Loss of Membrane Components in the Pathogenesis of Antibody-Induced Spherocytosis

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Research Article

The infusion of hyperimmune agglutinating antibodies into man or animals causes spherocytosis and hemolysis. The mechanism of spherocytosis was studied in rats given rabbit anti-rat red cell antiserum intravenously. During the 18 hr after antibody infusion, a time before the onset of reticulocytosis, hematocrits fell from 40.6 to 27.6%. However, no change occurred in mean cell volume, mean cell hemoglobin content, or the red cell concentrations of potassium or adenosine triphosphate (ATP). There was a progressive loss of membrane constituents and membrane surface area which followed first order kinetics. At 18 hr membrane cholesterol had decreased 23.5%, phospholipid 26.3%, protein 4.7%, and surface area (calculated from a measure of osmotic fragility) 14.2%. There was no change in the per cent composition of the various phospholipids. Similar changes occurred in animals splenectomized before receiving antibody.

These studies demonstrate that spherocytosis induced by heterologous agglutinating antibodies in vivo results from a loss of surface area with no accompanying change in cell volume or in the concentration of the major intracellular constituents. It is caused by a process acting at the cell surface leading to the loss of lipid-rich, protein-poor components of the red cell membrane.

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INTRODUCTION

Almost 75 years ago Bordet (1) observed that by im-
munizing one species with red cells of another species,
serum could be obtained which caused red cells to agglu-
tinate and lyse in vitro. In the early years of this cen-
tury a number of laboratories, including those of Banti
(2), Chauffard and Troisier (3), and Muir and M'Nee
(4), investigated this problem. They establish that
the degree of hemolysis produced by this serum in vivo
always exceeded the amount predicted from studies in

vitro. Moreover, the hemolysis which occurred in vitro
was rapid and was not associated with spherocytosis,
while that which followed the injection of serum in vivo
occurred slowly and was associated with the appearance
of red cells which were spherocytic and osmotically
fragile. The factor which led to hemolysis in vitro was
shown to be complement (1); however, the mechanism
of spherocytosis and hemolysis in vivo was unknown.

In man, most agglutinating antibodies are reactive in
the cold and, in general, produce little or no spherocy-
tosis in vivo (5). However, spherocytosis is seen when
hyperimmune anti-A or anti-B agglutinins of maternal
origin are transferred to newborns (6) or when high
titers of hyperimmune isoagglutinins are inadvertently
administered in "dangerous universal donor" transfu-
sions (7-9). Ham and Castle observed that the intra-
venous administration of concanavalin A, a plant lectin
which induces agglutination in vitro, caused spherocyto-
sis and hemolysis in experimental animals in vivo (10),
and they postulated the same sequence of events in the
hemolysis associated with the red cell agglutinin in horse
antiserum against pneumococcus Type XIV (11).

Spherocytosis, the morphologic equivalent of a de-
creased red cell surface area-to-volume ratio, can result
from either an increase in volume (e.g. with colloid os-
motic swelling) or a decrease in surface area. Because
volume is a cubed geometric function whereas surface
area is a squared function, cleavage of red cells into two
or more hemoglobin-containing fragments leads to
spherocytosis. Castle, Ham, and Shen induced spherocy-
tosis during prolonged incubation (erythrostasis) of red
cells in vitro, and they proposed that agglutination led
to erythrostasis in vivo, and that the metabolic damage
which ensued resulted in spherocytosis (12). Indeed,
prolonged nembutal anesthesia with the attendant eryth-
rostasis in the spleens of dogs was capable of causing
mild spherocytosis in vivo. More recently it has been
shown that during prolonged incubation under conditions
leading to glucose exhaustion, red cells undergo a disc-

sphere transformation upon ATP depletion (13, 14) and thereafter lose membrane lipids and surface area (15, 16). In order to gain insight into the mechanism of spherocytosis and red cell destruction, the present studies were undertaken to define the cellular changes occurring during the induction of spherocytosis by heterologous agglutinating antibodies in vivo.[1]

