The Combined Study of Agglutination, Hemolysis and Erythrophagocytosis

With Special Reference to Acquired Hemolytic Anemia

By James A. Bonnin and Lawrence Schwartz

Some seventy years ago, Ehrlich, in 1881, first described erythrophagocytosis in a case of paroxysmal hemoglobinuria. Since then his observations of in vivo erythrophagocytosis in the peripheral blood have been confirmed and extended by numerous other investigators. Without attempting an exhaustive review of the literature, some of the conditions in which this phenomenon has been described are: paroxysmal cold hemoglobinuria, incompatible blood transfusions, hemolytic disease of the newborn, pernicious anemia, sickle cell anemia, acquired hemolytic anemia, plasma cell leukemia, following splenectomy, in chemical intoxications such as potassium chlorate poisoning, protozoal infestations, and in a variety of bacterial infections including pneumococcal sepsis and subacute bacterial endocarditis.

Erythrophagocytosis has also been studied in vitro by various investigators. Hektoen was able to demonstrate it by the addition of incompatible human serum to mixtures of erythrocytes and leukocytes. Boerner and Mudd developed a method of quantitating the phenomenon. Wright and co-workers have recently described a method employing tissue culture macrophages. Other observers have described the occurrence of erythrophagocytosis in vitro in cases of paroxysmal cold hemoglobinuria and similar in vitro observations have recently been made in cases of acquired hemolytic anemia.

Since many of these articles were written, a considerable amount of knowledge has accumulated on the nature and serologic characteristics of the antibodies of acquired hemolytic anemia. The early work until 1940 was summarized in an excellent review by Dameshek and Schwartz. Then in 1946, Boorman, Dodd, and Loutit and Mollison applied the direct antiglobulin reaction of Coombs, Mourant, and Race to cases of acquired hemolytic anemia, thus demonstrating the presence of an incomplete type of antibody. Dacie, in 1949 and 1950, showed that cold hemolysins against normal red cells could be demonstrated in certain sera containing cold hemagglutinins, if the pH of the serum-cell mixture, normally approximately 8, was adjusted by acidification to the physiologic level or below. In 1951 Dacie and de Gruchy illustrated the value of...
trypsinized erythrocytes and the corpuscles from cases of paroxysmal nocturnal hemoglobinuria (PNH corpuscles) as indicators of hemolysis. They reviewed nineteen cases of acquired hemolytic anemia of various types and recorded the corresponding antibody patterns. Thus it has been possible to classify the antibodies of acquired hemolytic anemia under the following headings: high titer, cold autoantibodies; lytic and nonlytic, warm autoantibodies; and the cold autohemolysins of the Donath-Landsteiner type.

It was the purpose of the present investigation to examine various types of hemagglutinating and hemolytic antibodies under different experimental conditions, and to correlate their serologic characteristics with their ability to produce erythrophagocytosis in vitro. A technic for the combined demonstration of erythrophagocytosis, agglutination, and hemolysis was developed and applied to this study. Erythrophagocytosis was found to be produced only by those antibodies which were either actually or potentially hemolytic, and with a few exceptions the conditions necessary for the production of the two phenomena were the same for any given antibody.

**Materials and Methods**

**Sera**

These were obtained from blood defibrinated at room temperature, or from clotted blood separated at 37°C. Where possible they were freshly obtained. Others had been stored at −20°C for periods up to three years. Where sera were acidified, 10 per cent by volume of N/4 HCl was added. The pH of such sera was estimated by means of a glass electrode and found to be approximately 6.7.

**Erythrocytes**

These were always freshly prepared either from the heparinized or, more commonly, from the defibrinated blood of normal subjects, except in the case of PNH cells which were sometimes stored in acid-citrate-dextrose solution at 4°C for short periods. Group 0 corpuscles were used throughout except when dealing with anti-A. In experiments requiring trypsinized erythrocytes, a technic based on that of Morton and Pickles was employed. All corpuscles were washed three times in large volumes of isotonic saline and finally prepared for use as a 30 per cent suspension in the same medium. Tanned erythrocytes, where required, were prepared by the method of Peck and Thomas.

**Preparation of Leukocyte Suspensions**

Suspensions of leukocytes were obtained, relatively free of red cells, from the heparinized blood of normal subjects of blood groups compatible with those of the red cell donors, often from the same subject. They were prepared by a technic of differential sedimentation which was a modification of that described by Boerner and Mudd. This technic was employed because it involved a minimum of chemical or mechanical manipulation of the leukocytes and yielded highly motile and phagocytic cells, free of clumps and relatively free of admixed erythrocytes. It was impossible to satisfy these conditions with buffy coat preparations made by centrifugation where there was usually much clumping of the leukocytes and admixture with red cells.

