SUPPRESSION OF BLOOD GROUP AGGLUTINABILITY OF HUMAN ERYTHROCYTES BY CERTAIN BACTERIAL POLYSACCHARIDES*

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Conditioned hemagglutination, that is, modification of the agglutinative properties of erythrocytes by adsorption of bacterial antigens, has been widely explored during the past decade. This technique has been applied to a variety of bacterial antigens and it is now well established that most bacterial polysaccharides, when admixed with washed red cells are readily and firmly adsorbed on the surface conferring on the erythrocytes an additional antigenic specificity, that of the adsorbed bacterial antigen (2).

While this technique has aroused great interest and has gained general acceptance, most investigations have been directed largely towards specific antigenic components adsorbed on the erythrocyte and the numerous practical applications of the test. On the other hand, it apparently has not been generally appreciated that the fixation on the red cell surface of bacterial antigens might exert an effect on the reactivity of the red cell due to its native antigens, as well as other properties of the cell related to the general phenomenon of agglutination.

While studying the basic phenomenon underlying fixation of certain bacterial antigens on red cells, Ceppellini and De Gregorio (3, 4) observed that crude extracts of Vi strains of *Salmonella typhosa* interfered with agglutinability of human red cells by anti-blood group sera. However, it was not established with certainty whether the inhibitory effect was due to the Vi antigen itself or to some other bacterial component.

The inhibitory action of bacterial polysaccharides on viral hemagglutination has been reported by a number of workers. The situations in which the effect was observed were as follows: *Klebsiella* type B polysaccharide on hemagglutination by mumps virus (5); fractions derived from Vi strains of *S. typhosa* on hemagglutination by influenza virus (6); *Klebsiella* polysaccharides on hemagglutination by influenza virus (7); and *S. typhosa* polysaccharide preparations on hemagglutination by myxo- and polyoma viruses (8).

The objective of the present work was to determine the identity of the bacterial component responsible for the suppression of blood group agglutination and to ascertain the mechanism of this phenomenon. The studies reported here show that this attribute is shared by a number of capsular polysaccharides

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* A preliminary report of these findings was presented at the 42nd Meeting of the American Association of Immunologists, Philadelphia, April, 1958 (1).

which impart to the red cell surface some of their peculiar physicochemical characteristics. As a consequence there is produced a non-specific stabilization of erythrocyte suspensions.

Materials and Methods

Capsular Polysaccharides.—The Vi antigens from *Escherichia coli* 5396/38, *Paracolobactrum ballerup* 7851/39, and *Salmonella typhosa* Ty 2 were those prepared by Webster *et al.* (9). Capsular polysaccharides were isolated from cultures of types A and B *Klebsiella pneumoniae* by the method of Goebel and Avery (10). The Type III pneumococcus polysaccharide was kindly provided by Dr. M. Heidelberger. This preparation was distinctive in that it was the only one, of a total of 9, that adsorbed on red blood cells for conditioned hemagglutination (11).

Somatic Lipopolysaccharides.—These were prepared from *S. typhosa*, *Haemophilus pertussis*, *Proteus vulgaris*, and *Shigella paradysenteriae* by our colleague Dr. M. E. Webster, employing a modification of the Boivin procedure. The polysaccharides from *Pseudomonas aeruginosa* (12), *Serratia marcescens* (13), and from *Pasteurella tularensis* (14) were contributed by Dr. L. Ginger, Dr. M. J. Shear, and Dr. G. G. Wright. These preparations had been previously examined with regard to their uptake by erythrocytes for hemagglutination by specific antibacterial sera (11).

In this report polysaccharides which depress or inhibit agglutination of erythrocytes by blood group antisera or viruses will be designated as "active" polysaccharides. Preparations which, although adsorbed and effectively contributing their own serological specificity to the red cell, do not affect blood group or virus hemagglutination will be referred to as "inactive" polysaccharides.

Antisera.—The majority of bacterial antisera were prepared by intensive immunization of rabbits with the various microorganisms from which the aforementioned polysaccharides had been obtained. The pneumococcus antiserum was a concentrated immune globulin of rabbit derivation prepared by the Lederle Laboratories, Pearl River, New York. The anti-A and anti-B sera contained natural human agglutininins; anti-H was extracted from *Ulex europaeus*; anti-M and anti-N were commercial products prepared in rabbits; and anti-P contained a natural horse agglutinin. "Anti-human" serum was a potent unadsorbed serum prepared by immunization of rabbits with human erythrocytes. Other specific antisera for the remaining blood group agglutinogens were of human origin obtained from "immunized" individuals (pregnancy or transfusion). Sera containing heterophile antibody were obtained from patients with infectious mononucleosis. The anti-sheep amboceptor was commercially prepared.

Viruses.—Influenza, Newcastle, and mumps viruses were lyophilized allantoic fluids harvested from infected chick embryos.

Erythrocytes.—The majority of tests were conducted with erythrocytes from human donors, collected and preserved in modified Alsever solution. Sheep cells, similarly collected and preserved, were utilized in a few instances. The preserved cells generally were used between 3 and 12 days after bleeding.