METHODS

Preparation and administration of rabbit anti-rat red cell serum. Rabbits were injected with 1 cc of freshly obtained thrice-washed rat red cells in saline at a cell concentration of 10% at weekly intervals for 4 wk. Antibody was prepared on two occasions separated by 14 months utilizing four different rabbits on each occasion. The injection sites for the first immunization were divided between footpads, skin, and peritoneal cavity. Thereafter the cell suspension was administered intraperitoneally. Antiserum was harvested 5 days after the fifth immunization and was heated to 56°C for 2 hr to decrease its hemolytic activity in vitro (17). To measure its agglutinating capacity, two drops of antiserum were mixed with two drops of freshly obtained, washed rat red cells in saline at a hematocrit of 2% and examined after centrifugation for 45 sec. The end point of agglutination was read macroscopically. Agglutination titers ranged from 1:1280 to 1:5120. Hemolytic titers were measured in similar fashion but with the addition of two drops of fresh rat serum as a source of complement. Hemolysis was never present beyond a 1:2 dilution of antiserum. Antiserum was diluted with saline to an agglutinating titer of 1:640 and was administered by tail vein infusion over the course of 1 min into Bartonella-free, Sprague-Dawley rats weighing 150–250 g, each rat receiving 1 ml of antiserum per 100 g of body weight. Blood was obtained from rats by means of cardiac puncture into heparinized syringes at various times up to 18 hr after antibody infusion, a time which preceded the release of newly formed reticulocytes. Each rat provided blood on only one occasion. One group of animals was splenectomized 5 days before the infusion of antibody.

Analysis of red cells. Hematocrit was measured by the micromethod after centrifugation for 10 min. Red cells were counted in a Coulter model B electronic counter (Coulter Electronics). Hemoglobin was measured using Drabkin's solution. ATP was assayed with the firefly-lantern extract as modified by Keitt (18) from Beutler and Baluda (19). Red cell osmotic fragility, corrected for the effect of pH and tonicity, was measured by the method of Emerson, Shen, Ham, Fleming, and Castle (20). Red cell surface area was calculated from the cells' spherical volume derived from a direct measure of osmotic fragility using the formula of Castle and Daland (21) as described previously (22). This calculation is made from the spherical volume of the cell, and it depends on predictable changes in cell volume in hypotonic salt solutions of known tonicity. It is based on the Boyle-van't Hoff law which considers the red cell as a perfect osmometer. The formulation of Castle and Daland (21) is in good agreement with the more recent findings of Hoffman, Eden, Barr, and Bedell (23) and Savitz, Sidel, and Solomon (24). Since spherical vol-

ume, the maximum volume attainable before hemolysis, depends on (a) the intracellular content of osmotically active material and (b) the surface area of the limiting cell membrane, it serves to reflect the latter only if there has been no change in the former. Thus its use requires that the red cell population measured be a single population distributed about a mean; that the volume and content of osmotically active solute be the same in all samples of red cells measured; and that the red cells be exposed to hypotonic solutions for a period of time shorter than that which would itself cause an appreciable change in fragility due to cation changes. All of these conditions were met. Red cell potassium concentration was calculated from the measure of its concentration in whole blood and serum using a flame photometer.[2] Red cell cholesterol (25) and lipid phosphorus (26) were measured in quadruplicate portions of thrice-washed red cells extracted with 80 vol of isopropanol and chloroform (27). The antioxidant 2,3-di-*tert*-butyl, 4-methyl phenol (BHT) (28) was added to duplicate extracts which were evalporated and chromatographed on thin layer plates coated with Silica Gel G (29), and the individual phospholipids were measured after quantitatively recovering the gel and digesting it with H_2SO_4 and H_2O_2 (26). Red cell ghosts were prepared in duplicate (30), and the content of cholesterol (25) and protein (31) was measured in duplicate from each ghost preparation. There was no loss of lipid during ghost preparation. Protein content was corrected for hemoglobin persisting in ghosts, as measured by the benzidine reaction (32). Hemoglobin protein accounted for less than 2% of the total ghost protein. Protein per red cell ghost was calculated from a measure of the cholesterol: protein ratio of ghost preparations and of the cholesterol content per red cell.

RESULTS

18 hr after the intravenous infusion of rabbit anti-rat red cell antibody into rats there was a decrease in hematocrit of approximately one-third (Table I). The percentage of reticulocytes was within the normal range. There was no change in mean cell volume or mean cell hemoglobin content, and the red cell concentrations of both potassium and ATP were within the normal range.

