A HEMOLYTIC SYSTEM ASSOCIATED WITH ENTERITIS IN RABBITS

I. Nature of the Cell Change and the Serum Factors Concerned*

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A hemolytic system, which appears in rabbits in association with an enteric disorder, has been described in a preliminary report (1). Commercial rabbit growers regard "mucoid" enteritis as their most serious problem since losses ascribed to this disorder among the young rabbits may reach 25 per cent. A minority of the affected animals we observed produced a mucoid or a bloody rectal discharge, but watery diarrhea was more common. Some of the animals recovered, but the majority died with severe saline depletion. The bowel mucosa was not ulcerated nor was there evidence of inflammatory change in the tissues to suggest bacterial invasion. In our study of 200 young rabbits in 28 litters, 60 per cent with signs of enteritis had a red cell abnormality which was present in only 15 per cent of those without evidence of enteritis. The characteristics of the red cell change and the serological factors in the hemolytic system are summarized below.

The abnormality of the cells occurred most commonly in weanling rabbits and did not appear until the animals were 5 to 8 weeks of age.[1] The cell "lesion" disappeared gradually in surviving animals.

The cells of the affected rabbits were susceptible to hemolysis in normal rabbit sera and to agglutination in heat-inactivated normal rabbit sera. Agglutinins for the abnormal cells were also present in other mammalian sera.

The serum factors necessary for hemolysis and agglutination of the abnormal cells were commonly absent from the sera of animals with abnormal cells.

The abnormal factor of the cells was present in stroma prepared from the abnormal cells and was found to be heat-stable. Since a filtrate of one of several mixed bacterial cultures of bowel organisms produced a similar abnormality in

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[1] "Cell" will be used interchangeably with red cell. The term "weanling" is used to denote rabbits between 5 and 8 weeks of age which take solid food, but which may or may not continue to suckle.

normal rabbit cells, it was postulated that the lesion of the cells of affected rabbits may result from the adsorption of a bacterial polysaccharide or that a bacterial enzyme may act to uncover or alter a cell antigen of polysaccharide nature.

Additional observations of this hemolytic system are presented in this communication. Since the abnormal cells resemble the polyagglutinable cells which are occasionally observed in patients with acute infections, they will be referred to as "polyagglutinable cells" and the disease as the "polyagglutinable-cell disease."

Materials and Methods

Determination of the Agglutinability of Polyagglutinable Red Cells.—The heat-inactivated sera of some normal adult rabbits were found to have sufficiently high titers of agglutinins for polyagglutinable cells ("polyagglutinins") to be useful in the routine testing of cells.[2] Since isoantibodies for normal rabbit red cells are not present in normal rabbit sera, preliminary treatment of the sera with normal cells was not necessary. Cells to be tested for polyagglutinability were washed 3 times in physiological saline and a 2 per cent suspension of cells was prepared. 0.1 ml of the suspension was added to 0.1 ml of an undiluted, normal rabbit serum that had been inactivated by heating at 56°C for 30 minutes. The mixture was centrifuged promptly in a 10 × 75 mm tube for 1 minute at 1500 RPM, and after gently resuspending the cells the degree of agglutination was graded on a 1+ to 4+ scale.

Normal goat sera from which heterologous agglutinins had been absorbed with normal rabbit cells were found to have high titers of agglutinins for polyagglutinable cells. Since this reagent could be obtained in large quantities it was used routinely in screening tests for polyagglutinability of cells. The technique employed was the same as that described for normal rabbit sera except that the goat sera were diluted 1:4 with physiological saline.

Determination of Sensitivity of Polyagglutinable Red Cells to Hemolysis.—One sample of 0.05 ml of a 50 per cent suspension of polyagglutinable cells was added to 0.5 ml of fresh normal rabbit serum. Another sample was added to 0.5 ml of distilled water. The mixtures were incubated at 37°C for 30 minutes and then centrifuged at 1500 RPM for 1 minute. 0.2 ml of the supernatant fluid from each mixture was added to 2 ml of water and the optical density was read at 540 mμ in a Coleman spectrophotometer, using 0.2 ml of unincubated serum in water as a control blank. The per cent hemolysis was calculated by using the concentration of hemoglobin in the control blank to represent 100 per cent hemolysis.

Methods Concerned with Complement and Properdin.—Complement (C′) determinations were carried out by the methods of Kabat and Mayer (2). Properdin-free serum (R.P.) and properdin were prepared by the methods of Pillemer *et al.* (3).