Coating of Erythrocytes with Polysaccharides.—For uptake of polysaccharides, erythrocytes were washed 3 times with 10 volumes of 0.85 per cent sodium chloride, made up to a 10 per cent suspension, and mixed with an equal volume of polysaccharide dissolved in saline at the desired concentration. Such mixtures were incubated for 2 hours at 37°C, washed 3 times to remove unadsorbed polysaccharide, and, unless otherwise indicated, made up to a 1 per cent suspension for test. As given in the experimental section, the quantities of polysaccharide refer to a 1 per cent suspension of erythrocytes, *e.g.* where a 10 per cent red cell suspension

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was mixed with an equal volume of 100 micrograms polysaccharide per ml, the cells (adjusted to a 1 per cent suspension) are referred to as having been treated with 10 micrograms of polysaccharide per ml.

Bacterial Hemagglutination.—All bacterial polysaccharides used in this work were shown to be fixed on red cells as evidenced by conditioned hemagglutination with appropriate homologous antibacterial sera. The details of the technique and its application have been reviewed by Neter (2).

Blood Group and Virus Hemagglutination.—For blood grouping the standard techniques were employed for different systems and kinds of antibodies; *cf.* Race and Sanger (15). Except for special situations the procedure employed for the titration of antisera was as follows: a 5 per cent red cell suspension was used mixed with an equal amount of antibody dilution, incubated at the appropriate temperature, centrifuged for 1½ minutes at 140 *g*, and agglutination read macroscopically after gentle shaking of the tube. Various degrees of agglutination were expressed by number with 5 representing maximum agglutination (a single clump), and 1 as fine agglutination, barely visible to the naked eye. For virus hemagglutination the technique employed was the widely used procedure described by Salk (16).

EXPERIMENTAL

The availability of isolated Vi antigen, freed of other bacterial components, enabled us to demonstrate promptly that this bacterial polysaccharide was, indeed, the agent in bacterial extracts responsible for the suppression of blood group agglutinability. The type of effect on hemagglutination brought about by coating red cells with Vi antigen is presented in Table I. Untreated human group A erythrocytes were agglutinated to a titer of 1:512 by the anti-A serum. When these erythrocytes were coated with different amounts of Vi antigen, the titer of the antiserum decreased progressively until agglutination had been completely suppressed; *i.e.*, even undiluted serum failed to agglutinate these cells. The possibility that the bacterial preparations neutralized the anti-A agglutinins was disproved early in these studies.

Screening of Bacterial Polysaccharides for Activity.—It was not practical to examine in detail each individual agglutinating system; instead human anti-A or influenza virus were used, against human group A erythrocytes, in a dose 4 times the dilution representing the agglutination end point; *i.e.*, 4 units. For each polysaccharide, levels of 250, 50, and 10 μg were used to coat the test red cells. This provided a sensitive test; under these conditions products which did not inhibit could be excluded with assurance as being inactive.

The results of this survey are summarized in Table II; SSS III, *Klebsiella* types A and B, polysaccharides, and Vi antigens derived from *E. coli*, *P. ballerup*, and *S. typhosa* were active at each of the levels tested. In contrast the other products, which are somatic antigens (endotoxins) of various Gram-negative bacteria, did not inhibit hemagglutination to any significant degree.

The inhibitory action was studied quantitatively with 3 Vi products, inasmuch as they had previously been shown to differ primarily as regards their physical state; the *coli* and *ballerup* polysaccharides were more highly polymerized than the product from *S. typhosa* (9). Human group A erythrocytes

were treated with dilutions of these Vi antigens ranging from 1 to 100 μg and set up with doubling dilutions of the appropriate reagents for each of 3 agglutinating systems. Some of the results of these tests are given in Table III where the values represent the reciprocal of the altered endpoint of the agglutinating agent toward cells treated with Vi antigen at the indicated levels. Similar tests with SSS III showed that on a weight basis its activity was comparable to that of the *S. typhosa* Vi polysaccharide. For each of the 3 systems the magnitude of the effect was proportional to the concentration of polysaccharide used for coating the erythrocytes and inversely proportional to the titer of the agglutinating agent.

TABLE I

Vi Inhibition of Blood Group A Agglutination

Treatment of human group A_1 red cells with *coli* Vi antigen	Agglutination of Vi-coated cells								
	Dilutions of anti-A serum (human group B)								
	1	1:2	1:4	1:8	1:32	1:64	1:128	1:256	1:512
μg									
0	5	5	5	5	5	5	5	4	1
1	5	5	5	5	5	5	4	2	1
3	5	5	5	5	5	5	2	1	0
7	5	5	5	5	5	5	1	0	0
10	5	5	5	5	2	1	0	0	0
20	5	5	3	2	0	0	0	0	0
40	2	1	0	0	0	0	0	0	0
80	0	0	0	0	0	0	0	0	0

Test conditions: 2 hour incubation at 20°C; read macroscopically after 1½ minutes at 1000 RPM.

Agglutination: 5, 4, 3, 2, 1 represent decreasing strength of agglutination; 0 = no agglutination.