The technic employed was as follows: Approximately 10 ml. of venous blood were placed in bottles containing 1 mg. of heparin. The blood was at once centrifuged at 2500 to 3000

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*In experiments comparing suspensions made from heparinized, citrated, oxalated, and defibrinated blood, phagocytic activity was independent of the type of anticoagulant. These findings are similar to those of Zinkham and Diamond. Suspensions prepared from*
rpm for ten minutes separating it into three layers, a supernatant of plasma, a lower layer of packed red cells, and an intermediate buffy coat containing the bulk of the leukocytes. With a Pasteur pipet the upper half of the plasma layer was removed and discarded. The tip of the pipet was introduced to the bottom of the tube disturbing the buffy layer as little as possible and three fourths or more of the red cell layer removed. The result was a blood specimen of smaller volume, rendered artificially anemic, but containing almost all the original leukocytes. Such specimens proved to have very rapid erythrocyte sedimentation rates and afforded excellent differential sedimentation of erythrocytes and leukocytes.

The specimens were then vigorously resuspended, transferred to tubes of narrow diameter which produced a relatively long column facilitating subsequent separation, and allowed to sediment under gravity for 30 minutes at 37°C. The resulting supernatant leukocyte-rich plasma was removed with a Pasteur pipet to be used as the leukocyte suspension.

The volume thus obtained was approximately 2.5 ml., with a leukocyte count of some 40,000 cell/cu.mm., and an erythrocyte count of less than 20,000 cell/cu.mm. The suspensions were used as soon as possible after preparation to ensure the highest possible viability. The usual time from drawing the blood to use of the leukocytes was from 45 to 60 minutes, although excellent results were obtained after 3 hours on occasions. It should be emphasized that scrupulously clean glassware completely free of all traces of detergents was found necessary for viability and phagocytosis.

Complement

In nearly all cases it was found necessary to add fresh normal compatible human serum to the test sera to supply complement, and 1 volume was usually added to an equal volume of test serum. On occasions, a dilution as high as 1 in 8 was used. The titer of complement was determined according to the method described by Dacie and de Gruchy.36 Where necessary the heat labile fractions of complement were destroyed by heating sera to 56°C for 30 minutes. It is known37 that the hemolysins are stable under these conditions, whereas complement activity is lost.

Where the role of complement was studied, it was necessary to remove any complementary activity of the leukocyte suspensions. In these experiments the leukocyte suspensions were centrifuged and the supernatant plasma withdrawn. The cells were then washed once in isotonic saline and resuspended in an equal volume of their own heat-inactivated serum or in saline.

Determination and Recording of Agglutination and Hemolysis

These were determined and recorded by the methods and criteria previously described by Dacie and de Gruchy.36

The Combined Titration of Agglutination, Hemolysis, and Erythrophagocytosis

Serial doubling or fourfold dilutions of the test sera were made in compatible fresh normal sera in 8 mm. tubes, using approximately 0.2 ml. volumes delivered from a marked Pasteur pipet. Control tubes were set up containing normal serum only, and a serum known to cause erythrophagocytosis used as a positive control. To each tube, 1 drop (approx. 0.03 ml.) of a 30 per cent suspension of red cells of appropriate type was added. The temperature and time allowed for sensitization of the erythrocytes by antibody was dependent upon the type of antibody and the purpose of the experiment.

For antibodies active in the cold, one hour was allowed either at 2°C. or at 18 to 20°C. The tubes were then examined for agglutination, and transferred to a water bath at 37°C.

Blood treated with dextran to hasten differential sedimentation were unsatisfactory, showing decreased phagocytosis, rouleaux of erythrocytes, and morphologically poor condition of the leukocytes. Heparinized blood was used for standardization and convenience.
Fig. 1.-- Erythrophagocytosis as seen on fixed Romanowsky stained smears. Note phagocytosis, often multiple, by both granulocytes and monocytes. Ingested erythrocytes range from fully hemoglobinized to clear vacuoles. A small vacuole, probably not phagocytic, is shown in the monocyte at upper right. Note also red cells and agglutinates adjacent to, or adherent to, neutrophils and monocytes.

and shaken gently to disperse any red cell clumps. Hemolysis was recorded after incubation at 37 C. for one hour.

For antibodies active at 37 C. the tubes were placed directly at this temperature. Agglutination and hemolysis were estimated after incubation for one hour.

In all cases, after sensitization had progressed for a predetermined period, usually 10 to 15 minutes, one drop subsamples were transferred to a duplicate set of tubes kept at 37 C. into which one drop of the leukocyte suspension had been placed. A further 10 minutes was allowed to elapse for erythrophagocytosis to take place.* The contents of the tubes were thoroughly mixed and one very small drop from each was placed on a glass slide and carefully spread. The resulting films were stained with May–Grünwald–Giemsa.

It was found that the majority of the engorged leukocytes were carried with the spreader to the tail of the films. If this tail was completely smeared, the phagocytic cells disintegrated, but if spreading was discontinued just prior to this, a fine tongue of material remained unspread which contained many excellently preserved phagocytic cells.