Preparation of Red Cell Stroma.—Red cells were washed 3 times in physiological saline and hemolyzed in 10 to 20 volumes of distilled water. The stroma was sedimented at 17,000 g in the Spinco ultracentrifuge.

Preparation of Bovine Serum Albumin–anti-Bovine Serum Albumin (BSA–anti-BSA) Precipitate.—The BSA–anti-BSA precipitate was prepared at optimal proportions by adding anti-BSA rabbit serum to a solution of BSA. The mixture was incubated at room temperature for 15 minutes and the precipitate was washed once in physiological saline.

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[2] For convenience the term "polyagglutinins" will be used to designate agglutinins that are "specific" for polyagglutinable cells.

RESULTS

Agglutination of Polyagglutinable Red Cells.—On occasion weanling rabbits were observed to develop polyagglutinable cells within a period of 24 hours. After full development the cell lesion usually disappeared in a gradual manner over a period of several weeks. Reappearance of the lesion occurred occasionally and some animals were observed to develop polyagglutinable cells after complete reversion of the initial lesion to normal. The intensity of the lesion varied widely among different animals and in some instances polyagglutinability remained minimal.

The intensity of agglutination of polyagglutinable cells from various affected rabbits in the test sera of rabbits and goats was roughly parallel and varied directly with the susceptibility of the cells to hemolysis in normal rabbit sera. When the lesion was most pronounced, all of the cells were agglutinated and large clumps of cells did not break up readily with agitation.

Agglutination of polyagglutinable cells was not greater in the anti-rabbit globulin goat serum than in the sera of normal goats. The activity of the anti-rabbit globulin goat serum was not inhibited by the addition of rabbit gamma globulin.

Nature of the "Polyagglutinin".—The concentration of the polyagglutinin in heat-inactivated normal rabbit sera varied considerably. The sera of some adult rabbits agglutinated cells with the most intense lesions to a dilution of 1:32. Many sera had lower titers but none was found that was completely devoid of agglutinating activity.

The sera of animals with polyagglutinable cells were usually lacking in agglutinins for polyagglutinable cells of other rabbits as well as for their own cells. As the cell lesion disappeared in such animals, the polyagglutinin appeared. However, the polyagglutinin did not reach higher titers in the sera of these recovered animals than it did in normal animals. Neither was the polyagglutinin in the sera of normal rabbits increased by giving the animals intravenous injections of polyagglutinable cells.

An agglutinin for polyagglutinable cells was found to persist in the sera of other species of animals after absorption of such sera with normal rabbit cells to remove agglutinins for the latter (Table I). Normal goat and swine sera contained somewhat higher titers of polyagglutinin than did normal rabbit sera. The sera of some dogs and of some humans also contained polyagglutinin but to a lesser titer than the rabbit, goat, or swine. One absorption with an equal volume of polyagglutinable cells was usually sufficient to remove all polyagglutinin from heterologous sera. Normal rabbit cells and the cells of rabbits that had recovered from the polyagglutinable lesion did not absorb polyagglutinins from homologous or heterologous sera. Neither were polyagglutinins adsorbed by normal rabbit cells that had been treated with papain or trypsin. Moreover,

treatment of polyagglutinable cells with these enzymes did not alter their behavior toward normal serum or their power to adsorb polyagglutinin.

In an attempt to ascertain the role of complement in polyagglutination and hemolysis, normal rabbit sera were incubated with BSA–anti-BSA precipitate at 20°C for 1 hour followed by incubation at 5°C for 24 to 48 hours. The absorbed sera were devoid of hemolytic activity for sheep cells sensitized by rabbit amboceptor and for polyagglutinable rabbit cells. The polyagglutinin was also decreased, but heating such sera to 56°C for 20 minutes activated their agglutinating power to values comparable to those of heat-inactivated sera that had not been absorbed with BSA–anti-BSA precipitate. The agglutinating activity

TABLE I

Agglutination of Polyagglutinable Rabbit Cells in Sera of Other Species

Serum source	Agglutination in dilutions of sera				
	Neat	1:2	1:4	1:8	1:16*
Rabbit	4+‡	3+	3+	1+	0
Man	3+	2+	1+	0	0
Dog	2+	2+	1+	0	0
Swine	3+	4+	3+	3+	2+
Goat (normal)	4+	4+	3+	2+	1+
Goat (anti-rabbit globulin goat serum)	4+	4+	3+	2+	1+

* The sera were heat-inactivated and absorbed with normal rabbit cells. Dilutions of sera were made in buffered isotonic saline.