Inhibition of Blood Group Agglutinability.—To determine whether the inhibitory effect of the Vi antigen varied with different blood group systems, erythrocytes from donors known to possess a variety of antigenic combinations were treated with *coli* Vi antigen (125 μg/ml of 1 per cent red cell suspension) and retested with potent blood group-typing sera according to the appropriate technique. The following antisera were used: human natural anti-A and anti-B; rabbit anti-M and anti-S; horse natural anti-P; human immune anti-D, C, c, E, Fy^a, and K; lectins of anti-A and anti-H specificity obtained from Lima and Ulex. In all cases agglutination was now completely suppressed.

To determine whether this inhibition was quantitatively similar for the different blood groups a selected number of antigens were reexamined under

conditions of test where the titer of the different antibodies was equalized to 8 agglutinating units, using human A B serum as diluent. These antibodies were tested against erythrocytes sensitized with decreasing amounts of Vi polysaccharide in order to determine the minimum inhibitory dose of Vi for each blood group system. Erythrocytes treated with 2 to 4 μg of *coli* Vi were no longer agglutinated by the test antisera (Table IV), showing that all blood

TABLE II

Effect of Various Bacterial Polysaccharides on Hemagglutination

Polysaccharides fixed on erythrocytes		Agglutinating systems					
		Anti-human serum (rabbit)			Influenza virus (PR-8)		
Source	Type	Polysaccharide treatment of red cells					
		250 μg	50 μg	10 μg	250 μg	50 μg	10 μg
Escherichia coli 08	Somatic	+	+	+	+	+	+
Escherichia coli 0127	Somatic	+	+	+	+	+	+
Haemophilus pertussis	Somatic	+	+	+	+	+	+
Pasteurella tularensis	Somatic	+	+	+	+	+	+
Proteus vulgaris	Somatic	+	+	+	+	+	+
Pseudomonas aeruginosa	Somatic	+	+	+	+	+	+
Salmonella typhosa 0901	Somatic	+	+	+	+	+	+
Serratia marcescens	Somatic	+	+	+	+	+	+
Shigella paradysenteriae	Somatic	+	+	+	+	+	+
Diplococcus pneumoniae Type III	Capsular	−	−	−	−	−	−
Escherichia coli 5396/38	Capsular	−	−	−	−	−	−
Klebsiella pneumoniae A	Capsular	−	−	+	−	−	+
Klebsiella pneumoniae B	Capsular	−	−	−	−	−	+
Paracolobactrum ballerup	Capsular	−	−	−	−	−	−
Salmonella typhosa Ty 2	Capsular	−	−	−	−	−	+

+ Indicates discernible agglutination by 4 agglutinating units; *i.e.*, no inhibition.
− Indicates no discernible agglutination by 4 agglutinating units; *i.e.*, inhibition.

group receptors tested were equally susceptible to inhibition by Vi polysaccharide.

Both natural and immune antibodies, and human, animal, or plant reagents, were made equally ineffective. Because there is some basic difference between the saline agglutinins and the so-called incomplete antibodies it was desired to compare them. Accordingly, two sera of anti-D specificity, one of the saline type and the other of the incomplete variety, showing the same titer against Rh-positive cells (when tested in saline and albumin respectively), were also titrated with these cells which had been coated with Vi polysaccharide. In both cases, in addition to saline agglutination, the three techniques in common

use for detecting incomplete antibodies were applied; namely, 20 per cent bovine albumin as suspending medium, pretreatment of the cells with ficin, and the indirect Coombs test. Treatment of the cells with 50 μg of Vi antigen suppressed the activity of both varieties of antisera. The characteristic effect of Vi was manifest despite the varied serological techniques employed. It was noted that treatment of the red cells with ficin (or trypsin) neither prevented

TABLE III

Inhibition of Hemagglutination by Different Vi Polysaccharides

Hemagglutinating systems	Titers following treatment of red cells with Vi antigen			
	Vi concentration*	Source		
		E. coli	*P. ballerup*	*S. typhosa*
Isoanti-A (titer,‡ 512)	100	0	0	16
	40	0	0	64
	10	1	2	128
	3	32	16	256
	1	64	128	512
Rabbit anti-human (titer,‡ 4000)	100	0	0	32
	40	0	0	128
	10	0	0	512
	3	64	32	2000
	1	128	256	4000
Influenza virus (titer,‡ 640)	100	0	0	80
	40	0	0	320
	10	20	40	640
	3	160	80	640
	1	320	320	640

* Amount of Vi used for coating, expressed as μg/ml of a 1 per cent red cell suspension.

‡ The titer for each system refers to that obtained with untreated human type A, erythrocytes.

the adsorption of Vi antigen nor brought about its release. The fact that incomplete antibodies *per se* do not clump the red cells made possible the coating of erythrocytes with Vi polysaccharide *after* they had been sensitized with the anti-D antibody. Also, in this case the subsequent use of techniques for the detection of incomplete antibodies did not bring about agglutination.