The mean osmotic fragility (50% lysis) of red cells was increased from an initial value of 0.41 g/100 ml sodium chloride to 0.25 g/100 ml at 18 hr. Red cells appeared to be uniformly affected, as indicated by osmotic fragility curves showng a single cell population (Fig. 1). Osmotic fragility expresses the surface area-to-volume ratio. Because there was no change in cell volume the observed change in osmotic fragility indicates that there was a loss of cell surface area. It was calculated from these data that red cell surface area had decreased by 13–18%.

An analysis of red cell membrane constituents is shown in Table II. There was a loss of both cholesterol and phospholipid from the red cell membrane. At 18 hr, the cholesterol loss averaged 23.5% and the phospholipid loss 26.3%. The difference between the loss of cho-

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[1] These studies have been reported in abstract form; Cooper, R. A. 1969. Loss of membrane components in the pathogenesis of antibody-induced hemolysis. *Clin. Res.* 17: 322.

[2] Instrumentation Laboratory, Inc, Lexington, Mass.

Table I

Characteristics of Rat Red Cells 18 Hr after Injection of Rabbit Anti-Rat Red Cell Antibody

	Control ±SE*	n‡	Antibody ±SE*	n‡
Hematocrit, %	40.6 ±1.1	(37)	27.6 ±1.4	(35)
Reticulocytes, %	1.3 ±0.6	(13)	1.6 ±0.8	(11)
Mean cell volume, u^3	60.9 ±1.9	(37)	60.4 ±2.4	(35)
Mean cell hemoglobin, $uug/cell$	20.2 ±0.8	(37)	20.7 ±0.8	(35)
Potassium, $mEq/liter$ cells	115.6 ±1.8	(45)	115.6 ±3.0	(27)
ATP, $mmoles/liter$	1.6 ±0.1	(13)	1.6 ±0.1	(10)

* Standard error of the mean, 95% confidence limits.
‡ n, number of animals.

lesterol and the loss of phospholipid per cell, although small, was highly consistent, being seen in 25 of 30 animals, and was statistically highly significant ($P < 0.005$). However, there was no difference in the per cent composition of the various phospholipids separated by thin-layer chromatography. The ghost content of protein was decreased by 4.7% at 18 hr, and this difference was statistically significant ($P < 0.05$).

The time course of changes in membrane cholesterol and phospholipid and the associated changes in membrane surface area, as calculated from measurements of osmotic fragility, is shown in Fig. 2. Five separate animals were used for each intermediate experimental point and 10 were used for the initial (control) and final points. There was a progressive loss of cholesterol, phospholipid, and surface area. The changes in these three parameters appeared to follow first order kinetics. Hematocrit decreased by 11% of its initial value over the first 15 min, by 19% at 2 hr, and 32% at 18 hr.
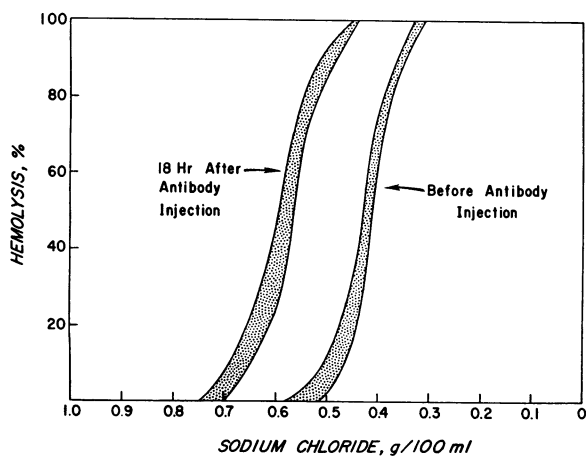

Figure 1   The osmotic fragility of red cells from normal rats and rats who 18 hr previously received rabbit anti-rat red cell antibody by intravenous infusion. Stippled areas represent the range of 31 normals and 36 experimental animals.

Spleen weights were increased by 90% at 18 hr (from 192 mg/100 g body weight in controls to 366 mg/100 g in experimental animals). However, in three animals splenectomized 5 days before the injection of antibody, changes in osmotic fragility and in red cell membrane cholesterol and lipid phosphorus content at 18 hr were identical with those observed in animals with intact spleens. Hematocrits fell from an initial value of 41.3–27.4% at 18 hr, a decrease of 34% of the initial value.