‡ 4+ = 75 to 100 per cent agglutination; 3+ = 50 to 75 per cent; 2+ = 25 to 50 per cent; and 1+ = 1 per cent to 25 per cent agglutination.

of the precipitate-absorbed sera was also increased by allowing them to stand for several hours at room temperature.

Neter *et al.* (4) have discovered a heat-stable inhibitor in human and animal sera which inhibits the agglutination of red cells coated with the "heterogenetic antigen" of Gram-positive bacteria. The possibility that some inhibitor of agglutination was involved in the present system was considered. Apparently, the precipitate-absorbed sera did not contain an inhibitor of agglutination since dilution of heat-inactivated normal rabbit sera with precipitate-absorbed sera did not reduce their agglutinating activity. Moreover, treatment of heat-inactivated normal rabbit sera with BSA–anti-BSA precipitate did not reduce the polyagglutinating activity of the sera. The agglutinating activity of normal sera was decreased to the same degree by sera of rabbits with polyagglutinable cells as by saline.

When normal rabbit plasma was heated to 56°C for 20 minutes, it showed agglutinating activity for polyagglutinable cells equal to that of heat-inactivated

serum. The use of heparin, oxalate, or citrate as anticoagulants did not alter the polyagglutinating activity of normal plasma.

Susceptibility of Polyagglutinable Red Cells to Hemolysis in Normal Rabbit Sera.—Susceptibility of polyagglutinable cells to hemolysis in normal rabbit sera varied directly with the susceptibility of the cells to agglutination in heat-inactivated normal sera. Whereas the cells from some affected rabbits exhibited only slight hemolysis after incubation in normal sera, the cells of other rabbits underwent 90 per cent hemolysis in the same sera. 40 to 50 per cent hemolysis was usual for cells from rabbits with recently acquired polyagglutinable cells.

TABLE II

The Influence of pH on the Hemolysis of Polyagglutinable Cells by Normal Rabbit Serum

pH*	Samples of polyagglutinable cells		
	No. 1	No. 2	No. 3
6.8	0‡	29	
7.0	6.6	31	16
7.2	19.5	42	49
7.4	32.0	42	53
7.6	38.0	41	55
8.0	34.0		59

* The pH of 1 ml of normal rabbit sera was adjusted with mineral acid and 0.05 ml of cells were added. The mixtures were incubated 1 hour at room temperature.

‡ The figures represent per cent hemolysis.

The degree of hemolysis of polyagglutinable cells was maximal when the cells were suspended in 40 volumes of serum. A cell-serum ratio of less than 1:40 resulted in less than the possible maximum hemolysis. The readdition of fresh serum to such mixtures resulted in further hemolysis of residual cells. Hemolysis of susceptible cells occurred rapidly and was nearly complete after 5 minutes incubation at 37°C. In 3 preparations of polyagglutinable cells hemolysis was decreased by lowering the pH of the serum with mineral acid as shown in Table II. The effect of temperature of incubation on the degree of hemolysis was not pronounced above 20°C.

Hemolytic Factors in Normal Sera.—The hemolytic activity of sera of normal rabbits for polyagglutinable cells was apparently limited and only minor variations were noted among most of the animals studied. Dilution of the sera with 2 to 4 volumes of physiological saline usually eliminated hemolysis, regardless of what ratio of cells to diluted serum was used. Normal serum was seldom devoid of all hemolytic activity for polyagglutinable cells and only 2 such sera were encountered in a group of over 100 normal animals examined.

Heating normal rabbit sera to 56°C for 20 minutes destroyed their hemolytic activity. The addition of normal guinea pig sera to heat-inactivated rabbit sera in a proportion of 1:6 restored the hemolytic activity of the latter for sheep cells sensitized by rabbit amboceptor, but did not restore hemolytic activity for the polyagglutinable cells.

The hemolytic activity of normal sera for polyagglutinable cells was suppressed by oxalate and citrate anticoagulants but not by heparin. The removal of Mg^{++} and Ca^{++} from the sera with ion exchange resin also resulted in loss of hemolytic activity.[3] Activity was fully restored by the addition of Mg^{++} and Ca^{++} to give final concentrations of 0.001 M and 0.0025 M, respectively. When Ca^{++} was added to resin-treated serum containing polyagglutinable cells and the suspension was incubated for 10 minutes prior to the addition of Mg^{++}, hemolysis did not occur either prior to or following the addition of Mg^{++}. When the process was reversed and Mg^{++} was added first, only slight hemolysis occurred before the addition of Ca^{++}. Following the addition of the latter, the hemolytic activity was restored to the level produced by untreated sera.