Multiple Layer (*Lattice*) *Test.*—The fact that Rh-positive cells, previously sensitized with a strong incomplete anti-D serum, and then treated with Vi polysaccharide were not agglutinated upon addition of antiglobulin serum, suggested that the mechanism of the inhibition was not due to prevention of

antibody uptake. However, other possibilities such as the displacement or release of the previously fixed antibody, were not excluded. To clarify this issue it was decided to use the multiple layer technique as described by Coombs and coworkers (17). This technique seemed to be particularly suited for determining whether the Rh antibody was still present on Vi-coated erythrocytes. Rh-positive red cells were sensitized with 3 levels (1:4, 1:16, and 1:64) of an incomplete anti-D serum which gave a titer of 1:512 against normal erythrocytes. The cells were then washed, treated with 125 μg of *coli* Vi polysaccharide, washed, treated with antiglobulin (15 minutes at room temperature), washed 3

TABLE IV

Quantitative Comparison of Vi Inhibition of Hemagglutination for Different Blood Groups

Blood group	Type of antibody*	Amount of Vi used for coating, expressed as μg/ml of 1 per cent red cell‡ suspension						
		8	6	4	2	1	0.5	0
Anti-A	Saline§	0	0	0	0	4	5	5
Anti-B	Saline	0	0	0	0	4	5	5
Anti-C	Saline	0	0	1	3	4	5	5
Anti-E	Saline	0	0	0	1	4	5	5
Anti-P	Saline	0	0	0	3	4	5	5
Anti-D	Saline	0	0	0	2	4	5	5
Anti-D	Incomplete	0	0	0	0	2	5	5
Anti-K	Incomplete	0	0	0	0	0	3	3

‡ Red cells: human A_1, B, C, D, E e, P+, Kk.

* All reagents brought to 8 agglutinating units upon dilution with AB serum. Complete antibodies tested in saline; incomplete antibodies tested with antiglobulin serum.

§ Subsequent application of antiglobulin technique to red cells which failed to be agglutinated by complete antibodies did not bring about agglutination.

times, and treated with human gamma globulin (15 minutes at room temperature); then again washed 3 times and mixed with a fresh aliquot of the antiglobulin serum; this cycle was repeated until agglutination occurred.

In replicate experiments the agglutination of the Rh-sensitized cells appeared between the 4th and 5th globulin-antiglobulin cycles. This showed that the Vi treatment had neither prevented uptake nor displaced the anti-D antibody. In fact, in another similar experiment, the cells were first treated with Vi and then the anti-Rh antibody. The antiglobulin serum had a titer of 1:1024 against Rh-positive cells fully sensitized with incomplete anti-D and, when used in the experiment, was diluted 1:20. The concentration of the human gamma globulin (10 μg/ml) was selected as representing 4 times the minimal amount able to neutralize the 1:20 antiglobulin serum. To avoid building up of globulin-antiglobulin chains not specifically founded on the anti-Rh antibody,

TABLE V

Summary Protocol of Multiple Layer (Lattice) Experiment

Sequence of treatments		Reagents applied to red cells in different experimental and control tubes								
		1	2	3	4	5	6	7	8	9
This cycle was repeated 5 times	1	Vi	Vi	Vi	Vi	Vi	Vi	D1:4	D1:16	D1:64
	2	D1:4	D1:16	D1:64	None	D1:4	D1:4	Vi	Vi	Vi
	3	aG	aG	aG	aG	aG	r.s.	aG	aG	aG
	4	gg	gg	gg	gg	r.s.	gg	gg	gg	gg
Results of tests with these reagents at intermediate stages	Anti-Vi	+	+	+	+	+	+	+	+	+
	Anti-A	0	0	0	0	0	0	0	0	0
	Anti-G	0	0	0	0	0	0	0	0	0
Final result on addition of anti-G at the 5th repetition of treatment		+	+	+	0	0	0	+	+	+

D = anti-Rh_0, serum used for sensitizing the cells at the indicated dilution; aG = rabbit anti-human gamma globulin; gg = human gamma globulin; r.s. = normal rabbit serum.

\+ = agglutination; 0 = no agglutination.

all reagents used were repeatedly absorbed both with normal and with Vi-coated human red cells. To rule out non-specific reactions, the following controls were employed: (*a*) in the treatment of aliquots of the cells, normal rabbit serum was substituted for the human globulin or the immune antiglobulin, (*b*) cells were included which initially had not been sensitized with anti-D, (*c*) after

