Immunohemolytic Mechanisms in Vivo
The Mode of Destruction of Sensitized Red Cells in the Living Organism

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HEMOLYTIC REACTIONS after transfusion of incompatible blood, hemolytic disease of the newborn, paroxysmal cold hemoglobinuria, and acquired hemolytic anemia represent various types of immunohemolytic disorders. The presence of red cell antibodies or proteins acting as such in the blood is common to the whole group. For the sake of simplicity we call these substances antibodies, even though we cannot prove that they are immune antibodies in the strict sense in cases where the mechanism of immunization is unknown.

In hemolytic transfusion reactions, the antigen-antibody system responsible for the red cell destruction is usually obvious, and even in hemolytic anemia of the newborn the mechanism of immunization is well known, but the immunologic mechanism is obscure in the other two types of immunohemolytic disorders. In many cases there is an underlying disease, i.e. syphilis in numerous cases of paroxysmal cold hemoglobinuria, and leukemia or lymphosarcoma in some cases of acquired hemolytic anemia. Even disseminated lupus erythematosus or a virus infection may cause acquired hemolytic anemia, but in many cases we do not know whether there is an underlying disease, hence we call the anemia "idiopathic" to distinguish it from the "symptomatic" type. However, even if we are aware of an underlying disease, we do not know how this disease can give the impulse to production of antibodies against red cells.

The antibodies are demonstrable in different ways. In most cases of immunohemolytic disease, the red cells are coated with a globulin and give a positive Coombs test. In some cases the serum contains free agglutinins active in saline, but more frequently agglutinins active in large molecular diluents or against trypsin treated red cells are found. A hemolysin is demonstrable in serum obtained from patients with paroxysmal cold hemoglobinuria and also in a few cases of acquired hemolytic anemia. Recently, methods have been described by which the opsonic effect of red cell antibodies can be demonstrated in vitro, i.e. the ability to render cells susceptible to phagocytosis. Hence, all the classic actions of cell antibodies in vitro have been demonstrated in immunohemolytic disorders, yet a great deal remains to be learned about the hemagglutinin, hemolysins, and hemopsonins. The purpose of this review is to discuss the action of red cell antibodies in vivo: In what way are the "sensitized" (antibody coated) red cells destroyed in the living organism?
INTRAVASCULAR HEMOLYSIS

In cases where a marked hemoglobinemia is found, we may assume that lysis of red cells is taking place in the bloodstream. That is the case, for instance, in the hemoglobinurias, because the renal threshold for hemoglobin is as high as about 100 mg. per 100 ml. A positive Donath-Landsteiner test demonstrating a hemolysin active in vitro makes this assumption even more probable. Hence the mechanism of red cell destruction in paroxysmal cold hemoglobinuria is probably, at least in part, due to lysis of red cells in the streaming blood. Also the results of Björn-Hansen’s experiments are in good agreement with the concept of intravascular hemolysis in this disease. He isolated the blood of a patient’s arm for a while by means of a tourniquet and cooled and warmed the arm. After this procedure he found hemoglobinemia in the vessels of the arm, but not in the general circulation.

The presence of free hemoglobin (or methemalbumin) in the plasma is evidence in favor of intravascular breakdown of red cells, but it does not prove that the intravascular hemolysis is caused directly by amboceptor and complement. In this type of hemolysis, complement is used, and if it takes place intravascularly, the remaining complement in the serum will be low. Hence, if a large amount of hemoglobin and normal complement content is found in the serum, other hemolytic processes probably take part in the destruction of red cells, e.g. hemolysis caused by mechanical trauma acting on sensitized cells in the circulating blood. The cell surfaces are probably damaged, when the erythrocytes are coated with antibody, and this causes the mechanical fragility of the cells to increase, particularly if the cells stick together and are then violently separated in the circulation. The importance of this hemolytic mechanism in patients with a high titer of cold agglutinin has been stressed by Heilmeyer and Schubothe. The possible importance of intravascular red cell agglutination in immunohemolytic anemias will be discussed in detail below.