Normal sera stored for 24 to 48 hours at 5°C retained most of their hemolytic activity for polyagglutinable cells. However, the sera of some normal rabbits, when allowed to stand overnight at 5°C without removal of the clot, lost all hemolytic activity for polyagglutinable cells without measurable reduction in complement activity. It was also observed that the hemolytic activity of normal sera dropped when they were allowed to stand at room temperature for several hours.

The sera of animals with polyagglutinable cells lacked hemolytic activity for polyagglutinable cells. This was not due to lack of C′ since such sera possessed normal hemolytic activity for sensitized sheep cells. Furthermore, the levels of the various fractions of C′ were within the same range as those of normal sera, as shown in Table III.

The possibility that the lack of hemolytic activity of the sera of animals with polyagglutinable cells might be due to a deficiency of the heat-stable polyagglutinin was explored. However, polyagglutinable cells which had been incubated in 40 volumes of heat-inactivated normal serum containing normal concentrations of polyagglutinin did not hemolyze when they were resuspended in the sera of animals with polyagglutinable cells. Furthermore, the addition of heat-inactivated normal sera, containing polyagglutinin, to an equal volume of sera of animals with polyagglutinable cells containing normal levels of all fractions of C′, was not hemolytic for polyagglutinable cells, even though it was hemolytic for sheep cells sensitized with rabbit amboceptor. When normal rabbit sera were diluted with sera from animals with polyagglutinable cells, the hemolysis of polyagglutinable cells was not inhibited to a greater degree than by dilution with physiological saline.

[3] The resin used was Amberlite IRC-50.

Normal rabbit sera absorbed with BSA–anti-BSA precipitate did not hemolyze polyagglutinable cells or sheep cells sensitized with rabbit amboceptor. However, the hemolytic activity of precipitate-absorbed normal sera for polyagglutinable cells could not be restored by the addition of sera of animals with polyagglutinable cells even though the latter sera possessed the complement activity of normal sera.

Further Observations on the Nature of the Serum Factor Necessary for Hemolysis of Polyagglutinable Red Cells.—Properdin-deficient sera were made by incubating normal rabbit sera with zymosan at 15°C according to the method of Pillemer *et al.* (3). These preparations were apparently free of properdin since C'_3, which was present in close to normal concentration, was not diminished

TABLE III

*Determination of Total Complement and Complement Fractions in Normal Rabbit Sera and the Sera of Animals with Polyagglutinable Cells**

Complement or complement fractions	Range of complement levels	
	10 rabbits with polyagglutinable cells	10 normal rabbits
C'	8 to 32	8 to 32
C'_1	64 to 240	64 to 200
C'_2	8 to 32	8 to 32
C'_3	32 to 64	32 to 64
C'_4	16 to 100	16 to 64

* The figures presented represent extremes of variation among 10 affected and 10 normal rabbits. Both rabbit and guinea pig sera were used for determining the fractions of complement and the values are expressed in units of complement based on 100 per cent hemolysis.

by reincubation with zymosan at 37°C. The preparations did not have hemolytic activity for polyagglutinable cells, even though full complement activity remained and the heat-stable agglutinin was present. Tests were also made with eluates derived from zymosan that had been used to absorb properdin from normal serum. Although such eluates appeared to have properdin activity as measured by C'_3 adsorption assay, they did not restore the hemolytic activity of R.P. for polyagglutinable cells.

The sera of animals with polyagglutinable cells were then studied for properdin content. Such sera usually showed a loss of C' activity when incubated with zymosan at 37°C. Assay of properdin by the method of McNall (5) which demands an excess of C'_3 and other components of C' showed that the levels of properdin in the sera of animals with polyagglutinable cells were equal to those of normal rabbit sera.

Further experiments, presented in Table IV, showed that when normal

rabbit sera were treated with zymosan at 0°C the hemolytic but not the agglutinating factor for polyagglutinable cells was removed. Such treated sera evidently had normal levels of properdin since C'_3 was removed by further treatment with zymosan at 37°C.

