

The Immunologic Specificity of Antiserum for Trypsin-Treated Red Blood Cells and Its Reactions with Normal and Hemolytic Anemia Cells

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ERYTHROCYTES modified by sensitization with antibodies, by treatment with enzymes or viruses, or by storage have shown properties in common with the abnormal, short-lived erythrocytes from patients with certain hemolytic anemias. These are increased fragility, increased susceptibility to phagocytosis,¹ and agglutination in normal serum. The characteristic agglutination of suitably trypsinized red cells by the incomplete antibody found in the sera of hemolytic anemias has proved of definite clinical value.^{2, 3, 7} This investigation was initiated with the hope that a study of the nature of the changes involved in trypsinization might assist in clearing the mire of abnormal hemolytic processes.

Wheeler³ suggested that the effect of trypsin and other enzymes on erythrocytes, which permits their agglutination in saline by incomplete antibody, may be due to nonspecific physical or chemical changes of the cell surface which modify their suspension stability when sensitized. Ponder⁴ has postulated a "loosening" process involving the breaking of bonds and the appearance of less acidic ionized groups, and possibly the incorporation of trypsin into small parts of the protein-containing ultrastructure.

We have considered the possibility that the enzyme action may alter the cell surface so that subsurface antigen sites are available, producing, in effect, a new specificity. It was further postulated that this specificity might be detectable in the red cells of certain hemolytic anemias. This could account for the presence of antibody in these clinical states.

For these reasons, antisera to normal and trypsinized human red blood cells were prepared by immunization of rabbits. The antibody specific for the trypsin cell component was separated by proper absorption procedures. The specificity of this antibody was also studied with periodate-treated cells and with cells from patients with various types of hemolytic anemia.

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MATERIALS AND METHODS

Preparation of Antisera

Sixteen rabbits weighing approximately 2.5 Kg. were used for immunization, half receiving normal human red blood cells and the remainder equal amounts of the same blood after trypsinization as detailed below. In both groups each animal received a total of five injections, four intravenously and one intraperitoneally, of either a 4 per cent or a 40 per cent suspension of the appropriate red blood cells. Injections were given every other day. In order to test the possible role of the spleen in the production of antibody, splenectomized rabbits were also treated as above. The animals were bled seven days after the last injection and the serum inactivated before titration.

Preparation of the Red Blood Cells

The normal human blood cells (Nrbc) used throughout this work were group O, Rh+, MN. The cells were washed five times with physiologic saline and suspended in either a 4 per cent or a 40 per cent concentration. The trypsinized cells (Trbc) were similar suspensions of the same blood cells which had been treated with the enzyme as previously described.^{1, 2} The periodate-treated cells (Prbc) were prepared by the method used by Moskowitz and Treffers.⁵

Absorption of Antisera

Absorptions were made using cells of group O, Rh+, MN, either normal or trypsinized, and previously washed five times in physiologic saline. Fresh cells were used for each absorption period listed below. The ratio of packed cells to serum was 1:1 for the first and second absorption and 1:2 for all subsequent absorptions. The following procedures were used to determine the most favorable temperature and time for maximum absorption:

Method 1. Absorption 3X at 37 C. for 1, 1½, and 1½ hours.

Method 2. Absorption 3X at 37 C. for 4, 12, and 20 minutes.

Method 3. Absorption 5 to 7X at room temperature for 10 and 30 minutes followed by three to five absorptions for 1 hour each.

Method 4. Absorption 3X at 4 C. for 1 hour each time.

Titrations of Antisera

Agglutinin titers with normal and trypsinized cells in saline and albumin were determined with 2 per cent suspensions as previously described.^{1, 2} Tests for hemolysis were carried out by adding two units of guinea pig complement to various dilutions of antisera containing 2 per cent cell suspension.

The anti rabbit globulin serum used in attempts to detect antibody on the red cells was guinea pig serum pooled from several animals immunized with rabbit serum. The antiserum was absorbed with normal rabbit cells before use as a "developing" serum.

RESULTS

The sera of rabbits immunized with normal or trypsinized erythrocytes showed little specificity for the homologous cells. As would be expected, the individual titers varied moderately, usually being somewhat higher in rabbits receiving the larger amount of antigen. With but one exception, the titer for trypsinized cells was always slightly higher than for normal cells. These findings were unaffected by incubation at 37 C., or in the cold, or by immediate readings after mixing and centrifugation. No significant difference in the titers of sera of splenectomized rabbits was detected. In view of these findings, the sera were pooled on the basis of the kind of cells used to immunize. Each pool was tested

that such antibody is not capable of sensitizing normal cells. Furthermore, the anti-TE did not block agglutination of normal cells by homologous unabsorbed anti-Nrbc. Its agglutination of Trbc was not enhanced by the addition of albumin, nor was it hemolytic in the presence of guinea pig complement.