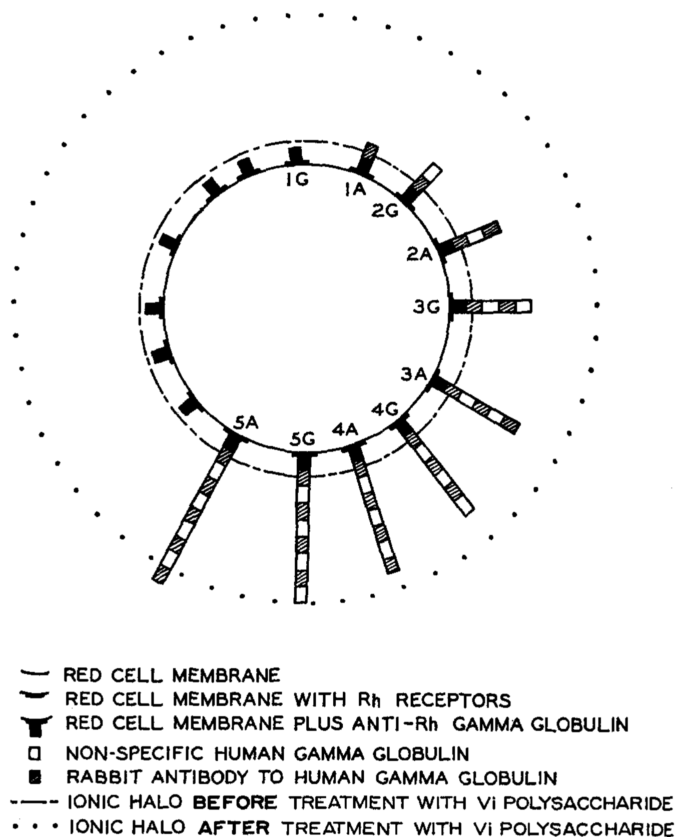

FIG. 1. Schematic diagram of multiple layer (lattice) experiment.

each globulin-antiglobulin treatment, a portion of the cells were titrated with an anti-Vi and an anti-A serum; the former agglutinated to titer while the latter was completely suppressed, showing that Vi was still present and in concentration sufficient to inhibit blood group agglutination. This experiment is summarized in Table V and is freely interpreted in Fig. 1. The finding that specific agglutination was obtained after 4 or more cycles, is attributed to the building up of a globulin-antiglobulin lattice, with the original anti-D antibody as the foundation, thus providing conclusive evidence for adsorption of anti-D antibody by Vi-treated Rh-positive red cells.

Absorption and Elution of Blood Group Antibodies.—The results of the multiple layer test, which showed that antibody was indeed fixed on Vi-treated erythrocytes, were further reinforced[1] by a direct measure of the antibody absorbed by and eluted from Vi-treated red cells. For this purpose aliquots of human anti-A (group B) serum were incubated (with repeated shaking) for 2 hours at room temperature with equal amounts of human erythrocytes of Group A_1, and these cells treated with different concentrations of Vi polysaccharide. As a control, similar absorptions were carried out with group O cells, Vi-treated and normal. Vi-treated group A_1 cells were able to absorb a significant amount of antibody as compared with group O controls, but, on a quantitative basis, consistently less than the A_1 untreated cells. The cells used for absorption were then washed, resuspended in equal volumes of A B serum[2] and then kept at 56°C for 20 minutes. A significant amount of antibody was recovered from the A_1 cells, both Vi-treated and normal, but not from the O cells; the amount of anti-A recovered from treated and normal A_1 cells did not differ significantly. In similar experiments with Rh-positive cells and anti-D incomplete serum (indirect Coombs test) Vi-treated and normal cells were found to absorb equal amounts of antibody.

Viral Hemagglutination.—Early in this work it became apparent that viral hemagglutination was sensitive to inhibition by the same polysaccharides which were "active" in suppressing blood group agglutination. Treatment of human or chicken red cells with as little as 1 μg of *coli* Vi significantly reduced the hemagglutination end point of influenza, mumps, and NVD viruses, and 40 μg suppressed agglutination entirely. Just as for blood group agglutinability, the extent of this inhibition was proportional to the concentration of Vi used for coating the red cells (Table III). These results are in accord with the

[1] It was observed that fixation of Vi on red cells had little, if any, effect on immune hemolysis. In titrations of rabbit anti-sheep hemolysin with an excess of complement, the titer was reduced from 1:28,000 for untreated cells to 1:10,750 for cells treated with 25 μg of *coli* Vi. In another experiment, sheep red cells were first sensitized with 4 100 per cent units of hemolysin, washed, and divided into 2 aliquots, one of which was coated with 25 μg of Vi; both suspensions gave the same titer of complement. Moreover, when aliquots of anti-sheep hemolysin, with a titer of 1:28,000 were absorbed with Vi-treated and normal erythrocytes respectively, the Vi-treated cells reduced the titer to 1:5120, while the untreated cells brought it down to 1:320.

Experiments such as these showed that, despite the presence of Vi polysaccharide, antibody in amount adequate to bring about hemolysis was able to reach receptors on the red cell surface. Unlike hemagglutination, where the second phase of the reaction is suppressed, antibody attached to receptors on the red cell permits complement to be oriented on the cell surface and lysis ensues. Diminished absorption of hemolysin by Vi-treated cells may be related to the known heterogeneity, in molecular size, of rabbit anti-sheep hemolysin (19).

[2] Since Vi was partly eluted along with the antibody, and might therefore interfere with the subsequent titration of the eluate, A B serum was used as the eluting medium; in this way uptake of Vi by the test cells was prevented by the inhibitor in human serum (18).

findings of MacPherson *et al.* (7) and Zhdanov *et al.* (8), who reported that capsular polysaccharide preparations from *Klebsiella aerogenes*, and *S. typhosa*, adsorbed on red cells, inhibited hemagglutination by a group of viruses.