TABLE IV

The Influence of Absorption with BSA–anti-BSA Precipitate, Polyagglutinable Cells, and Zymosan on the Capacity of Normal Rabbit Serum to Produce Hemolysis and Agglutination of Polyagglutinable Cells

Material used for absorption of normal rabbit sera	Per cent hemolysis	Reactions of polyagglutinable cells in the various sera*							
		Agglutination with unheated sera‡				Agglutination with heat-inactivated sera‡			
		Neat	1:2	1:4	1:8	Neat	1:2	1:4	1:8
1. None (control)	46	3+	2+	0	0	4+	3+	3+	1+
2. BSA–anti-BSA precipitate	0	1+	1+	0	0	4+	3+	2+	0
3. Zymosan, 0°C	0	3+	2+	0	0	4+	3+	2+	0
4. Zymosan, 17°C	0	3+	2+	0	0	4+	3+	2+	0
5. Polyagglutinable rabbit cells, 0°C	0	0	0	0	0	0	0	0	0
6. Normal cells, 0°C	43	3+	2+	0	0	4+	3+	1+	0

1. 1 ml of normal rabbit sera produced 46 per cent hemolysis of 0.05 ml of polyagglutinable cells. These proportions were used throughout.

2. 1 ml of the precipitate was incubated with 4 ml of normal rabbit sera for 1 hour at room temperature.

3. 3 mg of zymosan were added per ml of rabbit sera and maintained for 1 hour at 0°C in an ice bath.

4. Same procedure as 3, above, at 17°C.

5. 4 ml of normal rabbit sera were absorbed with 2 ml of washed polyagglutinable cells for an hour at 0°C.

6. Normal rabbit cells were used for absorption in place of polyagglutinable cells.

* Hemolysis and agglutination tests were carried out at 25°C.

‡ The dilutions of sera used ranged from neat to 1:8 and the range of agglutination employed was 1+ to 4+.

It was also demonstrated, as shown in Table IV, that polyagglutinable cells absorbed the hemolytic factor from normal rabbit sera at 0°C even though hemolysis did not occur at this temperature. In some experiments the heat-stable polyagglutinin was removed at 0°C at the same time that the hemolytic activity was removed. In other experiments in which a smaller proportion of cells to serum was used, the polyagglutinable cells did not remove all of the agglutinating activity of sera at 0°C though the hemolytic activity was removed. Whereas absorption of sera by polyagglutinable cells, zymosan, or BSA–anti-BSA precipitate at 0°C removed the hemolytic activity for polyagglutinable cells, only the BSA–anti-BSA precipitate reduced the level of

C'. In addition, it may be noted in Table IV that both normal sera and sera absorbed with the BSA–anti-BSA precipitate, or with zymosan, showed enhancement of agglutinating activity after heating at 56°C for 20 minutes.

When polyagglutinable cells which had adsorbed the hemolytic activity from normal sera at 0°C were washed at 0°C and suspended in sera derived from animals with polyagglutinable cells, hemolysis was slight. The hemolysis of these cells was also slight when they were suspended in normal sera from which the hemolytic activity for polyagglutinable cells had been absorbed by

TABLE V

*The Per cent Hemolysis of Polyagglutinable Cells in Normal and Resin-treated Rabbit Sera Following Incubation at 25°C with or without Preliminary Incubation at 0°C and with or without the Addition of Ca^{++} or Mg^{++}**

Rabbit sera	Ions added before first incubation		Temp. of first incubation period	Per cent hemolysis after a second incubation period (25°C)	Ions added before third incubation period		Per cent hemolysis after a third incubation period (25°C)
	Mg^{++}	Ca^{++}			Mg^{++}	Ca^{++}	
			°C				
1. Normal	−	−	25	27			
2. Normal	−	−	0	0.8			
3. Resin-treated	−	−	25	0			
4. Resin-treated	+	+	25	24			
5. Resin-treated	+	−	25	0	−	+	17
6. Resin-treated	−	+	25	0	+	−	0·8
7. Resin-treated	−	−	0	0	+	+	21
8. Resin-treated	+	+	0	5	−	−	5
9. Resin-treated	+	−	0	0	−	+	24
10. Resin-treated	−	+	0	0	+	−	2.7

* All incubation periods were for 30 minutes. When initial incubation at 0°C was carried out the reagents were chilled to this temperature prior to mixing. Hemolysis did not occur during the first incubation in the ice bath at 0°C. The figures presented designate the per cent hemolysis after a second and a third incubation at 25°C.

treatment with polyagglutinable cells at 0°C. It seemed possible that failure to produce more than a trace of hemolysis by the above trials might have been due to elution of the hemolytic factor during washing. However, when polyagglutinable cells were mixed with normal sera at 0°C and left at this temperature for 15 minutes, hemolysis either did not occur or was considerably depressed when the suspension was warmed and allowed to stand at 25°C or 37°C. The degree of inhibition of hemolysis induced by the initial incubation at low temperature appeared to depend on the properties of individual sera.