* This period was found optimal for phagocytosis by granulocytes in accord with earlier observations; although the optimum incubation for monocytes was 30 minutes. Longer periods of incubation yielded lesser degrees of phagocytosis, and particularly with highly lytic preparations vacuolization became extreme making interpretation difficult.
In observations on positive preparations it was noted that the phagocytic cells consisted of monocytes and granulocytes, chiefly neutrophils with occasional eosinophils. They contained from 1 to as many as 10 ingested erythrocytes, ranging from intact corpuscles through progressive stages of dissolution up to clear vacuoles approximately the size and shape of erythrocytes. These vacuoles appeared to arise both from the ingestion of ghost cells, and from continuing lysis and digestion within the phagocytes (see description of supravital observations). Interpretation of these large clear vacuoles as evidence of erythrophagocytic activity seemed justified since they were never seen in otherwise negative preparations whereas all stages of their formation could be traced in positive preparations, and because their origin could be observed supravitaly. They had previously been described by Boerner and Mudd\textsuperscript{28} and others.\textsuperscript{7} Clear vacuoles, considerably smaller in size than erythrocytes, were also seen from time to time in monocytes, and more rarely in granulocytes, in otherwise negative preparations, including even leukocyte suspensions which had not been incubated with erythrocytes. These were therefore disregarded. In positive preparations the red cells were noted to form clumps around or adjacent to polymorphonuclear leukocytes and monocytes (fig. 1).

To determine the degree of erythrophagocytosis, 100 consecutive polymorphonuclear leukocytes and 100 consecutive monocytes were counted and the phagocytic cells of each series expressed as separate percentages.

RESULTS

The results of this investigation are described under four headings: supravital observations on erythrophagocytosis, applicable to all types of antibodies causing erythrophagocytosis; a study of erythrophagocytosis and of the conditions necessary for its development using a variety of antibodies (summarized in table 1); observations on the role of complement; and some observations on hemolytic systems of nonimmune type.

Supravital Observations on Erythrophagocytosis

A drop of leukocyte suspension was placed beneath a coverslip on a glass slide, maintained at 37 C. on a warmed microscope stage. The leukocytes were only sluggishly motile but the majority at any one time, possessed from one to several small trigger-like pseudopodia which functioned somewhat similarly to the tentacles of a sea anemone.

A drop of sensitized red cell suspension was then permitted to run under the coverslip by capillarity. In the currents set up many erythrocytes came into contact with leukocytes. Certain of these, presumably those strongly sensitized, became adherent to pseudopodia, one or more red cells becoming thus affixed. At times the number of erythrocytes attached was so great as to enmesh the leukocyte in a clump of red corpuscles; at other times, previously agglutinated clumps were observed to become attached. Both processes probably contributed to the appearance of erythrocyte clumps surrounding many leukocytes in the fixed preparations. Only granulocytes and monocytes were observed to have this adhesiveness for the sensitized red cells, the lymphocytes floating quite free.

By retraction of the pseudopodia to which they were attached some of the corpuscles were drawn into concavities on the subjacent surface of the leukocyte. The adjacent protoplasm then flowed around and over the surface of the erythrocyte ingesting it. Other corpuscles, although similarly attached, were not phagocytosed. Leukocytes were seen to repeat the phagocytic process several
times in fairly rapid succession so that a given erythrophage might contain several ingested red cells.

Phagocytosis proceeded very rapidly and was found within several seconds after the addition of the sensitized erythrocytes. When turbulence had ceased, the slow migration of free leukocytes towards adjacent red cell clumps was occasionally observed but this accounted for a very small proportion of the total erythrophagocytosis. The more usual initiating step in the process appeared to be the fortuitous collision of erythrocyte and leukocyte with mutual adherence.

Once within the leukocyte, the vacuole containing the erythrocyte gradually lost its color and slowly decreased in size ending as a clear vacuole slightly smaller than a red cell. Clear vacuoles of quite similar appearance were also seen to result from the ingestion of erythrocyte ghosts.

Observations with Normal and Antierythrocytic Sera.

Normal sera

A total of one hundred tests for erythrophagocytosis, agglutination, and hemolysis were performed with fifteen different normal sera and compatible erythrocytes. Normal, trypsinized, and PNH corpuscles in unacidified sera, and normal corpuscles in acidified serum were examined. With normal and PNH corpuscles, almost all the tests were completely negative although an occasional serum produced 1 per cent or less of erythrophagocytosis. With trypsinized red cells, low grade positive tests were found somewhat more frequently but erythrophagocytosis never exceeded 1 to 2 per cent. In view of these findings a negative control test was always included with each experiment, and no result was considered positive which did not exceed 1 per cent erythrophagocytosis with normal and PNH corpuscles, and 3 per cent with trypsinized erythrocytes.

The low grade positive control tests found when dealing with trypsinized erythrocytes may have been due to the reversible antibody for trypsinized red cells present in many normal sera and described by Rosenthal and Schwartz.\(^\text{39}\) Phagocytosis of trypsinized human erythrocytes by tissue culture macrophages has been described by Wright and co-workers.\(^\text{29}\)

Sera containing antibodies

Acquired hemolytic anemia; cold autoantibody type: Three sera containing high titer cold agglutinins and acid hemolysins were examined. The results are indicated in table 1. Both hemolysis and phagocytosis of trypsinized and PNH corpuscles occurred in the unacidified serum. The normal corpuscles, on the other hand, were lysed and phagocytosed only after incubation in acidified serum.