The specificity of the anti-TE was also examined using periodate-treated cells. Moskowitz and Treffers⁵ and Stewart⁶ found that such cells produced specific antibody in rabbits. Periodate-treated red cells were agglutinated by unabsorbed anti-Trbc serum to the same titer as Trbc and Nrbc. Periodate-treated red cells were also agglutinated by anti-TE (fig. 2). However, homologous absorption of anti-Trbc removed all antibody for periodate-treated cells. On the contrary, anti-Nrbc serum still agglutinated periodate-treated cells after absorption with either Trbc or Nrbc. When anti-Trbc serum was absorbed with periodate-treated cells the antibody for these cells was completely removed while the antibody titer to the other two cell types was reduced.

ANTISERUM	ABSORBED WITH:	TEST CELLS	SERUM DILUTIONS										
			U	2	4	8	16	32	64	128	256	C	
ANTI-Trbc	Nrbc	Prbc	S*	S	+++	++	+	-	-	-	-	-	-
ANTI-Trbc	Trbc	Prbc	-	-	-	-	-	-	-	-	-	-	-
ANTI-Nrbc	Nrbc	Prbc	S	S	S	+++	+++	+++	-	-	-	-	-
ANTI-Nrbc	Trbc	Prbc	+++	+++	+++	-	-	-	-	-	-	-	-
ANTI-Trbc	Prbc	Prbc	±	-	-	-	-	-	-	-	-	-	-
ANTI-Trbc	Prbc	Nrbc	+	++	++	+	-	-	-	-	-	-	-
ANTI-Trbc	Prbc	Trbc	+++	+++	+++	++	+	±	-	-	-	-	-

FIG. 2.—Hemagglutination of periodate-treated cells (Prbc) by absorbed antisera to normal and trypsinized cells. *S = all cells in solid clump.

Before examining the reactivity of red cells of hemolytic anemia patients with the trypsin cell specific serum, both the absorbed anti-Nrbc (anti-NE) and anti-TE were tested with the cells of eleven normal individuals. Both the normal and trypsinized cells were used and the cell types included O, A₁, A₂, B, Rh+, and Rh-. The reactions in all cases were almost identical to those listed in figure 1 for normal and trypsinized cells and thus seemed to be independent of any of the above antigens. The anti-TE was also equally reactive with all samples of trypsinized cells, titers being either 1:256 or 1:512. None of the eleven samples of normal cells was agglutinated in undiluted anti-TE serum after eight absorptions and only trace agglutination occurred in sera absorbed five to seven times.

The red blood cells of nineteen patients with acquired hemolytic anemia were tested with anti-TE serum. The results are compared in figure 3 with data obtained with the antiglobulin (Coombs) test and with the trypsin cell test on the

The cells of all the cases studied were also reacted against the partially absorbed anti-NE. In general, they reacted like normal cells, since they usually gave higher titers than those obtained with trypsinized cells (fig. 1). These data indicate that both Nrbc and Trbc specificities may be present in the cells of these patients. Also, since both may be shown in sensitized and nonsensitized cells, the human antibody globulin present does not seem to interfere.

DISCUSSION

These results provide strong evidence for the existence of antigenic differences between normal and trypsin-treated human red blood cells. It was assumed that enzyme treatment of the intact erythrocyte probably involved the destruction of surface antigens of the cell with the consequent uncovering of deeper structures with antigenic capabilities. In antiserum to trypsinized cells, the modified normal antigens and the deeper structures no doubt account for the specificity of the anti-TE. Antigens of the normal portion of the cell produce the remainder. After absorption with normal cells, this serum (anti-TE) agglutinated trypsinized cells but could not be shown to sensitize or agglutinate normal cells. The fact that it agglutinated the cells of a majority of the cases of hemolytic anemia tested suggested that such cells show some changes similar to those produced by trypsin *in vitro*. The absorption data showing that both Prbc and Trbc specificities were removed by trypsinized cells, but not by the periodate-treated cells, indicated that some part of the change produced by trypsin was similar to the modification induced by periodate. Periodate-treated cells are agglutinated by incomplete anti-Rh antibody. Moskowitz and Treffers⁵ claimed that antibody specific for these cells could be demonstrated.