The inhibition of hemolysis induced by preliminary cold incubation was next studied with the use of resin-treated sera and the addition of Ca^{++} and Mg^{++}. The results are presented in Table V. It will be noted that when a preliminary

incubation at 0°C was carried out normal rabbit sera caused very little hemolysis of polyagglutinable cells upon subsequent incubation of the mixture at 25°C, as shown in Table V, line 2. The hemolytic activity of resin-treated sera to which Ca^{++} and Mg^{++} had been added was likewise inhibited in a similar manner when such sera were first incubated with the cells at 0°C as shown in Table V, line 8. However, when resin-treated sera were incubated with the cells

TABLE VI

*Absorption of the Hemolytic and Agglutinating Activity of Normal Rabbit Sera by Heated and Unheated Stroma of Polyagglutinable Cells**

Stroma preparation used for absorption	Reaction‡	Dilutions of stroma used for absorption							
		1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:640
Normal cells	Hemolysis	10	24	24	24	24	24	24	24
	Agglutination	3+	3+	3+	3+	3+	3+	3+	3+
Normal cells (heated)§	Hemolysis	13	24	24	24	24	24	24	24
	Agglutination	3+	3+	3+	3+	3+	3+	3+	3+
Polyagglutinable cells	Hemolysis	0	0	0	0	0	0	0	12
	Agglutination	0	0	0	0	0	0	1+	2+
Polyagglutinable cells (heated)§	Hemolysis	0	0	0	0	0	0	8	12
	Agglutination	0	0	0	0	0	0	2+	2+

* Sedimented stroma from 3 ml of cells was suspended in 1 ml of normal serum, and serial dilutions of these suspensions were made by transferring 0.5 ml to the next tube containing 1 ml of normal serum, etc. After incubation for an hour at 25°C and centrifugation, the supernatant sera were tested for their ability to hemolyze and agglutinate polyagglutinable cells.

‡ Hemolysis is expressed in per cent and agglutination in the range of 1+ to 4+.

§ A portion of the stroma was heated at 80°C for 20 minutes.

at 0°C without addition of Ca^{++} or Mg^{++}, hemolysis was not inhibited when these ions were added prior to the 3rd incubation carried out at 25°C as indicated in Table V, line 7. Apparently Ca^{++} but not Mg^{++} must be present during the preliminary period of cold incubation for inhibition of hemolysis to occur as shown in Table V, lines 9 and 10.

Influence of Zymosan Treatment of Normal Rabbits on the Capacity of Their Sera to Hemolyze Polyagglutinable Red Cells.—The intravenous injection of zymosan is reported to result in the reduction of serum levels of properdin without reduction in the levels of C′ (6). Each of 12 normal rabbits was given an intravenous dose of zymosan. The sera of all animals that received 25 or more mg of zymosan were completely devoid of hemolytic activity for polyagglutinable cells, even though hemolytic activity for sensitized sheep cells

was not reduced. Zymosan treatment did not reduce the titer of agglutinin for polyagglutinable cells. The effect of zymosan treatment on the *in vivo* destruction of transfused polyagglutinable cells is described in Paper II of this series.

Persistence of the Polyagglutinable Red Cell Abnormality in Preparations of Stroma.—Stroma derived from 5 ml of polyagglutinable cells was incubated with 5 ml of normal rabbit serum for 1 hour at 37°C. The stroma was then removed by centrifugation and the serum was tested for its hemolytic and agglutinating activity for polyagglutinable cells. Similar tests were conducted with heat-inactivated normal rabbit serum. The results presented in Table VI were consistent in demonstrating that stroma preparations of polyagglutinable cells were active in removing both the hemolytic and the agglutinating factors from normal rabbit serum and the agglutinating factor from heat-inactivated

TABLE VII

Absorption of Complement from Normal Rabbit Sera by Stroma of Normal and Polyagglutinable Cells

Material used for absorption	Per cent Hemolysis*					
	1:2	1:4	1:8	1:16	1:32	1:64‡
Stroma of normal cells§	100	100	100	68	15	0
Stroma of polyagglutinable cells	35	0	0	0	0	0
None (control)	100	100	100	100	55	8

* The absorbed supernatant sera were diluted and tested for hemolytic activity for sheep cells sensitized with rabbit amboceptor.