High concentrations of plasma hemoglobin have been noted in hemolytic episodes after transfusion of incompatible blood. If the hemolytic reaction occurs immediately after or during the transfusion and is accompanied by marked hemoglobinemia, then the mechanism of red cell destruction is probably that of intravascular lysis. The results of dog experiments performed by Young et al. favor this concept. After transfusion of incompatible blood to an immunized dog, the complement disappeared from the recipient’s serum, and the amount of available complement seemed to limit the rate of destruction of donated cells. On the other hand, Castle, Ham, and Shen described a severe hemolytic transfusion reaction without detectable hemolysin, and not accompanied by a fall in the complement titer of the recipient’s serum. Two phases in red cell destruction caused by incompatible plasma were distinguished by Ervin and Young in their case of hemolytic reaction occurring after transfusion of blood from a “dangerous universal donor”. There was probably first a direct intravascular hemolysis caused by complement, and later a slow mechanical destruction of sensitized cells.

In the cases of acquired hemolytic anemia investigated by Crosby and Damash, a marked degree of hemoglobinemia was usually found during periods
of violent red cell destruction, but some cases of severe hemolytic disease and relatively low plasma hemoglobin concentration were also seen. The authors pointed out that multiple mechanisms of red cell destruction may be present. One of the mechanisms in acquired hemolytic anemia is apparently intravascular hemolysis. This is particularly obvious when free auto- or iso-hemolysins in the serum are demonstrable, as in the cases described by Dameshek and Schwartz. Dacie and de Gruchy, using trypsinized red cells or cells from patients with paroxysmal nocturnal hemoglobinuria, demonstrated abnormal iso-hemolysins in thirteen cases. They also determined the plasma complement content in their cases and found normal values in most patients, but significantly decreased values in a few cases. As mentioned, the latter finding may be due to complement consumption during the process of intravascular hemolysis.

In hemolytic disease of the newborn, there is usually a high plasma bilirubin concentration, but no hemoglobinemia, hence the red cells are probably not destroyed by lysin and complement in the blood vessels. Hemolysins are not as a rule detectable in these cases by the ordinary methods. A weak and slow hemolytic effect of the serum may be demonstrated by the method of Hill, Haberman, and Jones, but the finding does not prove that there are real immune hemolysins acting in vivo.

As emphasized by Dameshek and Schwartz, experimental hemolytic anemia is similar to immunohemolytic anemia in many respects. Most authors who have produced hemolytic anemia in animals by injections of anti red cell serum, agree that the antibodies destroy more red cells in vivo than in vitro. When red cells, amboceptor, and complement are mixed in a test tube and incubated, almost the entire hemolysis takes place during the first hour, even if the absolute end point is not reached so soon. After injection of anti red cell serum into an animal, there is an immediate rapid hemolytic phase accompanied by hemoglobinemia and hemoglobinuria, followed by a prolonged hemolysis which goes on for several days. A larger number of cells is generally destroyed during the second hemolytic phase than during the first. This biphasic red cell destruction curve was first described by Banti, who also compared the effect of immune antibodies and that of distilled water in vivo. After injection of distilled water, he found only the first phase, and no marked anemia was produced. The first hemolytic phase after injection of anti red cell serum is probably caused directly by amboceptor and complement in the blood, but the prolonged phase is caused by other means. This explains why the complement titer is not much lowered in this type of experimental immunohemolytic anemia. Wasastjerna compared the hemolytic effect of anti red cell serum, saponin, and streptolysin and found the action of the immune serum to be quite different from that of the other two hemolysins. The immune serum destroyed much larger quantities of red cells in vivo than in vitro, whereas saponin and streptolysin had a stronger effect in vitro. It was concluded that the living organism takes part in the destruction of red cells which have been sensitized by immune serum injected into the animal.