Sera from rabbits immunized to normal cells, if first diluted before absorption with Nrbc, have a specificity for trypsinized cells and only a trace agglutination for homologous cells. This suggests that *in vivo* destruction of normal erythrocytes may create conditions in which the antibody forming mechanism is stimulated by antigenic groups similar to those made manifest by *in vitro* trypsinization. The fact that some antigens present on the normal cell are removed during *in vitro* trypsinization is indicated by the failure of trypsinized cells to remove antibody for normal cells from anti-Nrbc, and a similar failure of absorption to show any distinct specificity for normal cells in anti-Trbc serum. From these results it appears that three types of antibody are involved: (1) antibody specific for antigens common to both types of cells; (2) antibody specific for antigens uncovered or modified by trypsinization; (3) antibody specific for antigen(s) present on the normal cell but not on the treated cell. Obviously anti-Trbc would contain only types (1) and (2), whereas anti-Nrbc could conceivably contain all three types of antibody.

It may be implied from the above discussion that antibody specific for antigens uncovered or modified by trypsin would react only with treated cells. However, this is opposed to the finding that undiluted anti-Nrbc, after homologous absorption, gives approximately equal titers with both types of cells, although normal cell antibodies should have been removed. This necessitates a modification of the concept of correspondence between *in vitro* enzymation and *in vivo* destruction of the normal human cell in rabbits. In the former process the enzy-

matic treatment is allowed to proceed to some arbitrary point before immunization. In the latter the degradation of the cell is very likely carried out in the immediate vicinity of the site of antibody formation, thus affording the opportunity for the formation of antibodies with dual specificities. Such dual specificity is found frequently in the case of immunization with proteins containing prosthetic groups; antibodies being present with specificities directed simultaneously to protein groupings and haptene groups. In the present situation the dual specificity would involve the antigenic groups on the surface of the red cell and the modified or uncovered groupings produced by trypsin. In vitro trypsinization leads first to agglutinability by incomplete antibody and then to agglutinability by the classical "T" agglutinins of Thomsen. This would support the idea of at least two well-defined steps in the process of cell digestion. The same steps can be observed in cells treated by other procedures, e.g., treatment with periodate ions, *Vibrio cholerae* filtrates, or papain. Animals immunized with cells subjected to various methods of treatment produce antibodies specific for cells treated by the same process as the immunizing antigen. Again this can be attributed to arbitrary termination of the procedures. Sera from animals immunized with normal untreated cells show practically identical reactivity for all types of treated cells. From this, it is likely that in vivo modification takes place in discrete steps resulting in the production of antibodies with dual specificity. Thus, part of the reactive site would be directed toward chemical groupings exposed by a variety of digestion procedures, with each procedure accompanied by some other unique change.

That the modified cell antigens may play a role in the production of the immunologic phase of hemolytic anemias is suggested by the presence of trypsin cell specificity in the cells of hemolytic anemia patients, together with the previously reported fact that at least some of the antibody in the serum of hemolytic anemias was specific for trypsinized cells.⁷ Although the original mechanism by which such modifications could occur in vivo is unknown, their appearance could initiate the process of autoimmunization with the production of antibodies with dual specificity for normal and treated cells. The different serologic manifestations obtained—agglutination of treated cells as opposed to sensitization of normal cells—probably depend on the different manner in which the antibodies combine with each type of cell. The antigenic relationship of red cells treated with trypsin or periodate also explains why both types of cells react with incomplete antibody in anti-Rh and hemolytic anemia serum. It also suggests that further study of modified cells may be useful in the investigation of hemolytic disease.

SUMMARY

1. Rabbits were immunized with both normal (Nrbc) and trypsinized (Trbc) human red blood cells and the antisera examined with normal, trypsinized, periodate-treated, and hemolytic anemia cells.
2. Absorption studies showed the presence of a fraction in both anti-Trbc and anti-Nrbc that was specific for trypsinized cells.
3. This T cell specific fraction from anti-Trbc serum (anti-TE) did not agglutinate or sensitize normal red blood cells, but agglutinated periodate-treated

cells. This latter specificity was shown to be a part of the modification produced by trypsinization.

4. Anti-TE also agglutinated the cells of fifteen of nineteen patients with acquired hemolytic anemia and three of thirteen cases of hereditary spherocytosis.

5. Antibody for trypsinized and normal cells was also detected in antiserum to normal cells. Absorption data suggested the presence in this antiserum of antibody with a dual specificity for both types of cells.

6. The role of the antigenic modifications produced by trypsin in red cell immunization and in hemolytic anemia is discussed.

ADDENDUM

Since the completion and submission for publication of this study, similar observations by Finch and Ross have been published in abstract (*Clin. Res. Proc.* 1: 13, 1953).

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