When quantitative tests were carried out by titration of acidified serum versus normal corpuscles, erythrophagocytosis was detected to a greater dilution than hemolysis and to approximately equal titer with agglutination. The relative numbers of granulocytes and monocytes showing phagocytosis varied with the dilution of the antibody; at high dilutions only the monocytes were phagocytic. Similar findings have been noted throughout this work and are discussed elsewhere.

Paroxysmal cold hemoglobinuria; cold autohemolysins of the Donath-Land-
steiner type: The sera of two patients were available (table 1). In contrast to the sera containing high titer cold agglutinins, hemolysis and erythrophagocytosis of normal erythrocytes occurred in unacidified serum. Acidification, if anything, slightly decreased the degree of phagocytosis. Although hemolysis and agglutination were slightly enhanced by the use of trypsinized erythrocytes, phagocytosis was unaffected.

Titration experiments showed that trypsinized and PNH erythrocytes were more sensitive indicators for hemolysis than normal corpuscles, and that trypsinized cells were more readily agglutinated. For erythrophagocytosis there was little to choose between the cell types. Again it was noted that phagocytosis occurred to a higher titer than lysis.

Acquired hemolytic anemia; warm autoantibody type: In addition to the preceding two types of antibodies whose activities are potentiated at low temperatures, there exist a group of warm antibodies associated with cases of acquired hemolytic anemia which are at least equally active at 37°C as at cooler temperatures, and which possess other characteristics at variance with the cold antibodies. These warm antibodies may be divided into a lytic group capable of hemolyzing PNH red cells and trypsinized corpuscles, or rarely normal cells if the serum is acidified, and a nonlytic group capable of causing agglutination under suitable circumstances, but not lysis.

Four sera of the lytic type and eight of the nonlytic variety were studied. The nonlytic warm antibodies failed to produce hemolysis or erythrophagocytosis under any conditions.

The four lytic sera produced both hemolysis and erythrophagocytosis of trypsinized and PNH red cells. One of the four sporadically produced slight lysis and phagocytosis of normal corpuscles when acidified. All of these sera acted as less potent lysins and opsonins than did the cold antibodies.

Erythrophagocytosis of trypsinized corpuscles occurred to a greater titer (128) than did hemolysis (32) or agglutination (32) (table 1). Monocytes were more actively phagocytic than the granulocytes and were the only type of cell active in the higher dilutions of antibody (1 in 128 versus 1 in 64).

Rh isoantibodies: Two potent anti-D sera and a serum containing an active anti-e present as an autoantibody in a patient with acquired hemolytic anemia were examined. Normal, trypsinized, and PNH corpuscles (D and e positive) were incubated in unacidified serum, and normal corpuscles in acidified serum for one hour at 37°C. Neither hemolysis nor erythrophagocytosis could be demonstrated, although the presence of agglutination and positive antiglobulin tests indicated that the antibodies had reacted with the erythrocytes.

Reversible agglutinin against trypsinized corpuscles: In 1950 Rosenthal and Schwartz described a factor (R.A.T.C.) present in low titer in most normal sera which agglutinated otherwise compatible trypsinized corpuscles. On continued incubation for about 1 hour at 37°C, this agglutination was reversed by another serum factor. Hurley and Dacie, in 1953, described a factor with similar characteristics present in very high titer in the serum of a subject with pernicious anemia in remission, which not only caused reversible agglutination of trypsinized corpuscles but also hemolysis and sensitization to antiglobulin serum. The serum of this patient was found to cause erythrophagocytosis as well.
as hemolysis (table 1). Erythrophagocytosis like hemolysis was restricted to
trypsinized corpuscles.

Since agglutination with this factor was reversed on continued incubation, the
effect of varying times of incubation upon erythrophagocytosis, agglutination,
and hemolysis was studied. Agglutination progressively diminished and had
almost disappeared at the end of 1 hour. Hemolysis and erythrophagocytosis, on
the other hand, increased in intensity during the incubation period.

Anti-A: Anti-A sera may be divided into naturally occurring (nonimmune)
and immune varieties. The immune sera are strongly hemolytic while the
naturally occurring types are minimally lytic or nonlytic.

Seven anti-A sera were investigated including three pairs of sera (table 2:
sera 1, 2, and 3, nonimmune and immune). Each pair represented a nonimmune
and an immune serum collected from the same group O individual both before
and after immunization with T.A.B. vaccine (typhoid, paratyphoid A and B)
containing A substance. The seventh (table 2; serum 4) was of the immune
type; it was obtained from the sensitized group O mother of a group A infant
with hemolytic disease of the newborn. Serum 4 had lost approximately half,
and the paired sera had lost all their complement activity through prolonged
storage, as shown by the complement titers in the table.