Inhibition of Conditioned (Bacterial) Hemagglutination.—A variety of bacterial polysaccharides appear to be adsorbed on red cells with approximately equal facility; the order in which these substances are applied may be varied at will (2).

Heretofore no interference of one polysaccharide with the activity of the others has been reported (11). However, the concentration of antigens employed was, as a rule, lower than those used in the present work in which 1 ml aliquots of a 10 per cent suspension of human type O washed erythrocytes were incubated with 1 ml (1 mg) of each of a number of individual bacterial polysaccharides. Following 2 hours' incubation at 37°C the cells were washed as usual, an aliquot of each suspension made up to 1 per cent and reserved for subsequent titration with the homologous antibacterial serum. The remainder of each of the cell suspensions was incubated with an equal volume of Vi antigen (300 μg/ml), washed, and diluted to a 1 per cent suspension for test. Thus the 1 per cent suspensions used in these tests had been exposed to 100 μg/ml of the various test polysaccharides and 30 μg/ml of Vi antigen. In some experiments the order was reversed and in still others both antigens were applied simultaneously. The reactivity of singly and doubly treated erythrocytes toward homologous antibacterial serum was then compared.

The results of the most pertinent experiments are summarized in Table VI which shows that the reactivity of bacterial antigens fixed on the red cell surface was markedly depressed by Vi antigen, just as were receptors indigenous to the red cell. The inhibition of conditioned hemagglutination generally required concentrations of Vi antigen higher than those which sufficed to affect blood group reactivity (taking into account the titer of the reagents). It is noteworthy that the bacterial polysaccharides which were markedly affected by Vi, were "inactive" with regard to inhibition of blood group hemagglutination.[3] In contrast, the capsular polysaccharides which were "active" with regard to blood group agglutination were practically unaffected by Vi; the implications of this finding for a general interpretation of the Vi effect is considered elsewhere in this communication.

Alteration in the Physical Properties of Polysaccharide-Treated Erythrocytes.—It remained to be determined, other than by immunological methods, whether

[3] For its special bearing in the interpretation of the phenomenon of "O inagglutinability" in viable *Salmonella typhosa* we have previously reported on the reciprocal interference of Vi and O *Salmonella* antigens when adsorbed on erythrocytes simultaneously or in either sequence (20). Reduction in O agglutinability occurred, which became more pronounced as the level of Vi antigen was increased in relation to the amount of O. The possibility is recognized that treatment with high dosage of different polysaccharides may lead to a partial displacement as suggested by Spaun (21). However, the fact that inhibition of hemagglutination, by polysaccharides such as Vi, is common to systems as varied as blood groups and viruses as well as conditioned bacterial hemagglutination, points toward a common mechanism essentially independent of the nature of the receptors and of the agglutinating agents.

the fixation of "active" polysaccharides on red cells brought about changes in the physical properties of the red cell surface. The techniques available for ascertaining such changes were sedimentation rate, acid agglutination, and electrophoretic mobility of the treated cells.

(*a*) *Sedimentation Rate.*—

Saline at pH 7, human plasma from subjects with high and low sedimentation rate and a dextran preparation of 160,000 molecular weight (dextravan, British Drug House) were employed as suspending media. Erythrocytes, treated as usual with different concentrations of various bacterial polysaccharides and washed, were made up to 20 per cent suspensions in these media, dispensed into Wintrobe tubes, and allowed to settle at room temperature. Sedimentation, in millimeters, was recorded at intervals between 30 minutes and 24 hours.

TABLE VI

Effect of Vi Polysaccharide on Conditioned (Bacterial) Hemagglutination

Treatment of erythrocytes	Hemagglutination titers of specific antibacterial sera tested with erythrocytes coated with polysaccharide derived from					
	Salmonella typhosa	*Pseudomonas aeruginas*	*Serratia marcescens*	*Escherichia coli*	*Diplococcus pneumoniae* Type III	*Klebsiella pneumoniae* Group A
Homologous polysaccharide	2560	240	480	960	7680	3840
Homologous polysaccharide followed by Vi (*coli*)	80	5	0	15	3840	3840

The rate of sedimentation of normal as well as polysaccharide-treated cells varied considerably with the different suspending media, being slowest in saline and most rapid in the dextran. However, in a general way, the relative effects of the various polysaccharides on sedimentation were comparable for all suspending media. Since the constitution of plasma is variable, *viz.*, content of fibrinogen, and presence of small amounts of antibodies for the polysaccharides under test, most of the comparisons were made with dextran. Only those polysaccharides which had been shown to affect specific agglutination likewise stabilized the cell suspension (Table VII). Vi polysaccharide (*coli*) was by far the most effective agent in modifying the sedimentation rate of red cells. Thus, cells treated with as little as 3 μg of Vi (per ml of 20 per cent suspension) were still in suspension 24 hours later. Indeed these Vi-treated cells stayed in suspension over a period of days until they eventually lysed.

(*b*) *Acid Agglutination.*—

Normal and polysaccharide-treated erythrocytes were suspended in a final concentration of 1 per cent in buffered saline at pH values extending from pH 3 to 8 by 0.2 pH increments as described by MacPherson (7). Agglutination patterns were read after standing for 1 hour at room temperature.