DISCUSSION

These studies demonstrate that the spherocytosis induced by heterologous agglutinating antibodies in vivo results from a loss of membrane surface area with no accompanying change in cell volume or hemoglobin content or in the cellular concentrations of potassium or ATP. Thus, the sequence of cellular changes attendant upon erythrostasis (12), with a transformation from disc to sphere coincident with the decline of ATP stores (13, 14), does not account for the spherocytosis observed. Moreover, spherocytosis does not result from a loss of hemoglobin containing fragments (33, 34) or from colloid osmotic swelling. Rather, it is caused by a process acting at the cell surface leading to the loss of 20–30% of the membrane's content of lipid with an accompanying loss of protein equal to only 5% of the protein content of membrane ghosts. That a similar process may be operative in man is indicated by the deficiency of both cholesterol and phospholipid in red cells of patients with gamma Coombs hemolysis, despite the presence of reticulocytes which would tend to increase lipid values (35).

Surface area was calculated from measurements of osmotic fragility. Since the structure of red cell membranes is unknown it is also unknown which components of the membrane contribute to its surface area. Varying the cholesterol content of human red cell membranes in vitro over a 100% range (from 25% less than normal to 75% greater than normal) causes a 24% variation in surface area (from − 8% to + 16%) (36). Because cholesterol contributes approximately 28% to the sur-

TABLE II

Characteristics of Rat Red Cell Membranes 18 Hr after Injection of Rabbit Anti-Rat Red Cell Antibody

	Control ±SE	n	Antibody ±SE	n	Difference
Cholesterol, $\mu g/10^8$ cells	13.04 ±0.18	(28)	9.98 ±0.26	(30)	23.5%
Phospholipid, $\mu g/10^8$ cells	29.28 ±0.35	(28)	21.58 ±0.45	(30)	26.3%
Lysolecithin, %	5.1 ±0.4	(7)	5.6 ±0.9	(10)	NS*
Sphingomyelin, %	10.1 ±1.4	(7)	9.5 ±1.3	(10)	NS
Phosphatidyl choline, %	52.0 ±2.8	(7)	52.0 ±2.0	(10)	NS
Phosphatidyl serine, %	10.4 ±1.4	(7)	10.7 ±1.4	(10)	NS
Phosphatidyl ethanolamine, %	22.3 ±2.4	(7)	22.2 ±1.7	(10)	NS
Protein, $\mu g/10^8$ cells	72.8 ±4.2	(12)	69.4 ±3.8	(18)	4.7%
Mean osmotic fragility NaCl, g/100 ml	0.414 ±0.002	(31)	0.592 ±0.022	(36)	
Surface area‡ % of Control	100.0 ±0.5	(31)	85.8 ±1.2	(36)	14.2%

* NS, not significant.
‡ Calculated from mean osmotic fragility.

face area of a monolayer derived from red cell lipids (37), it was inferred from the observed 24% variation in red cell surface area that lipid alone contributes to red cell surface area. This is consistent with the view that membrane lipids exist as a bilayer (32, 33). While lipids in this model are relatively uniform over the cell surface, protein need not be uniformly dispersed. The large lipid loss compared with the small protein loss could reflect this uneven distribution. However, if lipid alone contributes to surface area, than a 25% loss of the membrane lipid should have caused a 25% decrease in cell surface area, whereas the observed decrease was only 14.2%. It is possible that as a portion of the membrane was lost the surface pressure changed within the bilayer permitting a greater surface area for the remaining lipid. On the other hand, membrane lipids may exist not as bilayers but as lipoprotein subunits (38), and protein, which accounted for 63% of the membrane by weight in these studies, may also contribute to cell surface area. This may account for the observed compartmentalization of membrane phosphatidyl choline (39, 40). It appears that, if this is the case, the portion of the membrane lost during the induction of spherocytosis consisted of lipoprotein components rich in lipid, phospholipid, in particular, and poor in protein. The resulting osmotic fragility reflects the contribution of these particular lipoprotein subunits to red cell membrane surface area.

The current studies show that there was a considerable increase in spleen weight in those animals receiving antibody. However, splenectomy had not demonstrable beneficial effect. It is probable that this was due to the large amounts of antibody infused. Jandl and Kaplan showed that with increasing amounts of antibody coating rat red cells, there is an increasing contribution of the liver in the process of red cell destruction, whereas the spleen predominates only when small amounts of antibody are present (17). Beneficial effects of splenectomy were

shown clearly by Wasastjerna who induced a chronic hemolytic anemia in guinea pigs by the daily injection of small amounts of rabbit anti-guinea pig red cell antibody (41).