Two main points are illustrated in the results. Firstly, a high degree of erythro-
phagocytosis resulted from the action of the immune anti-A sera, while it was
virtually absent with the nonimmune. Secondly, this erythrophagocytosis was
present although complement activity had been completely lost on storage,
and hemolysis was absent (see also the section on the role of complement and table 5).

Rabbit anti-human red cell antibody: Jordan and co-workers failed to observe
erythrophagocytosis when rabbit anti-sheep red cell serum was incubated with
fresh whole sheep blood, although agglutination and hemolysis occurred. These
findings suggested the possibility that homologous antibody and possibly
homologous complement might be necessary for phagocytosis. Accordingly a
system using normal human erythrocytes and leukocytes, and heterologous
antibody and complement in the form of normal rabbit serum (containing anti-
human red cell antibody) was tested in the usual fashion (table 1). Human
complement was excluded by suspending the leukocytes in heat-inactivated
serum.

Both hemolysis and erythrophagocytosis were obtained. Thus, in this system
at least, heterologous antibody and heterologous complement were capable of
opsonizing human corpuscles for human leukocytes.

Tanned erythrocytes: Reiner, Fischer, and Kopp, in 1929, and others have
described the ability of tannic acid to agglutinate and opsonize erythrocytes,
and to hemolyse them in the presence of complement. Tanned erythrocytes,
suspended in normal human serum, were studied for erythrophagocytosis,
agglutination, and hemolysis by the standard technic. It was found that tannic
acid behaved like an artificial antibody causing agglutination, hemolysis, and
phagocytosis of tanned corpuscles (table 1).

Observations on the Role of Complement

The importance of complement in erythrophagocytosis has been stressed by
Jordan and co-workers, who demonstrated that the opsonic activity of anti-A
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Sensitization temperature</th>
<th>pH</th>
<th>Agglutination (titer)</th>
<th>Hemolysis (titer)</th>
<th>Erythrophagocytosis (titer) %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>normal R.B.C.</td>
<td>trypsin R.B.C.</td>
<td>PNH R.B.C.</td>
</tr>
<tr>
<td>Normal serum</td>
<td></td>
<td>37 C.</td>
<td>8</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Acquired Hemolytic anemia Cold autoantibody</td>
<td></td>
<td>18 C.</td>
<td>8</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+++</td>
<td>+++</td>
<td>0 &lt; 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+++</td>
<td>+++</td>
<td>0&lt; 1</td>
</tr>
<tr>
<td>Paroxysmal cold hemoglobinuria</td>
<td></td>
<td>2 C.</td>
<td>8</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td>Donath-Landsteiner antibody</td>
<td></td>
<td></td>
<td>+ (4)</td>
<td>+ (16)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+++</td>
<td>+++</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>++</td>
<td>++</td>
<td>42</td>
</tr>
<tr>
<td>Acquired hemolytic anemia Warm autoantibody</td>
<td></td>
<td>37 C.</td>
<td>8</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td>(nonlytic)</td>
<td></td>
<td></td>
<td>++</td>
<td>++</td>
<td>0 &lt; 1</td>
</tr>
<tr>
<td>Acquired hemolytic anemia Warm autoantibody (lytic)</td>
<td></td>
<td>37 C.</td>
<td>8</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+++</td>
<td>+++</td>
<td>0 &lt; 1</td>
</tr>
<tr>
<td>Anti-D and e</td>
<td></td>
<td>37 C.</td>
<td>8</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>+++</td>
<td>0</td>
</tr>
<tr>
<td>Antitrypsized R.B.C., R.A.T.C. factor</td>
<td></td>
<td>37 C.</td>
<td>8</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td>Anti-A (nonimmune)</td>
<td></td>
<td>37 C.</td>
<td>8</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Anti-A (immune)</td>
<td></td>
<td>37 C.</td>
<td>8</td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Rabbit anti human</td>
<td></td>
<td>37 C.</td>
<td>8</td>
<td></td>
<td>42</td>
</tr>
<tr>
<td>Tannic acid plus normal serum</td>
<td></td>
<td>18 C.</td>
<td>8</td>
<td></td>
<td>6</td>
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</table>

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TABLE 2.—Agglutination, Hemolysis, and Erythrophagocytosis by Anti-A Sera of Nonimmune and Immune Types

<table>
<thead>
<tr>
<th>Anti-A serum</th>
<th>Agglutination</th>
<th>Hemolysis</th>
<th>Erythrophagocytosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ + +</td>
<td>0</td>
<td>1 1 0</td>
</tr>
<tr>
<td>1: Nonimmune</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immune</td>
<td>++ +</td>
<td>0</td>
<td>30 53 0</td>
</tr>
<tr>
<td>2: Nonimmune</td>
<td>+ +</td>
<td>0</td>
<td>0 1 0</td>
</tr>
<tr>
<td>Immune</td>
<td>++ +</td>
<td>0</td>
<td>26 55 0</td>
</tr>
<tr>
<td>3: Nonimmune</td>
<td>+ + +</td>
<td>0</td>
<td>0 0 0</td>
</tr>
<tr>
<td>Immune</td>
<td>++ + +</td>
<td>0</td>
<td>32 42 0</td>
</tr>
<tr>
<td>4: Immune</td>
<td>+ + +</td>
<td>++</td>
<td>10 34 50</td>
</tr>
</tbody>
</table>

All sensitizations were at 37 C., pH 8 for 5 to 10 minutes. Normal group A corpuscles were used.

