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Erythrocyte-Modifying Capacity of *Shigella dysenteriae* (SHIGA) Antigen and Its Polysaccharide Component.* (20933)

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Studies carried out since 1947 have established the fact that antigenic components or products of numerous bacteria as well as of certain rickettsiae, fungi, and protozoa can be adsorbed by red blood cells, thus rendering these modified erythrocytes specifically agglutinable by homologous microbial antibodies. Some of these antigens are polysaccharides, others are proteins. The present study was undertaken to determine the erythrocyte-modifying capacity of *Shigella* (*S.*) *dysenteriae* (Shiga) antigen. Such an investigation appeared to be of particular promise, since this antigen has been studied in great detail(1-7) and both the whole antigen complex (a polysaccharide-phospholipin-protein (or protein-like) complex) and its polysaccharide component were available. It is the polysaccharide that gives the whole complex and, therefore, this microorganism its main serological specificity.

Material and methods. The whole somatic *Shigella dysenteriae* (Shiga) antigen as well as its polysaccharide fraction were prepared recently by Dr. W. T. J. Morgan of The Lister Institute of Preventive Medicine, London, England, according to methods described in 1937(3). Solutions containing 50 mg of the antigens per 100 ml of phosphate buffer† (8) were prepared. The following diagnostic antisera were used: 1) two *S. dysenteriae* (Shiga) (rabbit) antisera;‡ 2) one polyvalent *S. dysenteriae* and *S. paradysenteriae* (horse) antiserum.‡ In addition, 2 *S. sonnei* (rabbit) antisera,‡ 2 group-specific *Escherichia* (*E.*) *coli* 026 and 0111 (rabbit) and pneumococcus types 4 and 32 (horse) antisera§ were employed for control purposes. In order to remove erythrocyte antibodies, all sera were first absorbed with the respective untreated red blood cells which were used in the *S. dysenteriae* hemagglutination and hemolysis tests. Red blood cells were modified as described previously(9). The *S. dysenteriae* (Shiga) antigen (2 ml) in appropriate dilution was added to the sediment of thrice washed red blood cells; the cells were mixed, incubated for 30 minutes at 37°C, washed 3 times in physiological saline solution, and resuspended in 2 ml of the same diluent. The following procedure was employed for the demonstration of erythrocyte-modification. The antisera (0.2 ml) in serial dilutions were mixed with an equal volume of the modified red blood cells. The mixtures were incubated for 30 minutes at 37°C and the resulting hemagglutination was read after centrifugation at 1500 R.P.M. for 2 minutes. In the hemolysis experiments guinea pig complement‖ (0.1 ml of a 1:20 dilution) was added to the mixtures of antiserum and modified red blood cells. The tubes were incubated at

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† Difco Laboratories, Detroit, Mich.
‡ Divisions of Laboratories and Research, N. Y. State Department of Health, and of Public Health Laboratories, Kansas State Board of Health.
§ Department of Health, N. Y. City.
‖ Carworth Farms, New City, N. Y.

37°C and the resulting hemolysis was read after 30 minutes. The hemagglutination inhibition tests were carried out as follows: The antigens (0.1 ml) in serial dilutions were mixed with *S. dysenteriae* antiserum (0.2 ml) in appropriate dilution; the mixtures were kept for 30 minutes at 37°C. Red blood cells modified by *S. dysenteriae* antigen or polysaccharide, respectively, were then added and the resulting hemagglutination was read after incubation at 37°C for 30 minutes and centrifugation at 1500 R.P.M. for 2 minutes.

Results. Red blood cells of sheep, man (blood group 0), rabbit, and guinea pig modified with *S. dysenteriae* antigen complex and its polysaccharide fraction, respectively, were agglutinated by 3 different *S. dysenteriae* antisera in dilutions ranging from 1:25 to 1:1600. The specificity of this reaction is attested by the facts that these modified blood cells were not agglutinated by 2 *Shigella sonnei,* 2 pneumococcus and 2 *E. coli* antisera in dilutions of 1:25 and higher; furthermore, the *S. dysenteriae* antisera failed to agglutinate untreated red blood cells as well as erythrocytes modified with crude *S. sonnei* antigen.