Mechanisms which may be operative in the genesis of the membrane modification reported herein include (a) agglutination per se, (b) complement, and (c) gamma globulin coating the red cell surface. That agglutination per se may be responsible is suggested by studies which showed that concanavalin A, a plant lectin, agglutinates rat red cells in vitro and induces spherocytosis and hemolysis in rats in vivo (11). However, it does not agglutinate sheep red cells in vitro and has no effect when injected into sheep (42). Red cells coated with agglutinating antibodies develop finger-like processes causing contiguous cells to become interlinked (43, 44). Possibly the adhesion and disjunction of these processes dur-
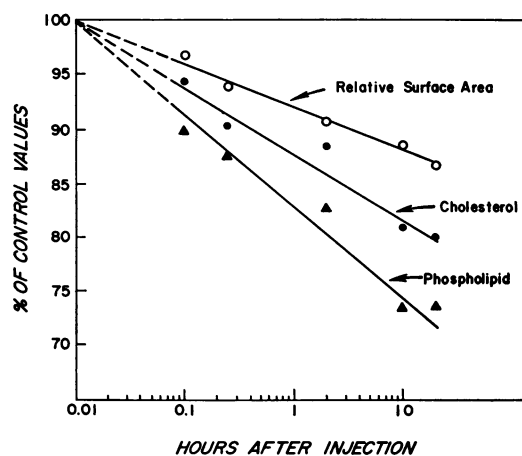

FIGURE 2 Change in the lipid composition and surface area of rat red cells after the intravenous injection of rabbit anti-rat red cell antibody. Surface area was calculated from a measure of osmotic fragility.

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ing the turbulence of flow in vivo results in a loss of membrane at the junction points. However, data supporting such a mechanism are lacking.

The antibody used in these studies was a strong agglutinin, but, in high concentration, exhibited weak hemolytic activity in vitro. However, it appears unlikely that complement played a significant role in the red cell membrane modification or cell destruction reported herein. In vitro, complement causes lysis without spherocytosis and without significant changes in the lipid content of the posthemolytic stroma (45, 46). Serum from animals injected with antibody is not hemolytic when tested with fresh red cells in vitro and does not induce spherocytosis upon continued incubation (1, 12), although it retains its ability to agglutinate red cells. When rat red cells coated with rabbit anti-rat red cell antibody were injected into normal mice, cell destruction was rapid (47). However, prior treatment of the antibody with pepsin, which destroys its complement-fixing properties, or prior decomplementation of mice resulted in a rate of red cell destruction during the 20 min of observation which was not appreciably faster than that observed in controls. In similar studies, but in rats, Vitale and coworkers observed rapid destruction of red cells coated with hemolytic antibody, whereas the pepsin-treated antibody, which retained its agglutinating properties, led to the destruction of only 15% of the injected red cells in 2 hr (48). In both of these studies, small numbers of antibody-coated red cells were injected into otherwise normal animals, whereas in the present study the animals' entire red cell population was coated by a large excess of antibody infused intravenously. Nonetheless, the survival of red cells in the present study, using heat-treated agglutinins with low hemolytic titers, was similar to that seen with pepsin-treated antibody: approximately 19% of the circulating red cells were destroyed within the first 2 hr (as reflected by changes in hematocrit).

Boyden has demonstrated that sheep red cells will adhere to normal guinea pig macrophages previously sensitized with guinea pig anti-sheep red cell antibody (49). Using homologous human systems it has been shown that human red cells coated with antibodies of the IgG class, subclasses IgG_1 and IgG_3 in particular, adhere to and are sphered by human mononuclear cells, and that spherocytosis persists after they are released from the mononuclear attachment sites by papain (50–52). Complement in the absence of IgG fails to induce adherence (50). It is an IgG agglutinin which is responsible for the spherocytosis reported herein (48) as well as in the spherocytosis resulting from hyperimmune anti-A and anti-B in man. In contrast, cold agglutinins (e.g. in cold agglutinin disease) which do not induce spherocytosis are IgM antibodies (5). Thus, it is likely that the

phenomenon observed in the present studies was induced by the interaction between IgG-coated red cells and macrophages in vivo. However, proof that this is true remains to be established, and the mechanism whereby membrane lipoprotein might be removed under these conditions remains to be explored.

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