* The absence of complement activity was due to prolonged storage.

TABLE 3.—Role of Thermolabile Serum Components in Hemolysis and Erythrophagocytosis by an Antibody of the Donath-Landsteiner Type

<table>
<thead>
<tr>
<th>Cold phase</th>
<th>Warm phase</th>
<th>Hemolysis</th>
<th>Erythrophagocytosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>mono.</td>
</tr>
<tr>
<td>Fresh test serum</td>
<td>Fresh normal serum</td>
<td>+ + +</td>
<td>13 32</td>
</tr>
<tr>
<td>Fresh test serum</td>
<td>Inactivated* normal serum</td>
<td>0</td>
<td>0 0</td>
</tr>
<tr>
<td>Inactivated* test serum</td>
<td>Fresh normal serum</td>
<td>0</td>
<td>0 0</td>
</tr>
<tr>
<td>Inactivated* test serum</td>
<td>Inactivated* normal serum</td>
<td>0</td>
<td>0 0</td>
</tr>
<tr>
<td>Fresh test serum</td>
<td>Fresh test serum</td>
<td>++</td>
<td>7 24</td>
</tr>
</tbody>
</table>

* Inactivation by heating to 56 C. for 30 minutes.

sera was decreased, and that of Donath-Landsteiner sera completely abolished, in its absence. Our own observations confirm and extend these findings, showing that in the antibodies studied complement (or a heat-labile factor(s) present in normal serum) was necessary for optimal, and in most cases for any, opsonic activity.

High titer cold autoantibodies and antibodies of Donath-Landsteiner type: Both types of sera were studied in identical fashion, except that the former were acidified, and the latter tested unacidified. The standard tests were modified and carried out in two phases, a cold or sensitizing phase at 4 C., and a warm or hemolytic and phagocytic phase at 37 C. The test erythrocytes were washed in cold saline between the two phases. The leukocytes were washed and resuspended in inactivated normal serum in these and subsequent experiments on the role of complement. The four possible combinations of the presence or absence of heat-labile serum components in the cold and warm phases respectively were tested.

The findings were the same for both types of sera. The presence of thermolabile serum components was found to be necessary in both cold and warm phases for either hemolysis or erythrophagocytosis (table 3). The antibody itself, on the other hand, was required only in the cold phase. Erythrocytes, sensitized by the test sera in the cold phase, and washed and then resuspended...
TABLE 4.—Role of Thermolabile Serum Components in Agglutination, Hemolysis, and Erythrophagocytosis by Antiehrocytic Sera

<table>
<thead>
<tr>
<th>Serum</th>
<th>Agglutination</th>
<th>Hemolysis (C%)</th>
<th>Erythrophagocytosis (C%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mono. neut.</td>
<td></td>
</tr>
<tr>
<td>Warm autoantibody (fresh)</td>
<td>±</td>
<td>++</td>
<td>17</td>
</tr>
<tr>
<td>Warm autoantibody (inact.*)</td>
<td>+ + +</td>
<td>0</td>
<td>17</td>
</tr>
<tr>
<td>R.A.T.C. factor (fresh)</td>
<td>+ +</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>R.A.T.C. factor (inact.*)</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Rabbit anti human (fresh)</td>
<td>±</td>
<td>+ + +</td>
<td>92</td>
</tr>
<tr>
<td>Rabbit anti human (inact.*)</td>
<td>+ +</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

* Inactivation by heating to 56° C. for 30 minutes.

TABLE 5.—Role of Thermolabile Serum Components in Agglutination, Hemolysis, and Erythrophagocytosis by Anti-A Sera

<table>
<thead>
<tr>
<th>Anti-A serum</th>
<th>Agglutination</th>
<th>Hemolysis (C%)</th>
<th>Erythrophagocytosis (C%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mono. neut.</td>
<td></td>
</tr>
<tr>
<td>5: Fresh</td>
<td>±</td>
<td>++</td>
<td>62</td>
</tr>
<tr>
<td>Inact.*</td>
<td>+ + +</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>6: Fresh</td>
<td>±</td>
<td>+ +</td>
<td>36</td>
</tr>
<tr>
<td>Inact.*</td>
<td>+ +</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>7: Fresh</td>
<td>+ + + +</td>
<td>trace</td>
<td>4</td>
</tr>
<tr>
<td>Inact.*</td>
<td>+ + + +</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

* Inactivation by heating to 56° C. for 30 minutes.

in normal serum in the warm phase, were lysed and phagocytosed to the same degree as controls incubated in the test serum in both phases.