**INTRAVASCULAR AGGLUTINATION OF RED CELLS**

In hemolytic disease of the newborn and in acquired hemolytic anemia, abnormal red cell antibodies are usually demonstrable as agglutinins and rarely
as hemolysins. In many cases of acquired hemolytic anemia, autoagglutination has been found in whole blood samples from the patient\textsuperscript{5, 11} and the same has been noted after transfusion of plasma from “dangerous universal donors.”\textsuperscript{7-17} Hence, it seems very likely that agglutination of the red cells also takes place intravascularly. The main objection to this assumption seems to be the probability that the red cell clumps might very soon plug too many blood vessels and cause extensive thrombosis and death. As a matter of fact, thrombophlebitis is often seen in immunohemolytic disease.\textsuperscript{5} According to Heilmeyer and Schuboth\textsuperscript{17} and to Baumgartner,\textsuperscript{28} cold agglutinins especially tend to cause intravascular agglutination and circulatory disturbances.

There is ample experimental evidence in favor of intravascular hemagglutination in immunohemolytic disorders. Ham and Castle\textsuperscript{29} produced hemolytic anemia in animals by injections of concanavalin A, a substance which in vitro acts as a strong agglutinin and has no hemolytic effect in the test tube. Later, Castle et al.\textsuperscript{22} produced hemolytic anemia in dogs using an anti dog erythrocyte serum in a dilution which had only a hemagglutinating effect in vitro, but no hemolytic influence. As already mentioned, Wasastjerna\textsuperscript{26} compared the hemolytic effect in guinea pigs of anti red cell serum, saponin, and streptolysin and found the first mentioned substance to have a much stronger and more prolonged hemolytic effect in vivo than the same amount of hemolytic units of the latter two substances. Because saponin and streptolysin are hemolysins and not agglutinins, it is possible that the strong hemolyzing effect of immune serum in vivo is attributed to its agglutinating power.

In 1902, Kraus and Sternberg\textsuperscript{26} observed agglutination of red cells in whole blood samples from animals treated with antieerythrocyte serum. Later, Bessis and Freixa,\textsuperscript{31} Castle et al.,\textsuperscript{22} and Wasastjerna\textsuperscript{26} made similar observations. The behavior of the red cells in the vessels of treated animals was studied by means of a stereoscopic microscope in 1950 by Day and Perry,\textsuperscript{32} and in 1951 by Wasastjerna.\textsuperscript{26} Day and Perry used a magnification of 20 × and observed the vessels of sclera and mesenterium of rats injected with anti red cell serum, and Wasastjerna studied the conjunctival vessels of guinea pigs treated in the same way, using a magnification of 60 ×. The technics and magnifications used in both these experimental works permitted no detailed observations, but a marked degree of intravascular red cell clumping was reported.

In Wasastjerna’s experiments, the agglutinating power of the immune serum seemed to be stronger in vivo than in vitro, because the smallest dose causing hemolytic anemia and red cell agglutination in vivo corresponded to an antibody concentration which did not agglutinate the red cells in defibrinated guinea pig blood in vitro. An explanation of this finding might be the presence in vivo of some substance which is able to enhance the agglutination of red cells. In dog serum, Young et al. found a heat labile component which enhances the agglutinating power of dog anti-A and anti-E serum.\textsuperscript{21}

In order to observe details of the phenomenon of intravascular agglutination, Wasastjerna et al.\textsuperscript{33, 34} induced immunohemolytic anemia in hamsters by injections of anti hamster red cell rabbit serum. The circulation in the hamster cheek pouch was then observed microscopically at high magnification, and intravascular agglutination of red cells was always found, if the dose of immune serum injected was large enough to cause anemia. A high degree of intravascular agglutination
was found in many animals which were still in good condition and which later recovered from their anemia. The red cell clumps in the vessels were sometimes very large, but they were friable and broke up when they had to pass through capillaries and other narrow passages. This reversibility of the phenomenon explains why the agglutination did not have a disastrous effect on the circulation and the general condition of the animals.