Those polysaccharides which affected specific agglutination and the sedimentation rate of the erythrocytes also decreased acid agglutination. In effect, such polysaccharide-treated cells were stable at a lower pH than controls or cells coated with "inactive" polysaccharides. It is noteworthy that Vi-treated cells are stable in solution down to pH 4.4, a value which lies in the range of the isoelectric point of the Vi polysaccharide, while normal cells are stable only as low as pH 5.6, which is in the range of the isoelectric point of the normal red cell surface.

(*c*) *Electrophoretic Mobility.*—Human erythrocytes, both untreated and treated with 50 μg of *coli* Vi were made up to a 1 per cent suspension in 0.1 M phosphate buffer, pH 7.4, containing 0.26 per cent NaCl. The U tubes (0.075 cm² cross-sectional area) of the Tiselius apparatus were filled with the suspensions and a current of 13.5 ma applied. The migration for normal cells was 0.003 cm/minutes, while that for the Vi-coated cells was 0.012 cm/minutes toward the anode.[4] This was clear evidence of a marked increase in negative charge of the red cell surface as a consequence of the presence of Vi.

TABLE VII

Effect of Polysaccharides on Rate of Sedimentation of Erythrocytes

Treatment of erythrocytes		Sedimentation hours		
Bacterial polysaccharides	μg/ml of 20 per cent suspension*	1	4	18
		mm	*mm*	*mm*
None		84	86	86
Typhoid O	1000	84	86	87
SSS-III	1000	1	1	86
Kleb. pneumoniae type B	200	1	1	87
Vi (*coli*)	12	0	0	4
Vi (*coli*)	6	0	1	6
Vi (*coli*)	3	0	1	10

* Suspending medium "DEXTRAVAN" (BDH).

DISCUSSION

The results of this investigation have shown that by adsorption of certain bacterial polysaccharides on erythrocytes their characteristic agglutinative properties can be completely suppressed. The "active" polysaccharides, which inhibited hemagglutination, are viscous, highly polymerized capsular antigens, characterized by their content of sugar acids (uronic acid), and carrying a

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marked negative charge. The Vi polysaccharide consists entirely of repeating units of *N*-acetyl *d*-galactosaminuronic acid (22); SSS III is made up of *d*-glucuronic acid + *d*-glucose (23); the *Klebsiella* polysaccharides contain *d*-glucuronic acid + glucose + fucose (24). The "inactive" products on the other hand, are the somatic (endotoxic) components of Gram-negative bacteria, which are high molecular weight lipopolysaccharide complexes, with a weak negative charge. The "active" acidic polymers are believed to be long thread-like molecules, whereas the "inactive" somatic complexes are probably globular. It is noteworthy that the capsular polysaccharides are taken up directly by erythrocytes; in contrast, prior treatment of the somatic antigens with heat or alkali is required to effect their fixation on red cells. The nature of the attachment of these antigens to the red cell surface is not known.

The active polysaccharides differ considerably as regards their capacity, on a weight basis, to inhibit. The relative potencies of the Vi products, which were maintained in a consistent fashion in the various hemagglutinating systems tested, correspond to differences in their physical and immunogenic attributes (25). Additional information has recently been obtained which confirms the correlation between specific viscosity of the Vi polysaccharides and their capacity to suppress hemagglutination (26). Preparations of Vi antigen isolated from *E. coli* by chemical fractionation (Webster) and by electrophoresis (Jarvis), displayed the same specificity and analytical values. However, the specific viscosity of Vi-J was 3 times that of Vi-W, and its capacity to suppress anti-A hemagglutination was also 3-fold greater than Vi-W; *i.e.*, specific viscosity and suppressive activities of Vi-J were increased in exactly the same ratio.

Hemagglutination by antibody (and by analogy, hemagglutination by virus as well) is regarded as occurring in two distinct stages: (*a*) uptake of antibody by specific receptors, (*b*) subsequent clumping of the sensitized cells. The latter stage is variously interpreted as the result of an antigen-antibody lattice (the antibody being bivalent) which "bridges" the cells (27), or, as primarily involving changes in electrical charge and therefore "solubility" of the cell surface, as suggested by Gard (28). The former hypothesis is now generally accepted, at least for some kinds of agglutinating antibodies. However, no explanation has been given why albumin in the suspending medium brings about agglutination by some kinds of incomplete antibodies (*e.g.* Rh, but not Kell). For the present it appears that the "completeness" or "incompleteness" of the agglutination phenomenon is the result of interplay between three major factors: (*a*) the valency and size of the antibody molecule; (*b*) the location of the receptors on the red cell surface, and the physical status of the cell surface itself; (*c*) the characteristics of the suspending medium. The observations of Moskowitz and Carb (29) may be pertinent, chiefly as regards (*b*). They reported the suppression of ABO blood group agglutinability following formalin treatment of erythrocytes; here too the uptake of antibody was not significantly affected.