**Warm autoantibodies, rabbit anti-human antibody, R.A.T.C. factor:** Heat-inactivation of these sera caused complete loss of opsonic activity in the case of the R.A.T.C. factor and virtually complete loss of activity with the other two antibodies (see table 4).

**Anti-A:** It has been shown in the previous section that anti-A sera of the immune type produced considerable erythrophagocytosis although complement activity had been completely lost in storage, and hemolytic activity was absent (table 2). The role of complement and of hemolytic activity was further investigated using three different anti-A sera, one being strongly hemolytic, another moderately, and the third virtually nonlytic. Opsonic and hemolytic activities were compared using unheated and heat-inactivated samples of each serum (table 5).

The varying degrees of hemolytic activity of these three sera are shown in the table. It will be noted that erythrophagocytosis was approximately proportional to lytic activity. Heat-inactivation abolished hemolysis and decreased erythrophagocytosis. It may be seen by comparing these results with the observations on anti-A in the preceding section (table 2) that a greater degree of phagocytosis persisted after loss of complement activity through storage, than after heat inactivation. In summary, it would seem that erythrophagocytosis caused by
STUDY OF AGGLUTINATION, HEMOLYSIS AND ERYTHROPHAGOCYTOSIS

anti-A is related to the immune and therefore potentially hemolytic character of the antibody (see previous section). It is, however, only partially dependent on the presence of complement, and thus may occur in the absence of actual hemolysis.

Tanned erythrocytes: When tanned corpuscles were resuspended in heat-inactivated serum or saline, hemolysis was abolished. Phagocytosis, on the contrary, occurred independently of the presence of unheated serum, and was, in fact, more intense in heated serum and in saline.

Hemolysis in Other Systems

Two systems in which erythrocytes were lysed without the action of antibody or complement were studied in an effort to determine whether nonspecific damage or lysis was sufficient to cause opsonization. In the first, normal cells were partially hemolysed by hypotonic saline; in the second, PNH corpuscles were hemolysed in acidified normal serum. Erythrophagocytosis was sought by the usual technic but was not observed in either instance.

DISCUSSION

Three aspects of the subject warrant further consideration: firstly, the mechanism of erythrophagocytosis; secondly, the roles of antibody and complement; and finally, the relationship between hemolysis and erythrophagocytosis.

Mechanism of Erythrophagocytosis

Opsonization appears to be a change affecting the erythrocytes and not the leukocytes. Red cells, once sensitized, could be removed from further contact with free antibody, washed, and still be phagocytosed by normal leukocytes. The initial, or at least the most obvious, effect was the development of an adhesive-ness between erythrocyte and leukocyte. Once affixed, there was an associated effect whereby the leukocyte protoplasm streamed over the red corpuscle and engulfed it. This resembled the behavior of a liquid in contact with a particle with a wettable surface, and suggested a change in the surface tension relationships of the two types of cells.

Monocytes appear to possess greater avidity as erythrophages than do granulocytes, and in titrations were phagocytic in dilutions of antibody at which the latter were inert. With relatively weak opsonins such as the lytic, warm auto-antibodies, phagocytosis was seen predominantly in monocytes at all dilutions.

Role of Antibody and Complement

With most of the antibodies studied, erythrophagocytosis was dependent upon the presence of thermolabile components of fresh serum. Heat-inactivation resulted in complete loss of opsonic activity in the case of the high titer cold auto-antibodies, Donath-Landsteiner antibodies, and the R.A.T.C. factor. Both types of cold antibody required the presence of thermolabile serum components in both cold and warm phases of the reaction. This is in accord with the observation, that complement must be present for these antibodies to produce sensitization to antoglobulin serum, as well as to produce hemolysis. The R.A.T.C. factor has itself been shown to be thermolabile. The lytic warm autoantibodies and the rabbit anti-human red cell antibody retained slight opsonic activity after heating.
It is clear that all the foregoing antibodies possess little or no opsonic activity by themselves, and require potentiation by heat-labile components of fresh serum. These requirements resemble those for hemolysis, and it is possible that an important function of these antibodies in opsonization as well as in hemolysis is to sensitize the erythrocyte to the definitive action of complement.

The immune anti-\(A\) sera behaved somewhat differently. Heat-inactivation or loss of complement through storage had much less effect in decreasing erythrophagocytosis. Here the antibody itself possesses appreciable opsonic activity. This activity is, nevertheless, enhanced by the action of complement.

**Relationship of Hemolysis and Erythrophagocytosis**

Throughout this work a parallel was noted between hemolysis and erythrophagocytosis. The two phenomena were found to be affected in a similar manner by temperature, pH, type of erythrocyte, and, in most cases, by the presence or absence of thermolabile components of fresh serum.

In our experiments erythrophagocytosis could only be elicited with antibodies that were actually or potentially hemolytic. Neither Rh antibodies nor those warm autoantibodies which were nonlytic caused phagocytosis under the conditions examined. In the case of anti-\(A\), which when heat inactivated produced phagocytosis in the absence of hemolysis, only the immune or potentially lytic variety was active. These findings are in agreement with those of van Loghem who reported erythrophagocytosis with anti-D.