A series of experiments was carried out to determine whether red blood cells modified by *S. dysenteriae* complex or its polysaccharide fraction, respectively, are lysed in the presence of homologous antibodies and complement. Lysis occurred with modified sheep red blood cells but not with modified human red blood cells. Furthermore, lysis of modified sheep red blood cells was effected in the presence of 2 *S. dysenteriae* antisera of rabbit origin, but not when an equine polyvalent antiserum was used. The titers of the 2 *S. dysenteriae* (rabbit) antisera producing agglutination and lysis were essentially the same.

The minimal amounts of the 2 antigens which suffice for modification of red blood cells and subsequent agglutination by homologous bacterial antiserum was then determined. In view of the observation(9,10) that heating of bacteria-free supernates and sterile filtrates of freshly harvested *E. coli* suspensions brings forth their erythrocyte-modifying capacity, the influence of heat upon the erythrocyte-modifying capacity of the 2 *S. dysenteriae* antigens was also investigated. The results are summarized in Table I.

TABLE I. Erythrocyte-Modifying and Hemagglutination-Inhibitory Capacities of *Shigella dysenteriae* Antigen and Its Polysaccharide Fraction.

S. dysenteriae antigens	Minimal amounts (μg) of antigens adequate for: Modification of sheep red blood cells*	Minimal amounts (μg) of antigens adequate for: Inhibition of hemagglutination by *S. dysenteriae* antiserum
Antigen complex		
Unheated	5	.0125–.025
Heated	1.3	.0125–.025
Polysaccharide		
Unheated	20	.006
Heated	5–10	.006

* Calculated for 1 ml of 1% erythrocyte suspension.

It can be seen that 1) relatively minute amounts (1.3 to 20 μg) of the antigens sufficed for modification; 2) the antigen complex was somewhat more effective than the polysaccharide fraction, and 3) previous heating in the water bath at 100°C for one hour enhanced the erythrocyte-modifying capacity of both materials.

The results of titration of the unheated and heated antigens as specific inhibitors of hemagglutination by *S. dysenteriae* antiserum are presented in Table I. It is interesting to note that as little as .006 μg of the polysacchharide can be demonstrated by this method and that hemagglutination is inhibited by smaller amounts of the polysaccharide than of the antigen complex.

Random human sera from blood donors, healthy pregnant women, and children suffering from various diseases other than intestinal infection were examined for *S. dysenteriae* hemagglutinins. Eighteen out of 60 sera contained such antibodies, the titers ranging from 1:5 to 1:80. Absorption tests with both the antigen complex and its polysaccharide fraction revealed that these antibodies were specifically removed from these sera.

It was found previously that electrolytes were required for the adsorption of crude *E. coli* antigens by red blood cells(11). The question presented itself as to whether electrolytes are indispensable also for the adsorption of purified *S. dysenteriae* antigen. Because

of the limited amounts of antigens available, experiments could be carried out only with heated *S. dysenteriae* antigen complex. Modification of sheep red blood cells was carried out in 5% glucose solution; the treated and washed erythrocytes were resuspended in saline solution. Lysis and agglutination did not occur in the presence of complement and/or homologous antibodies. These results suggest that electrolytes are required for the adsorption of the (heated) purified *S. dysenteriae* antigen complex.

Discussion. The results indicate that purified *S. dysenteriae* antigen complex and its polysaccharide component, which determines the serological specificity of the microorganism, are readily adsorbed by red blood cells. This finding parallels observations by Landy (12) who has demonstrated adsorption by red blood cells of 7 bacterial antigens (lipopolysaccharides and acidic polymers), namely, those isolated from *Salmonella typhosa, Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris, Pasteurella tularense, Serratia marcescens,* and *Diplococcus pneumoniae.* The minimal amounts of the polysaccharides studied by Landy which bring forth detectable modification of red blood cells (one ml of 1% suspension) range from .016 to 4 μg). The amounts of the *S. dysenteriae* complex and its polysaccharide component sufficient for detectable modification is of the same order of magnitude, though somewhat larger (1.3 to 20 μg). It is evident from the results of the present study that the phospholipin and protein components of the whole antigen are not indispensable for adsorption by red blood cells. Furthermore, the lipid fraction obviously did not prevent adsorption of the whole antigen by erythrocytes. In this connection attention may be called to the observation that certain human plasma fractions, which are rich in lipids, as well as lecithin and cholesterol, when added to crude *E. coli* antigen, interfere with modification of red blood cells and subsequent hemagglutination by homologous bacterial antiserum (13,14).

