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Requirement for Properdin in Hemolysis of Human Erythrocytes Treated with Tannic Acid.* (22908)

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The hemolysis of the abnormal erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNH) by normal human serum requires the properdin system, which consists of properdin, the components of complement, and magnesium(1). Normal human erythrocytes treated with tannic acid (TA cells) are also hemolysed by normal serum(2). The present report indicates that the hemolysis of appropriately treated TA cells also requires the properdin system.

Methods. Erythrocytes. Human group O blood was collected in an equal volume of Alsever's solution and stored at 4°C. The blood was stored 4 days before use and discarded after 10 days. The same donor was used throughout, although blood samples from other donors were equally satisfactory. The erythrocytes were washed 3 times in 0.15 M NaCl and suspended to 2% in barbital buffer containing 0.0005 M $MgCl_2$(3,4). *Tanning of cells.* A stock solution of 1% tannic acid in 0.15 M NaCl was stored at 4°C. It was diluted immediately before use in 0.15 M NaCl to concentrations optimal for tanning cells. The final concentration of tannic acid employed was usually 1:15,000. There was some variation from day to day, and concentrations from 1:10,000 to 1:80,000 were tested each day. The activity of the properdin system was demonstrated only for cells exposed to a relatively limited range of concentrations of tannic acid. Concentrations of greater than 1:10,000 caused clumping of erythrocytes, slight spontaneous hemolysis, and marked hemolysis in serum lacking properdin (RP). Concentrations of less than 1:20,000 usually failed to alter the cells. There was little difference in activity whether the tanning was carried out in silicone treated or ordinary glassware. Aliquots of 30 ml of erythrocyte suspension and tannic acid solution of optimal concentration, in separate 150 ml Erlenmeyer flasks, were placed in a 37°C water bath until the contents of each flask reached a temperature of at least 36°C. The tannic acid solution was then poured rapidly into the cell suspension with continuous and vigorous swirling for 5 to 10 seconds. The mixture was immediately transferred to test tubes and centrifuged for 1 minute at approxi-

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TABLE I. Hemolysis of Erythrocytes Treated with Tannic Acid.

Reagent	Hemolysis: Without added properdin	Hemolysis: With added properdin (10 u)
Human serum	+	+
Serum heated to 56°C	0	0
RP	0	+
R 1	0	0
R 2	±	+
R 3	0	0
R 4	0	0
Resin treated serum	0	0
Idem + Ca^{++}	0	0
" + Mg^{++}	+	+

mately 2000 r.p.m.; the cells were well packed. The supernatant fluid was decanted and the packed cells were washed once with a large volume of 0.15 M NaCl; they were centrifuged again at 2000 r.p.m. for 1 minute, and finally suspended to exactly 2% in barbital buffer. The cells were prepared daily, and kept at room temperature during the day of use. Cooling to 4°C and then rewarming resulted in spontaneous hemolysis. *Temperatures* lower than 37°C and periods of exposure to tannic acid of greater than 5 to 10 seconds resulted in inadequate sensitization of the cells. Cells were considered satisfactorily tanned when they were not hemolyzed in RP, or purified properdin, but were hemolyzed by fresh serum or RP to which properdin was added. *Test system.* To 0.25 ml of serum or serum reagent were added varying amounts of properdin, barbital buffer, and 0.05 ml of 2% TA cells, to give a final total volume of 0.50 ml. The mixture was incubated at 37°C for 30 minutes, the tubes centrifuged, and the amount of hemolysis determined in a photoelectric colorimeter. *Serum reagents.* Normal human serum, serum lacking properdin (RP), serum lacking specifically one of the components of complement (R1, R2, R3, R4), and purified properdin, were collected and prepared as previously described(4,5). Serum was treated with amberlite IRC-50 sodium cycle resin to remove Mg^{++} and Ca^{++} as previously described; it was tested immediately(6).