The circulating blood in patients suffering from immunohemolytic anemia has been investigated by a few authors. In 1950, Day and Perry reported a high degree of intravascular red cell clumping in a child with acquired hemolytic anemia, and a mild degree of clumping in a newborn infant with hemolytic anemia due to Rh-immunization of the mother. Zilliacus and Arjänäri examined the conjunctival blood vessels in twelve cases of hemolytic disease of the newborn and found marked red cell aggregation in ten of them. By means of a stereoscopic slit lamp microscope, Wasastjerna, Dameshek, and Komninos examined the circulating blood in fourteen patients suffering from acquired hemolytic anemia, and found definite red cell clumping in the conjunctival vessels of all these patients. The degree of red cell clumping varied considerably from case to case. The agglutination was moderate if the patients were in good clinical remission and pronounced in severely ill and anemic patients. The red cells of all these patients gave a positive Coombs test. However, a marked degree of intravascular red cell clumping was seen in other severely ill patients, for instance in cases of leukemia with negative Coombs test and no evidence of hemolytic disease. Knisel et al. found intravascular red cell clumping in patients suffering from a variety of diseases. Present techniques for intravascular blood studies in man give only a rough idea of the distribution of red cells in the blood vessels, and no qualitative difference between the red cell clumping in the Coombs positive and the Coombs negative cases could be seen by Wasastjerna et al. Non-immunologic hemolytic disorders revealed no intravascular agglutination, or only a very moderate degree, even if the anemia was severe.
From the behavior of red cell antibodies in vitro, from the observations in experimental hemolytic anemia, and also, to some extent, from the direct observations of the circulating blood in patients, we may draw the conclusion that intravascular agglutination of red cells very probably takes place in immunohemolytic disorders in man. It is quite obvious that agglutinated red cells are more susceptible to mechanical trauma when passing narrow passages than are free cells, even if the clumps can be forced to break up as in the hamster experiments mentioned above. According to Castle, Ham, and Shen, the agglutination also brings about hemolysis via erythroagglutination, with local exclusion of serum, tissue ischemia, and release of injurious substances from autolysing tissues, adjacent to stagnating red cells. Even lyssolecithin may be responsible to some degree for the destruction of clumped red cells which naturally remain for longer periods than normal cells in sequestering organs, such as the spleen.

**Phagocytosis of Red Cells**

The three classic effects of cell antibodies upon the antigen (the cells), are lysis, agglutination, and the opsonic effect. The probable pathogenetic importance of hemolysins and hemagglutinins has now been outlined, and the hemosponins, i.e. the antibodies which render red cells susceptible to phagocytosis, remain to be discussed.

Leukocytes containing ingested red cells are sometimes seen in stained smears of the peripheral blood from patients with immunohemolytic disorders. The phagocytes seen are usually monocytes or neutrophilic granulocytes, but phagocytizing cells, which are not normally present in the peripheral blood, have also been noted. Erythrophagocytosis, either in the peripheral blood or in the spleen and other tissues, has been described in hemolytic transfusion reactions, paroxysmal cold hemoglobinuria, hemolytic anemia of the newborn, and acquired hemolytic anemia.

Zinkham and Diamond recently showed that red cells from patients with acquired hemolytic anemia are susceptible to phagocytosis by leukocytes in vitro. They described a simple method to demonstrate this phenomenon for diagnostic purposes. Baumgartner and Müller described a method for demonstrating the opsonic action of iso- and autoantibodies in vitro.

**Animal experiments** support the concept of erythrophagocytosis as a method of the organism to rid itself of sensitized red cells. In 1902, Levaditi described extensive phagocytosis of red cells in the spleen and other organs of guinea pigs after injection of anti guinea pig blood serum. Later, erythrophagocytosis in the spleen and the circulating blood in experimental hemolytic anemia has been the subject of close studies by Dudgeon et al., Baumgartner, Bessis and Freixa, and Wasastjerna. The ability of spleen macrophages to ingest the sensitized cells but not the normal ones was demonstrated by Brandt et al.

