Zeitschrift f. Hygiene 150, 1—9 (1964)

From the Laboratory of Bacteriology, Children's Hospital,
Buffalo, New York, U.S.A. and
Department of Pathology and Bacteriology, Canton General Hospital,
Lucerne, Switzerland

Hemagglutination by Mixtures of Enterobacterial Antigen and Shigella Sonnei Antiserum*

By
E. A. GORZYNSKI, H. BRODHAGE, and E. NETER

(Received February 26, 1964)

The indirect, passive, or conditioned hemagglutination test has been employed for the demonstration of both O and Vi antibodies and of antibodies against certain common bacterial antigens. This method is based on the fact erythrocytes from various animal species readily take up these bacterial antigens. Although these antigen modified red blood cells do not undergo grossly visible changes, either agglutination or lysis, they are specifically agglutinated by the homologous bacterial antibodies. With erythrocytes from certain animal species, such as sheep, lysis takes place in the presence of antibody and complement. The hemagglutination inhibition test provides proof of the specificity of the reaction and, in addition, permits the quantitation of these antigens (cf. NETER[6]).

Antigens common to various enteric bacteria have been described by KUNIN[4,5] and by BRODHAGE[2,3]. Studies were carried out regarding the identity of these antigens by means of hemaglutination and hemagglutination inhibition tests. Utilizing a particular *Shigella sonnei* antiserum, the unexpected observation was made that hemagglutination of non-modified erythrocytes took place in mixtures of certain enterobacterial antigens and this antiserum. This hemagglutination reaction was then studied in detail, and the results are reported herewith.

Materials and Methods

Antigens were prepared from smooth strains of *Shigella sonnei*, *Salmonella typhimurium*, *Escherichia coli* serogroups O 111 and O 14, *Pseudomonas aeruginosa*, and *Staphylococcus* aureus. The bacteria were grown on brain veal agar in Kolle flasks and harvested in 25 ml of phosphate hemagglutination buffer (pH 7.3; Difco). The suspensions were heated in boiling water for one hour and the supernates (antigen) recovered after centrifugation at 23, 500 G for 30 minutes at 2°C. For control purposes supernates were prepared from uninoculated agar. Highly purified *Salmonella enteritidis* lipopolysaccharide, kindly supplied by Dr. E. RIBI, Rocky

* Study supported by Research Grant AI 00658, National Institute of Allergy and Infectious Diseases, U.S.P.H.S.

Mountain Laboratory, Hamilton, Montana, U.S.A., and *E. coli* O 14 lipopolysaccharide obtained through the courtesy of Dr. O. Lüderitz, Max-Planck Institute, Freiburg, Germany, were also used.

S. sonnei antiserum, prepared by Institute Sérothérapique et Vaccinal Suisse, Berne, was studied in detail, and all experiments were carried out with this reagent unless indicated otherwise. Another *S. sonnei* antiserum was obtained from the Division of Laboratories and Research, New York State Department of Health, Albany, New York. Other antisera were prepared at Children's Hospital by intravenous immunization of rabbits with heat-killed suspensions. The hemagglutination test was carried out as described previously[8].

Hemagglutination test

Briefly, a 2.5% erythrocyte suspension obtained from healthy subjects of blood group O were washed three times in phosphate buffer. Antigen was added to the erythrocyte sediment in volume to make a 2.5% cell suspension. The mixtures were incubated in a waterbath at 37°C for 30 minutes. The cells were again washed three times, in order to remove excess antigen. To antiserum in serial two-fold serial dilutions (0.2 ml) was added an equal volume of treated (antigen modified) erythrocytes. The mixtures were incubated in a waterbath for 30 minutes at 37°C and the resulting hemagglutination read grossly after centrifugation at 1300 G for 2 minutes.

Hemagglutination inhibition test

The test was carried out by mixing serum in serial two-fold dilutions (0.2 ml) with equal volume of supernate (antigen) of various bacteria. The mixtures were incubated in a waterbath at 37°C; antigen modified erythrocytes were then added. The mixtures were again incubated, and the resulting hemagglutination read as described above.

Hemagglutination by mixtures of enterobacterial antigen and antiserum

This test was carried out as the hemagglutination inhibition test just described with the exception that unmodified, washed erythrocytes were employed.