In evaluating possible mechanisms of the inhibitory effect of polysaccharides there are two major alternatives. The active polysaccharides prevent the uptake of antibody by the cell, or, the antibody is fixed on the cell as usual, but the polysaccharide has changed the physical status of the cell surface in such a way that the fixed antibody no longer can bring about clumping of the cells.

The present work has provided data which have a bearing on both hypotheses. Thus, it was found that the Vi-treated cells do adsorb antibody and virus particles. For instance, with hemolytic systems no significant difference was found between the reactivity of Vi-treated and normal cells. Moreover, inhibition of the Coombs reaction was obtained when the red cells had fixed the incomplete anti-D antibody before treatment with Vi. Proof that the anti-D had not been displaced by the polysaccharide was obtained by means of the multiple layer antiglobulin technique. In fact, Vi-treated cells were agglutinated to titer when multiple layers of globulin and antiglobulin were applied alternatively (Table V). This would seem to rule out the possibility that the "active" polysaccharides blanket or otherwise hinder red cell receptors. The results of this test are of particular significance for the understanding of the phenomenon because they indicate that in the presence of Vi a higher layer of globulin (alternating human globulin and anti-human globulin) is necessary to bring about agglutination. It is therefore visualized (Fig. 1) that the action of Vi consists primarily of extending the ionic halo which normally keeps the cell in "solution" (Gard, 28) or, according to the lattice theory, there is a wider gap to be bridged by the globulin.

It is noteworthy that Vi depressed conditioned agglutinability involving a variety of bacterial polysaccharides except for SSS III and *Klebsiella* type A, the only other products tested which share the physicochemical characteristics of Vi. This observation suggests that the level of accessibility of the receptors, relative to that of the Vi molecule, may be decisive. As regards antibody uptake by Vi-treated cells, in some systems such as rabbit anti-human hemolysin and incomplete anti-D, uptake was quite unaffected, while in other systems such as group A "non-immune" anti-A the amount of antibody fixed by the Vi-treated cells was somewhat diminished. It should be noted, however, that incomplete anti-D and some non-immune anti-A differ in their physical characteristics, chiefly as regards molecular size (30). It is a reasonable assumption that diminished uptake involves only 19 S antibodies, but this remains to be determined experimentally. Finally, attention is directed toward the parallelism in the inhibition by Vi of immune hemagglutination and the altered physical properties of the cell as shown in experiments on sedimentation rate, acid agglutination and the altered physical properties of the cell as shown in experiments on sedimentation rate, acid agglutination, and electrophoretic mobility. The relationship between these two sets of phenomena is probably based on the orientation assumed by these acidic polymers when fixed on the red cell surface: the extension of the highly charged Vi polysaccharide into the surrounding

medium has two major consequences; *viz.*, stabilization of the cell suspension and the inhibition of lattice formation.

There exist naturally occurring situations which bear a resemblance to the coating of erythrocytes with polysaccharides obviously not indigenous to the red cell surface; for example, the Lewis antigens in man (31), the J antigen in cattle (32), and the R antigen in sheep (33). The human Lewis antigens are not known to affect the general agglutinability of the red cell; on the other hand the so-called inagglutinability of cells of some individual oxen (Coombs) may be related to the uptake *in vivo* of a soluble substance not unlike the "active" polysaccharides alluded to in this report.

The distinctive stabilizing effect of the Vi polysaccharide may be used to advantage in a number of hematological and immunological problems where it is desirable to avoid the second stage of agglutination. In the test for the rheumtoid factor and for the detection of antigenic specificities of human antibodies, agglutinating antibody could be used in place of incomplete antibody for coating the carrier erythrocytes. In the study of hemolytic systems it may be desirable to avoid agglutination without impairing uptake of antibody. In fact, Vi inagglutinability may provide a convenient means of transforming a "complete" antigen-antibody reaction to one which is "incomplete." Finally, an even broader application of this effect may be foreseen for stabilizing a variety of mammalian cells such as platelets, leucocytes, and isolated tissue cells (34) which, because of their tendency to clump spontaneously, are subjected to serologic tests with great difficulty. In addition to such practical applications there is little doubt that this unique action of the Vi polysaccharide could provide a useful approach to achieving a better understanding of the basic mechanism of agglutination and other cell-surface phenomena.

SUMMARY

Erythrocytes coated with bacterial capsular polysaccharides, notably the Vi antigen, were no longer agglutinated by antibodies directed against the various antigens native to the red cell surface. These effects could not be attributed to prevention of antibody uptake even though in some systems the uptake of antibody was diminished. In fact, agglutination by Rh-incomplete antibody was brought back to the original titer only after the sensitized Vi-coated cells had been subjected to ten alternating exposures to globulin and antiglobulin. Hemagglutination by Newcastle, mumps, and influenza viruses was also suppressed. Erythrocytes coated with Vi polysaccharide assumed the distinctive physicochemical attributes of this acidic polymer which results in a stabilization of the erythrocyte suspension as manifested by increased electrophoretic mobility and a striking decrease in the rate of sedimentation. Among the possible models for explaining the nature of the Vi effect on immune agglutination, the data favor interference with lattice formation.

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