‡ Dilutions of absorbed sera.

§ Stroma prepared from 5 ml of normal cells and from 5 ml of polyagglutinable cells were incubated with respective aliquots of 2 ml of normal sera.

normal rabbit serum. Stroma preparations derived from normal cells lacked this property.

The active factor in the stroma of polyagglutinable cells was tested for heat stability by the following procedures.

The sedimented stroma derived from 6 ml of polyagglutinable cells was suspended in 2 ml of physiological saline and divided into 2 aliquots. One aliquot was heated to 80°C for 20 minutes. Serial doubled dilutions of the heated and unheated stroma preparations were made in normal rabbit serum. The mixtures were incubated for 30 minutes at 37°C and the stroma removed by centrifugation. 0.1 ml of a 10 per cent suspension of polyagglutinable cells was added to each supernatant fluid. Heated and unheated preparations of control stroma prepared from normal cells were similarly diluted with normal rabbit sera and tested.

Whereas both heated and unheated stroma of polyagglutinable cells inhibited the hemolytic and agglutinating activity of normal serum for polyagglutinable cells to dilutions of 1:256, similar preparations of stroma derived from normal cells did not possess such inhibitory activity. Subsequent trials

indicated that heating of stroma of polyagglutinable cells to 100°C for 20 minutes caused a slight decrease in their inhibitory activity.

Complement Fixation by Stroma of Polyagglutinable Red Cells.—The stroma from each of several preparations derived from 5 ml samples of red cells from normal rabbits and from rabbits with polyagglutinable cells was suspended in 2 ml of normal rabbit serum. Following incubation for 1 hour at 37°C, the stromata were sedimented and the supernatant fluids were assayed for C′ activity. The stroma of polyagglutinable cells removed all of the C′ activity from the serum, whereas the stroma of normal cells diminished it only slightly as presented in Table VII.

DISCUSSION

The present results lead us to propose that overt hemolytic anemia does not occur in the polyagglutinable-cell disease of rabbits because the sera of the affected animals are so readily depleted of agglutinating and hemolytic factors for polyagglutinable cells that excessive red cell destruction is prevented. This concept is based on the observation that the agglutinating and hemolytic factors which were lacking in the serum of animals with polyagglutinable cells promptly returned to normal levels after disappearance of the cell lesion. The serum factors are apparently present in limited quantities in normal serum since they can be readily removed by treatment with polyagglutinable cells or stroma derived from such cells.

The nature of the serum factors concerned with the agglutination and hemolysis of polyagglutinable cells has not been completely clarified. Although separate factors appear to be concerned with agglutination and hemolysis, respectively, the possibility that the agglutinin participates in hemolysis as well as agglutination cannot be ruled out on the basis of present data. The capacity of normal sera to agglutinate the cells of affected animals together with the observation that complement is fixed in the reaction between the stroma of such cells and normal rabbit serum indicates that at least one component is an antibody.

Evidence indicating that the agglutinin may not participate in hemolysis is provided by the observation that polyagglutinable cells that had adsorbed agglutinins from heat-inactivated normal sera were not hemolyzed when suspended in either the complement-containing sera of animals with polyagglutinable cells or normal sera which had been adsorbed with polyagglutinable cells or zymosan at 0°C. Similar evidence is provided by the observation that normal sera treated at 0°C with zymosan lacked hemolytic activity but not agglutinating activity. Although evidence is lacking that the agglutinin is essential to the hemolysis of polyagglutinable cells, this possibility cannot be discarded since it has not been possible to prepare a reagent from rabbit serum which is hemolytic for polyagglutinable cells and which at the same time lacks the agglutinin.

It is also notable that heating at 56°C for 20 minutes enhanced the poly-

agglutinating activity of normal sera and sera from which the hemolysin had been removed with antigen-antibody precipitates or zymosan. The reason for this enhancement is not known. The possibility that a heat-labile inhibitor of agglutination was present in normal and zymosan-absorbed serum was excluded. The relation of the agglutinin for polyagglutinable rabbit cells present in normal mammalian sera to the T agglutinin described by Friedenreich (8) is under investigation.