Absorption of antiserum

In this test the sediment of antigen modified erythrocytes, as prepared above, was mixed with the antiserum in suitable dilution. The mixture was incubated in a waterbath at 37°C for 30 minutes. The suspension was centrifuged. The supernate was used as absorbed antiserum. For control purposes unmodified erythrocytes were used for absorption of aliquots of the antiserum.

Results

Homologous and heterologous hemagglutinins in S. sonnei antiserum

In the first series of experiments titration of hemagglutinins against *S. sonnei* and several other enteric bacteria was carried out. The results

are recorded in Table 1, and they indicate that, in addition to agglutinins against the homologous *S. sonnei*, antibodies in rather high titers were present against *S. typhimurium* and *E. coli* O 111 and O 55. It is of note that the titer of antibodies against *E. coli* O 14 was considerably lower. For control purposes, hemagglutination tests were also carried out with

Table 1. *Hemagglutination by S. sonnei (Inst. Séroth. Suisse) antiserum of erythrocytes modified with various enterobacterial antigens*

Antigens	Antiserum	
	S. sonnei (Inst. Séroth. Suisse)	*E. coli* O 14 CH*
	Hemagglutinin titers (reciprocal)	
S. sonnei	1600	1600
S. typhimurium	3200	1600
E. coli O 111	1600	1600
E. coli O 14	200	3200
E. coli O 55	6400	3200

* Prepared at Children's Hospital, Buffalo, New York.

Table 2. *Absorption of hemagglutinins in S. sonnei antiserum by antigen modified erythrocytes*

S. sonnei antiserum	Indicator erythrocytes	
	S. sonnei	*S. typhimurium*
	Hemagglutinin titers (reciprocal)	
Unabsorbed	1600	3200
Absorbed with:		
Untreated RBC	800	1600
S. sonnei treated RBC	<200	<200
S. typhimurium treated RBC	800	100
E. coli O 14 treated RBC	800	1600

E. coli O 14 antiserum and revealed that antibodies against common enterobacterial antigen reacted to approximately the same degree with all five antigens tested. In order to identify the cross-reacting antibody further, hemagglutination inhibition tests were carried out with unabsorbed and absorbed *S. sonnei* antiserum. To this end, the antiserum was absorbed with untreated erythrocytes, and with *S. sonnei*, *S. typhimurium*, or *E. coli* O 14 treated red blood cells. Following absorption the remaining antibodies were titrated with red blood cells modified with either *S. sonnei* or *S. typhimurium* antigen. The results of this experiment are shown in Table 2.

It can be seen that *S. sonnei* treated red blood cells absorbed the antibodies reacting with *S. sonnei* and *S. typhimurium*. In contrast, no substantial reduction in antibody titer was effected by absorption with

E. coli O 14 treated red blood cells. Thus, it is suggested that the cross-reacting antibody is different from that directed against the common antigen of KUNIN. It can be noted, furthermore, that the antibody reacting against *S. typhimurium* was removed by absorption with either *S. sonnei* or *S. typhimurium* treated red blood cells, but not by *E. coli* O 14 treated erythrocytes. This finding lends further support to the above assumption that some of the cross-reacting antibodies present in this *S. sonnei* antiserum are not directed against the common antigen of KUNIN.

Hemagglutination by mixtures of enterobacterial antigen and S. sonnei antiserum

Numerous attempts were made without success to identify the antibodies in *S. sonnei* antiserum by means of hemagglutination inhibition tests. An explanation for the perplexing results was found when it was discovered that mixtures of several enterobacterial antigens and *S. sonnei* antiserum produced agglutination even of untreated erythrocytes.

Table 3

Hemagglutinating capacity of mixtures of Shigella sonnei serum and various supernates

S. sonnei serum	Mixtures of *Shigella sonnei* serum. Bacterial supernates				
	E. coli O 111	*E. coli* O 14	*S. sonnei*	*S. typhimurium*	Sterile agar wash
	Untreated erythrocytes				
	Hemagglutinin titers (reciprocal)				
Inst. Séroth. Suisse	>1600	<100	>1600	>1600	<100
N. Y. Dept. Health	< 100	<100	< 100	< 100	<100

For the sake of brevity, this type of hemagglutination is referred to as complex hemagglutination. The results of a representative experiment are recorded in Table 3.

