many polymorphonuclear neutrophilic erythrophages and clumping of red cells around these cells. 500X

Fig. 3.—Marked clumping of red cells around polymorphonuclear neutrophile. 2000X

Fig. 4.—A monocyte projecting a thin stream of cytoplasm around a red cell that is to be ingested. 2000X
DISCUSSION

It has been found that marked erythrophagocytosis occurs when the buffy coats of patients with acquired hemolytic anemia are incubated in vitro. The degree of in vitro erythrophagocytosis observed in these patients was quite striking. In a control series of 95 normal adults the erythrophagocytic index of the incubated buffy coat was never greater than 0.1 per cent, while in three of four patients with idiopathic acquired hemolytic anemia erythrophagocytic indices of 5 to 80 per cent were observed. All 4 of these patients had abnormalities of their sera and red cells as demonstrated by positive direct Coombs test, indirect Coombs test, trypsinized red cell test and increased mechanical fragility of incubated red cells. Only 3, however, showed in vitro erythrophagocytosis.

Other workers have shown that in vitro erythrophagocytosis occurs when red cells are damaged or altered by antibodies. When major blood group incompatibilities are produced in the test tube the red cells are altered by the anti-A or anti-B antibodies, and are then ingested by the surrounding leukocytes. In the case of paroxysmal cold hemoglobinuria this alteration of the red cells is the result of a cold hemolysin.

In our series of cases the factor or factors altering the red cells have not been as clearly defined. Even though all 4 patients had positive direct Coombs tests and increased mechanical fragility of incubated red cells, only 3 had in vitro erythrophagocytosis. This may indicate that factors other than those producing a positive direct Coombs test are responsible for in vitro erythrophagocytosis. There was a direct relationship, however, between the direct Coombs test and the degree of in vitro erythrophagocytosis when these two findings were present in the same patient. In Case E. H. in vitro erythrophagocytosis persisted until the direct Coombs test became negative. This suggests that in vitro erythrophagocytosis may be used as another method of demonstrating abnormalities of the red cells in patients with acquired hemolytic anemia.

Another point of interest in this study was the marked difference between the erythrophagocytic index of direct smears and that of the incubated buffy coats. In Case E. H. erythrophagocytes were found in incubated buffy coats for a period of two months after they were longer present on direct smears of peripheral blood; in Case L. P. the erythrophagocytic index of the incubated buffy coat

FIG. 5.—A monocyte drawing a red cell into its cytoplasm. 2000X

FIG. 6.—A polymorphonuclear neutrophilic erythrophage containing two red cells. Note clumping of red cells around the erythrophage. 2000X

FIG. 7.—An eosinophilic erythrophage containing one red cell. 2000X

FIG. 8.—A cluster of three polymorphonuclear neutrophilic erythrophages, two containing two red cells, and one containing one red cell. 2000X

FIG. 9.—A monocyctic erythrophage containing eight red cells. 2000X

FIG. 10.—A polymorphonuclear neutrophilic erythrophage filled with many red cells which have lost their structure. This cell is approximately three times the size of the polymorphonuclear neutrophile in figure 3. 2000X

FIG. 11.—A polymorphonuclear neutrophilic erythrophage filled with many red cells which have lost their structure. Note large size of this cell. 2000X

FIG. 12.—Nuclear remnants of a polymorphonuclear neutrophilic erythrophage. 2000X

Note: Figures 3 to 12 were obtained from smears of defibrinated blood of Case E. H. which had stood at room temperature (23 C.) for a period of 15 to 30 minutes.
IN VITRO ERYTHROPHAGOCYTOSIS

was 4.6 per cent, but erythrophages were never observed on smears of peripheral blood.

At present no direct information is available to explain this marked difference between the erythrophagocytic indexes of direct smears and incubated buffy coats. Indirect evidence, however, provides a possible explanation for this difference. In the test tube erythrophages containing two to eight red cells are three to four times the size of normal circulating monocytes and polymorphonuclear neutrophiles. These same cells are also very fragile, and frequently on smear only nuclear remnants of these cells remain. Therefore it seems possible that most of the peripheral erythrophages formed in vivo are broken up in the circulation or filtered out in the sinusoids of the liver and spleen, and the lung capillaries, and the likelihood of seeing these cells on direct smears of peripheral blood is very slight. Peripheral erythrophages formed in vitro, however, are spared from the mechanical trauma and the filtering systems of the circulation, and consequently appear in greater numbers on smears of blood incubated in the test tube.

Another explanation for the marked difference between the erythrophagocytic indexes of direct smears and incubated buffy coats is that the number of peripheral erythrophages (i.e., neutrophiles, monocytes, and eosinophiles which have ingested red cells) formed in vitro is greater than the number formed in vivo. In vivo, many of the damaged or altered red cells are removed from the circulation by the stationary macrophages of the reticulo-endothelial system, and it is only when these stationary macrophages are presented with a very large number of altered red cells that peripheral erythrophages are formed, e.g., during episodes of acute hemolysis in cases of paroxysmal cold hemoglobinuria, potassium chlorate poisoning, or transfusion reactions. In vitro there is no competition between the stationary macrophages and the peripheral phagocytes for the damaged or altered red cells. These red cells are exposed only to the peripheral phagocytes, and therefore the chances of observing peripheral erythrophages in the test tube are much greater.

All of these factors favor the appearance and preservation of erythrophages in the test tube, and also explain why in vitro erythrophagocytosis may be observed in other conditions where there is damage or alteration of the red cells—infec tions, toxins, etc.—even when erythrophages are not seen on direct smears of peripheral blood. On the other hand, it seems possible that even when in vitro phagocytosis is not demonstrable, the increased phagocytic activity of fixed tissue macrophages and peripheral phagocytes (in vivo) toward altered red cells in certain diseases may explain development of milder degrees of anemia not associated with obvious signs of excessive hemolysis.

SUMMARY

It has been found that marked erythrophagocytosis occurred when the buffy coats of 3 patients with idiopathic acquired hemolytic anemia were incubated at 37 C. for one hour. It is suggested that this technic may be used as another in vitro method of demonstrating abnormalities of the red cells in patients with acquired hemolytic anemia, as well as other conditions in which there is damage or alteration of the red cells.
REFERENCES

In Vitro Erythrophagocytosis in Acquired Hemolytic Anemia

WILLIAM H. ZINKHAM and LOUIS K. DIAMOND