The factors in normal rabbit sera which are responsible for hemolysis of polyagglutinable cells are probably complex and deviate from the classical system of factors concerned with complement hemolysis of sensitized red cells. The hemolytic activity of normal rabbit serum for polyagglutinable cells was obviously dependent on a heat-labile factor which was distinct from complement and probably from properdin as evidenced by the observation that it could be removed by treatment with either polyagglutinable cells or zymosan at 0°C without reducing the level of either C′ or properdin. This factor may be similar to the factor in normal human serum reported by Blum and Lepow (7) which reacts with zymosan at 0°C and which may be necessary for adsorption of properdin by zymosan. However, we have no evidence that the factor under present study plays any role in the adsorption of properdin. The results of the present experiments indicate that the hemolytic factor is not identical with properdin since normal sera rendered nonhemolytic for polyagglutinable cells by treatment with either polyagglutinable cells or zymosan at 0°C had normal levels of properdin. Moreover, the non-hemolytic sera of animals with polyagglutinable cells contained normal levels of properdin. Whereas it is true that sera rendered deficient in properdin (R.P.) did not hemolyze polyagglutinable cells, the failure of such sera to produce hemolysis was probably due to the removal of the "0° factor" by the zymosan treatment employed at 15°C to 17°C in the preparation of R.P. Although the present evidence does not establish with certainty the roles that properdin and complement may play in the activity of the hemolysin, it seems probable that complement is necessary. This is indicated by the observation that complement is fixed in the reaction between the stroma of polyagglutinable cells and normal rabbit serum.

The role of Ca^{++} and Mg^{++} in hemolysis of polyagglutinable rabbit cells presented some variations which may be of significance. The hemolytic activity of resin-treated sera was restored by the addition of Ca^{++} and Mg^{++}. However, hemolysis was inhibited by the addition of Ca^{++} to the suspension 10 minutes prior to the addition of Mg^{++}. It was also observed that incubation of normal sera with polyagglutinable cells at 0°C inhibited hemolysis upon subsequent incubation at 25°C. Further studies have shown that Ca^{++} is necessary during the preliminary cold incubation to produce this inhibition of hemolysis. This observation suggests that the induction of this inhibition of hemolysis which occurs during low temperature incubation results from progression of a reaction

involving Ca^{++} and concomitant inhibition of the reaction involving Mg^{++}. Apparently Ca^{++} reacting with components of C′ in the absence of Mg^{++} or at 0°C in the presence of Mg^{++} produces an irreversible complex which does not lead to hemolysis. In this respect the hemolysis of polyagglutinable cells appears to be different from most hemolytic reactions with human sera since the latter proceed to completion after an initial phase of cold incubation.

The red cell abnormality of polyagglutinable cells probably resides in a polysaccharide fraction of the red cell. This is based on the observations that the cell abnormality was not influenced by treatment with trypsin or papain, that it persisted in stroma derived from polyagglutinable cells and that it resisted heating to 80°C or 100°C. The failure of the serum factors of recovered animals to rise above normal levels together with the observation that the injection of polyagglutinable cells into normal animals did not result in an increase in the serum factors indicates that the red cell abnormality is not antigenic. The abnormality may be a polysaccharide fraction of the red cell altered or exposed by enzyme action.

SUMMARY

A disease characterized by frequent association of enteritis and polyagglutinable cells often develops in weanling rabbits. The red cell lesion renders the cells susceptible to agglutination and hemolysis in normal rabbit sera. The degree of red cell abnormality varies among different animals and disappears when the animals recover.

The abnormality of the red cells responsible for their polyagglutinability and susceptibility to hemolysis was resistant to the action of trypsin or papain and persisted in heated stroma preparations derived from polyagglutinable cells.

The factors necessary for agglutination and hemolysis of the polyagglutinable cells are present in normal rabbit sera but are lacking in the sera of affected rabbits. These factors returned to normal levels as the polyagglutinable cell lesion disappeared. The sera of rabbits with polyagglutinable cells contained normal levels of complement and properdin.

Whereas the agglutinating factor in normal sera is heat-stable at 56°C for 30 minutes, the hemolytic factor is heat labile. The hemolytic factor is apparently distinct from complement and properdin since it was adsorbed from normal rabbit serum by zymosan or by polyagglutinable cells at 0°C. However, complement was fixed when normal rabbit serum was reacted with stroma from polyagglutinable cells.

Hemolysis of polyagglutinable cells by normal rabbit serum at 25°C was inhibited by preliminary incubation of the mixture at 0°C prior to incubation at 25°C. Evidence was obtained which indicated that this inhibition was due to progression of a reaction involving Ca^{++} independent of a reaction involving Mg^{++}.

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