Perusal of the table shows that mixtures of the *S. sonnei* antiserum in high titer and antigens obtained from *E. coli* O 111, *S. sonnei*, and *S. typhimurium* resulted in hemagglutination of untreated erythrocytes. It is also evident that neither antigen from *E. coli* O 14 nor washing of sterile agar were reactive. The extraordinary characteristic of this *S. sonnei* antiserum is evident from the fact that a diagnostic *S. sonnei* antiserum prepared by the New York State Departement of Health failed to give complex hemagglutination with any of the antigens used. The observation that antigen from *E. coli* O 14 is not reactive in the system suggests that the common antigen described by KUNIN is probably not involved in this reaction. It is also evident that this complex hemagglutination reaction is not due to a reaction between *S. sonnei* anti-

bodies and the O antigen of *S. sonnei*, for antigenically unrelated microorganisms, such as *E. coli* O 111 and *S. typhimurium*, were reactive. Additional experiments revealed that antigen from *Pseudomonas aeruginosa* and *Staphylococcus aureus* did not give complex hemagglutination with the Swiss *S. sonnei* antiserum.

Table 4
Hemagglutination by mixtures of antigen and absorbed S. sonnei antiserum

S. sonnei antiserum	Antigens	
	S. sonnei	*S. typhimurium*
	Hemagglutinin titers (reciprocal)	
Unabsorbed	400	1600
Absorbed*		
Untreated	400	1600
S. sonnei	<200	<200
S. typhimurium	<200	<200
E. coli O 14	400	1600

* Absorption carried out with erythrocytes, untreated or antigen modified.

Table 5. *Effect of temperature on hemagglutinins in S. sonnei antiserum*

Antigens	*S. sonnei* antiserum		
	Unheated	30′ 56°C	30′ 75°C
	Hemagglutinin titers (reciprocal)		
	A. Hemagglutination by antigen-antibody mixtures		
S. sonnei	800	800	<200
S. typhimurium	1600	3200	<200
S. enteritidis	6400	3200	<200
E. coli O 111	6400	3200	<200
	B. Hemagglutination of antigen modified erythrocytes		
S. sonnei	3200	3200	400
S. typhimurium	3200	3200	<200
S. enteritidis	6400	3200	200
E. coli O 111	3200	3200	<200

In order to characterize further the antibodies responsible for this complex hemaglutination reaction, absorption experiments were carried out with untreated and with antigen modified erythrocytes. The results of this experiment are summarized in Table 4.

It may be noted that absorption with *S. sonnei* and *S. typhimurium* treated erythrocytes eliminates the complex hemagglutinating capacity of the antiserum, whereas *E. coli* O 14 absorption failed to reduce the

hemagglutinin titer. From these experiments it can be concluded that complex hemagglutination by *S. sonnei* antiserum and antigen is due to an antibody shared by several enteric bacteria, but not present in *E. coli* O 14. The antibody responsible for this reaction, therefore, is not of the Kunin type.

The next series of experiments was carried out to determine the heat stability of the antibody in *S. sonnei* antiserum responsible for agglutination of unmodified erythrocytes in the presence of antigen. To this end, the antiserum was heated for 30 minutes either at 56°C or at 75°C. Unheated antiserum was used for control purposes. This antiserum was

Table 6. *Influence of S. enteritidis lipopolysaccharide on hemagglutination by S. sonnei antiserum and S. enteritidis (crude) antigen*

Indicator	Antisera					
	S. sonnei			Salmonella group D		
	Antigens					
	S. enteritidis			Buffer *S. enteritidis*		
	Crude	Lp*	Lp followed by crude		Lp	Crude
	Hemagglutinin titers (reciprocal)					
Untreated erythrocytes	1600	<100	1600			
S. enteritidis (crude) treated erythrocytes				800	<100	<100

* Lp = Lipopolysaccharide.

then employed in both complex hemagglutination tests and hemagglutination tests of antigen-modified erythrocytes. The results are summarized in Table 5 and indicate that the antibody responsible for both types of hemagglutination reactions is destroyed at 75°C but not at 56°C.

Additional studies were carried out with highly purified lipopolysaccharide antigen. *S. sonnei* antiserum, and *Salmonella* group D antiserum for control purposes, were mixed with either crude or purified antigens or a mixture thereof. The antigen-antibody mixtures were incubated for 30 minutes at 37°C. To one series untreated erythrocytes, and to the other red blood cells previously modified with crude *S. enteritidis* antigen were added. The results are shown in Table 6.