Results. Hemolysis of TA cells in serum did not occur at 4° or 18°, and was optimum at 37°C. Hemolysis was greater at pH 7.4 than at pH 6.8 or 8.0. Therefore, the reaction was allowed to proceed at 37°C and pH 7.4. *Requirement for properdin.* Although marked hemolysis of TA cells occurred when they were incubated at 37°C in normal human serum, serum from which properdin had been removed (RP) failed to hemolyze TA cells (Table I). Addition of 10 units of purified properdin per ml of RP restored the hemolytic activity of the serum. Properdin alone was not hemolytic. Addition of varying amounts of properdin to RP caused a regular increase in hemolysis of TA cells (Fig. 1) proportional to the amount of properdin added. The RP employed in the experiment illustrated was slightly lytic. Properdin alone is not lytic. Addition of small volumes of various human sera to RP resulted in varying amounts of hemolysis. There was no correlation between the amount of hemolysis and the properdin level of the serum as determined by the zymosan assay(4). Variations in total complement in the various sera also did not account for the differences which were observed. Thus, while the TA cells are suitable for the assay of purified properdin, they are not suitable for the quantitative measurement of properdin in serum.

Requirement for complement. Serum from which C'1, C'3, and C'4 had been removed, or inactivated failed to hemolyze TA cells (Table I). Addition of 10 units of properdin per ml to these reagents failed to restore hemolysis. Some serums lacking C'2 activity

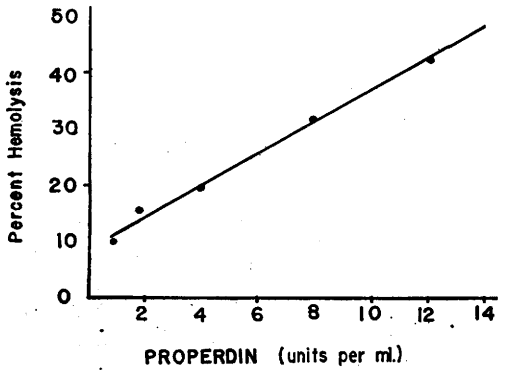

FIG. 1. Effect on hemolysis of tannic acid treated cells, of adding increasing amounts of properdin to RP.

caused slight hemolysis of TA cells which increased upon the addition of 10 units of properdin.

Requirement for Mg^{++}. Serum from which Mg^{++} and Ca^{++} had been removed did not hemolyse TA cells. Addition of 0.0005 M Mg^{++} to the resin-treated serum restored hemolytic activity. Addition of 0.005 M Ca^{++} did not restore hemolytic activity to the resin-treated serum.

Discussion. Under the conditions of the experiments described above, the hemolysis of normal human erythrocytes treated with weak solutions of tannic acid requires properdin, factors indistinguishable from serum complement, and magnesium. In this manner the mechanism of hemolysis of TA cells and PNH erythrocytes by human serum is similar. It differs from the hemolysis by isohemolysins and panhemolysins which require specific antibody and complement, but do not require properdin or magnesium(1).

However, differences exist between TA cells and PNH cells. TA cells spontaneously agglutinate, hemolyze spontaneously following exposure to cold, and lyse optimally in serum at pH 7.4. PNH cells do not spontaneously agglutinate, are stable in the cold, and hemolyze maximally in serum at pH 6.8.

Another slight difference is that C'2 is not an absolute requirement for the hemolysis of TA cells. Thus, the reaction of the properdin system with TA cells resembles its reaction with zymosan and other high molecular weight polysaccharides(7). This suggests that the surface presented by the tanned cell may resemble the polysaccharides which are known to interact with properdin(8).

Although attempts have been made to employ TA cells for the measurement of complement in serum, the methods used differed from those employed in the present study(9). It appears likely that the degree of alteration of the cell surface influences the susceptibility of the cell. Cells exposed to dilutions of tannic acid less than 1:10,000 do not require properdin for hemolysis, whereas cells exposed to 1:15,000 dilution of tannic acid required both complement and properdin. Cells exposed to higher dilutions are not hemolyzed.

The hemolysis of TA cells has not proven reliable as a simplified method for the assay of properdin in human serum. As mentioned above, other as yet undefined factors in normal serum also influence hemolysis. Thus there is no correlation between serum properdin level as measured by the zymosan assay and the ability of a serum to hemolyze TA cells. It is also unlikely that such a system would be suitable for the measurement of properdin in animal sera, since TA cells as well as PNH cells are hemolysed by heterolysins, independent of the properdin system.

Summary. 1) The hemolysis of normal human erythrocytes exposed to appropriate dilutions of tannic acid by human serum, requires the properdin system, which consists of properdin, complement, and magnesium. 2) Similarities and differences are pointed out between the properties and mechanism of hemolysis of tannic-acid-treated cells and erythrocytes from patients with paroxysmal nocturnal hemoglobinuria. 3) While the hemolysis of TA cells is suitable for the assay of purified properdin it is not reliable for the quantitative measurement of properdin in serum.

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