The finding that the polysaccharide of the *S. dysenteriae* antigen complex is twice as effective as an inhibitor of hemagglutination by *S. dysenteriae* antiserum than the whole antigen is in good agreement with the observation of Morgan (3,4) to the effect that the polysaccharide component represents 50 to 60% of the whole antigen. On the other hand, the reason why the whole antigen is somewhat more effective than the polysaccharide fraction in modifying red blood cells remains to be elucidated.

Another aspect of this study, which deserves brief comment, concerns differences in modification of red blood cells for agglutination and lysis. Whereas *S. dysenteriae* antiserum obtained from both rabbit and horse causes specific agglutination of modified sheep red blood cells, only the former produces lysis in the presence of guinea pig complement. It is unlikely that the simultaneous presence of *S. paradysenteriae* antibodies in the polyvalent equine antiserum is responsible for its inability to lyse modified sheep red blood cells. More plausible is the explanation that the source of the antibody determines whether lysis will take place. Wright and Feinberg (15) also observed differences in the ability of antibodies obtained from various animal species to effect lysis of sheep cells modified by *Bacterium tularense* antigen; the hemolytic titers of antisera from 5 species did not parallel their hemagglutinating titers. In this connection attention may be called to the observations of Horsfall and Goodner (16), made more than a decade ago, that certain lipids of horse and rabbit pneumococcus antisera are indispensable for agglutination and precipitation, although not required for the union betweeen antigen and antibody; the essential lipid of the horse antiserum is lecithin, that of the rabbit antiserum cephalin.

Aside from these differences of the antisera from different animal sources, the present study revealed that, as in the experiments with crude *E. coli* antigens (9), modified sheep red blood cells, in contrast to human erythrocytes, are readily lysed in the presence of homologous rabbit antiserum and guinea pig complement, although the modified red blood cells of both species are equally well agglutinated. The reason for the failure of this antibody-complement system to effect lysis of human red blood cells remains to be elucidated.

It has been established that red blood cells

may be modified either simultaneously or consecutively by more than one bacterial antigen (9,12,17) and that saturation with one antigen does not block the adsorption of another. These facts suggest that different receptors of red blood cells are operative in the adsorption of different antigens. It is conceivable that the agglutinability and lysability of modified sheep red blood cells depend, among other and secondary factors, upon number and spatial arrangement of the antigen molecules on the surface of the erythrocytes. Information on this question may be gained from experiments with purified polysaccharides of known molecular weight. The ratio of the minimal number of antigen molecules sufficient for modification for hemagglutination and that needed for modification for hemolysis may then be calculated. Obviously, in such experiments identical red blood cells and complement as well as antibodies from a single animal species will have to be used. On the other hand, the possibility will have to be considered that the hemagglutination and hemolysis systems measure 2 different antibodies, as suggested by Middlebrook(18) on the basis of results with red blood cells modified by tubercle bacillus antigens and tested with sera of tuberculous patients.

Summary. The erythrocyte-modifying capacity of the *S. dysenteriae* (Shiga) antigen complex and its polysaccharide fraction was studied with the following results: 1. Both materials modified red blood cells of sheep, man, rabbit, guinea pig for subsequent specific agglutination by homologous bacterial antibodies obtained from rabbit and horse. The antigens were somewhat more effective after being heated at 100°C for one hour. 2. In the presence of guinea pig complement and homologous bacterial (rabbit) antiserum lysis occurred of modified sheep red blood cells but not of human erythrocytes; lysis of modified sheep and human red blood cells was not effected by an equine antiserum. 3. The polysaccharide fraction could be measured as a specific inhibitor of hemagglutination in amounts as small as .006 μg and proved to be twice as effective as the antigen complex. In contrast, the whole antigen was somewhat more active in modifying red blood cells than the polysaccharide component. 4. Of 60 random human sera 18 contained *S. dysenteriae* hemagglutinins, which were specifically absorbed by either the whole antigen or its polysaccharide fraction.

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