It is evident that, in contrast to crude *S. enteritidis* antigen, the highly purified lipopolysaccharide failed to give complex hemagglutination, nor did the lipopolysaccharide inhibit this reaction with crude antigen. Both crude and lipopolysaccharide antigens neutralized the group specific O antibodies, as is evident from the marked reduction in titer

when tested with *S. enteritidis* modified red blood cells. From this and similar experiments it is obvious that complex hemagglutination by mixtures of antigen and *S. sonnei* antiserum does not occur with highly purified O lipopolysaccharide, and that the O antigen does not interfere with this reaction.

Table 7
Effect of heated (75° C) rabbit serum on S. sonnei erythrocyte modification

Inhibitory sera	*S. sonnei* antigen						
	Heated (75° C, 15′) serum as inhibitor						
	1:50	1:100	1:200	1:400	1:800	1:1600	Buffer
	Erythrocytes						
	*S. sonnei** antiserum hemagglutinin titers (reciprocal)						
Normal rabbit serum	<50	<50	<50	50	400	400	1600
S. sonnei (Inst. Séroth. Suisse)	100	200	400	400	800	800	1600

* New York State Department of Health.

Table 8. *Influence of S. sonnei antiserum on erythrocyte modifying capacity of E. coli O 111 antigen*

E. coli O 111 antiserum	*E. coli* O 111 antigen			
	S. sonnei antiserum*		Normal rabbit serum*	Buffer
	Inst. Séroth. Suisse	New York Dept. Health		
	Hemagglutination			
1:100	2	—	—	4
1:400	1	—	—	3
1:1600	—	—	—	2
1:6400	—	—	—	1
0	—	—	—	—

* 100°C 15′; dilution 1:100. 1 to 4 = Various degrees of hemagglutination. — = No hemagglutination.

In previous studies[9,10,11], it was shown that serum and certain fractions thereof, as well as lecithin, egg yolk, and some antibiotics prevent the attachment of lipopolysaccharide to erythrocytes. Additional studies have revealed that complex hemagglutination is effected by mixtures of human gamma globulin and a common antigen of gram-positive bacteria[7]. This complex hemagglutination is prevented by a heat stable factor in serum. With these considerations in mind, the possibility was considered that the Swiss *S. sonnei* antiserum may lack the inhibitor present in other sera. To elucidate this question, the inhibitory capacity of heated *S. sonnei* antiserum and normal rabbit serum was determined. *S. sonnei* antigen was mixed with various dilutions of the two sera that had been heated at 75° C for 15 minutes. The mixtures were then used for modification of red blood cells and tested with *S. sonnei* antiserum in serial dilutions obtained from New York State Health Departement. The results are shown in Table 7.

Perusal of the table indicates that the Swiss *S. sonnei* antiserum inhibited modification of erythrocytes to a significantly lesser extent than did normal rabbit serum used for control purposes. Additional experiments with *E. coli* O 111 antigen revealed similar results, as shown in Table 8. Thus, it is evident that the Swiss *S. sonnei* antiserum contained less inhibitor than did the New York State serum or normal rabbit serum, and it is conceivable that this low titer of the inhibitor accounts, in part at least, for hemagglutination by mixtures of antigen and this particular serum.

Discussion

The present investigation has revealed that a particular *S. sonnei* antiserum in the presence of one of several enterobacterial antigens causes agglutination of untreated erythrocytes. It can be assumed that this reaction is due to the action of a complex of antigen and antibody. The antibody involved is not directed against the O antigen of *S. sonnei*, but rather reacts with a common antigen of various enteric bacteria. The evidence presented also indicates that the antibody is not active against the common enterobacterial antigen discovered by Kunin[4,5]. The relationship of the antigen described by Brodhage[2,3], remains to be determined.

Hemagglutination by mixtures of antigen and antibody has been described previously. Boyden[1] observed that mycobacterial antigen and its homologous antibody cause agglutination of normal cells, and lysis when complement is also present. Similarly, hemagglutination can be effected by mixtures of human gamma globulin and a common antigen of gram-positive bacteria[7].