Hemolysis caused by systems other than antibody and complement, e.g., osmotic hemolysis or the lysis of PNH cells by acidified normal serum, was not accompanied by erythrophagocytosis.

It would seem, therefore, that erythrophagocytosis is the consequence of a specific type of injury to the erythrocyte, and that hemolysis and erythrophagocytosis as produced by antibody and complement are probably secondary to the same type of alteration of the red cell surface.

The relationship between these observations in vitro and events in vivo is not a straightforward one. For example, erythrophagocytosis has been described in the peripheral blood in cases of hemolytic disease of the newborn, many presumably due to anti-D, and we have ourselves recently seen a patient with acquired hemolytic anemia showing erythrophagocytosis in the peripheral blood, whose autoantibody possessed the characteristics of anti-\(e\). Nevertheless, we have been unable to demonstrate erythrophagocytosis with these antibodies in vitro under the conditions employed. It has been pointed out that red cells clumped and sequestered in the tissues may undergo changes due to the accumulation of metabolites and the action of tissue lysins. These changes may explain the hemolysis and erythrophagocytosis produced in vivo by antibodies incapable of producing these effects in vitro.

The converse problem is presented by the fact that many patients with intense hemolytic anemias show little or no erythrophagocytosis in freshly made smears of their peripheral blood, although erythrophagocytosis may be detected after incubation in vitro. It has been suggested that the distended erythrocytes are trapped in the capillaries, particularly in the lungs, thus explaining their paucity or absence from blood smears as well as the leukopenia observed in some 
STUDY OF AGGLUTINATION, HEMOLYSIS AND ERYTHROPHAGOCYTOSIS

patients during hemolytic episodes. Others have remarked upon the abnormal fragility of the erythrophages and have suggested that they may be destroyed mechanically in the circulation.

In hemolytic disorders, much erythrophagocytosis occurs in the fixed phagocytes of the reticuloendothelial system. Jordan and co-workers have discussed the function of phagocytosis in ridding the blood of injured erythrocytes. This method of disposal as opposed to intravascular hemolysis prevents the release of hemoglobin and other possibly injurious products of red cell destruction directly into the plasma.

SUMMARY AND CONCLUSIONS

1. A method for the combined demonstration of erythrophagocytosis, agglutination, and hemolysis was developed and applied to the study of a series of antieythrocytic antibodies under varying conditions of temperature and pH, using normal, trypsinized, and PNH corpuscles. Erythrophagocytosis was observed supravitaly and in fixed preparations.

2. Only those antibodies which were potentially or actually hemolytic produced phagocytosis under the experimental conditions employed. The conditions necessary for the production of the two phenomena were similar except in the case of anti-A. In titration experiments, erythrophagocytosis occurred in higher serum dilutions than did hemolysis.

3. All the antibodies producing opsonization required the presence of thermolabile components of fresh serum for optimal activity. Except with anti-A, appreciable erythrophagocytosis was not produced by heat-inactivated sera. Hemolysis produced by systems other than antibody and complement was unaccompanied by phagocytosis.

4. It is suggested that hemolysis and erythrophagocytosis may both result from the same type of alteration of the red cell surface.

SUMMARIO IN INTERLINGUA

1. Le objectivo del investigation hic presentate esseva le examine de varie typos de anticorpores hemoagglutinante e hemolytic sub diverse conditiones experimental e le establimento de un correlation inter lor characteristicas serologic e lor capacitate a producer erythrophagocytosis in vitro.

2. Un methodo pro le demonstration conjuncte de erythrophagocytosis, agglutination, e hemolys esseva disveloppate e applicate al studio de un serie de anticorpores del typo antieythrocytic sub varie conditiones de temperatura e de pH. Esseva emplate corpusculos normal, corpusculos trypsinisate, e corpusculos ab patientes con nocturne hemoglobinuria paroxysmal. Erythrophagocytosis esseva observate supravitalmente e in preparatos fixate.

3. Solo le anticorpores que esseva potentialmente o realmente hemolytic causava phagocytosis sub le conditiones experimental emplate. Le conditiones necessari pro le production del duo phenomenos esseva simile excepte in le caso de anti-A. Esseva constatate per experimentos de titration que erythrophagocytosis occurreva in seros plus altemente dilute que hemolys.

4. Omne le anticorpores que produceva opsonisation requireva pro lor optime activitate le presentia de componentes thermolabile de sero fresc. Excepte in le caso de anti-A, erythrophagocytosis in grado notable non esseva producite
per seros inactivate per calefaction. Hemolyse produce per systemas altere que anticorpo e complemento non esseva accompaniate per phagocytosis.

5. Il pale justificabile concluder que e hemolyse e erythrophagocytosis resulta del mesme typo de alteration del superficie del erythrocyto.

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The Combined Study of Agglutination, Hemolysis and Erythrophagocytosis: With Special Reference to Acquired Hemolytic Anemia

JAMES A. BONNIN and LAWRENCE SCHWARTZ

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