It has been quite convincingly proved that phagocytosis of antibody-coated red cells takes place in vivo in cases of immunohemolytic anemia in man and in experimental immunohemolytic anemia. However, the erythrophagocytosis cannot explain all aspects of red cell destruction in vivo. It cannot, for example, explain the spherocytosis. Immune hemolysins have very slight or no effect on the red cell shape and osmotic fragility in vitro but a pronounced effect...
Its VIVO. Hence, the marked spherocytosis in vivo after injection of anti red cell serum into an animal is mainly the effect of conditions in vivo upon sensitized red cells. In this respect, the sequestering of coated red cells in the spleen and other organs is an important mechanism. Even the hemoglobinemia, frequently found in immunohemolytic disorders, can hardly be explained by phagocytosis of red cells. Erythrophagocytosis is one of several mechanisms which cause destruction of sensitized red cells in vivo.

![Fig. 2.—Section of spleen (X 800).](image1)

**Fig. 2 and 3.**—Macrophages containing red cells in spleens of guinea pigs treated with anti red cell serum.

**The Role of the Spleen**

The unquestionable pathogenetic importance of the spleen with regard to the immunohemolytic mechanisms is revealed by the effect of splenectomy in acquired hemolytic anemia which is sometimes quite striking. However, the effect of splenectomy varies from case to case. Some patients are cured and others moderately improved, and in some, splenectomy has no effect whatever. The role of the spleen thus seems to differ from case to case. The antibody titer often decreases rapidly after splenectomy when the patients respond well to the treatment, but cases have been described in which the splenectomy has been of good clinical effect, even if the antibody titer remained high. In the first mentioned cases, abnormal antibodies are evidently produced by the spleen which is known to be an antibody producer of importance. Wright et al. found higher antibody titers in the splenic blood than in the peripheral blood. Wagley et al. incubated normal red cells with spleen tissue from patients with acquired hemolytic anemia.
and found the incubated cells to be heavily “coated” with an agglutinative
“substance.”

The clinical effect, sometimes noted without a change in the antibody titer,
has to be explained in other ways. In these cases the antibody coated red cells
are destroyed in the spleen. Emerson et al.50 and Young et al.51 have demon-
strated that spherocytes are selectively retained by the spleen; likewise clumps
of agglutinated red cells are probably retained there.29 In addition, erythrocyte
clumps tend to stagnate in other organs, e.g. in the liver.22 Some of the sequest-
ered cells are probably ingested by tissue macrophages;44 and other red cells
are lysed by lysolecithins,38 formed in the stagnant blood, or through metabolic
processes acting on the incubated cells.22, 29 Banti’s25 animal experiments showed
distinctly that splenectomized animals tolerate more anti red cell serum than
do normal animals. This finding was confirmed by Wasastjerna,29 who also found
that spherocytosis developed slowly after an injection of immune serum into the
blood and reached its maximum in about two days. It was more pronounced in
normal animals than in the splenectomized ones; hence it was partly produced
by the spleen. Saponin, on the other hand, produced spherocytosis of a much
higher degree in vitro than did the immune serum, but after injection of high
doses of saponin into animals there was only slight spherocytosis in their blood.

The spleen is probably one of the sources of autoantibodies in many cases of
acquired hemolytic anemia, but in other cases of immunohemolytic disease, the
spleen may take an active part in the red cell destruction, even if it does not pro-
duce autoantibodies. Red cells coated with antibody are ingested by splenic
macrophages; spherocytes and agglutinated red cells are sequestered in the spleen;
and these sequestered cells are lysed or made more spherocytic under the influ-
ence of lysolecithin, tissue lysins, and metabolic processes. However, this type
of red cell destruction is not limited to the spleen. It takes place in other organs
too, as for example the liver.22

CONCLUSIONS

As stated by Crosby and Dameshek,14 there are multiple mechanisms which
destroy the red cells in immunohemolytic disorders. The main mechanisms of
red cell destruction are probably (1) intravascular hemolysis; (2) intravascular
agglutination with mechanical trauma acting on the red cell clumps, and seques-
tration followed by hemolysis of agglutinated cells in the spleen and other organs;
and (3) phagocytosis of red cells coated with globulin (antibody?).

Sometimes one of the mechanisms may predominate, sometimes another, and
there may be other modes of red cell destruction, such as fragmentation,16, 52
which may contribute to the removal of sensitized cells from the blood.

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