At the present time, it is not clear why this complex hemagglutination does not take place with many other antigen-antibody systems, operative in indirect hemagglutination reactions. Indeed, when soluble antigen is added to homologous antiserum, agglutination of antigen modified erythrocytes is in fact inhibited. It is of interest to point out that the Swiss *S. sonnei* antiserum contained less inhibitor than other sera tested, and the lower titer of inhibitor, which prevents the attachment of bacterial antigens to erythrocytes, may be responsible, in part at least, for the above described complex hemagglutination phenomenon. Further studies are needed to elucidate the exact mechanism of complex hemagglutination, and to determine under what conditions immune sera are produced in either experimental animals or in man that are operative in this unusual hemagglutination reaction.

Summary

A study has been made of a special *S. sonnei* serum obtained from Inst. Séroth. Suisse and the following results have been obtained. 1. The

antiserum contains antibodies specific for *S. sonnei* and, in addition, antibodies reacting with crude antigens from other enteric bacteria in the hemagglutination test. 2. Mixtures of this antiserum in rather high dilutions and crude antigens from various enteric bacteria cause agglutination of normal erythrocytes. 3. The antibody responsible for this complex hemagglutination reaction is not directed against the O antigen nor the common antigen described by KUNIN, but rather against another antigen shared by certain enteric bacteria. 4. This particular antiserum has a lower content of an inhibitor which prevents erythrocyte modification than do normal rabbit serum or another *S. sonnei* antiserum.

Literature

[1] BOYDEN, S. V., and E. M. ANDERSON: Agglutination of normal erythrocytes in mixtures of antibody and antigen, and haemolysis in the presence of complement. Brit. J. exp. Path. **36**, 162–170 (1955).

[2] BRODHAGE, H.: Harnstoff-Extrakte aus gramnegativen Bakterien in der indirekten Hämagglutinationsreaktion. I. Harnstoff-Extrakte aus S. typhi, paratyphi-B, cholerae suis und Shig. sonnei. Z. Hyg. Infekt.-Kr. **148**, 94–104 (1961).

[3] — Harnstoff-Extrakte aus gramnegativen Bakterien in der indirekten Hämagglutinationsreaktion. II. Die serologische Beziehung zwischen dem „C-Antigen" und dem Rauh-Antigen. Z. Hyg. Infekt.-Kr. **148**, 208–213 (1962).

[4] KUNIN, C. M.: Separation, characterization, and biological significance of a common antigen in enterobacteriaceae. J. exp. Med. **118**, 565–586 (1963).

[5] — M. V. BEARD, and N. E. HALMAGYI: Evidence for a common hapten associated with endotoxin fractions of *E. coli* and other enterobacteriaceae. Proc. Soc. exp. Biol. (N. Y.) **111**, 160–166 (1962).

[6] NETER, E.: Bacterial hemagglutination and hemolysis. Bact. Rev. **20**, 166 (1956).

[7] — E. A. GORZYNSKI, H. ANZAI, and V. BOKKENHEUSER: Hemagglutination by mixtures of human gamma globulin and heterogenetic bacterial antigen. J. Immunol. **88**, 411–417 (1962).

[8] — — R. M. GINO, O. WESTPHAL, and O. LÜDERITZ: The enterobacterial hemagglutination test and its diagnostic potentialities. Canad. J. Microbiol. **2**, 232 (1956).

[9] — — O. WESTPHAL, and O. LÜDERITZ: The effects of antibiotics on enterobacterial lipopolysaccharides (Endotoxins), hemagglutination and hemolysis. J. Immunol. **80**, 66–72 (1958).

[10] — D. A. ZAK, N. J. ZALEWSKI, and L. F. BERTRAM: Inhibition of bacterial *(Escherichia coli)* modification of erythrocytes. Proc. Soc. exp. Biol. (N. Y.) **80**, 607 (1952).

[11] — N. J. ZALEWSKI, and D. A. ZAK: Inhibition of lecithin and cholesterol of bacterial *(Escherichia coli)* hemagglutination and hemolysis. J. Immunol. **71**, 145 (1953).

Dr. H. BRODHAGE,
Canton General Hospital, Lucerne, Switzerland
Prof. Dr. E. NETER,
Laboratory of Bacteriology, Children's Hospital, Buffalo 22. N.Y. 14222, U.S.A.