STUDIES ON HEMAGGLUTINATION AND HEMOLYSIS BY ESCHERICHIA COLI ANTISERA*

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Two recent developments have stimulated renewed interest in *Escherichia coli* infections of man. In the first place, through the important investigations of Kauffmann and his associates (1, 2) extensive knowledge has been gained on the antigenic pattern of this species. The discovery by Kauffmann of antigens as inhibitors of O agglutinability and the identification of the various antigens (the somatic O antigens, the envelope or surface L and B antigens, the capsular A antigens, and the flagellar H antigens) have brought order to the serological grouping and typing of *E. coli* and made possible studies on the relationship between antigenic types of *E. coli* and various infections.

The second discovery of major importance is the finding of the association of two serological groups of *E. coli*, namely, serogroups O111 and O55, with epidemic and sporadic diarrheal disease of infants. In 1923 and 1927, Adam (3, 4) reported on the recovery of several biochemical types of *E. coli* from patients with diarrheal disease. It remained for the British investigators Bray (5), Bray and Beavan (6), Giles and Sangster (7), Giles, Sangster, and Smith (8), Smith (9), as well as Taylor, Powell, and Wright (10) to establish the fact that in certain outbreaks of epidemic diarrhea two serogroups of *E. coli* are found in a high percentage of affected children and only rarely in healthy infants, children, and adults. These two antigenic groups have been identified as serogroups O111 and O55. The observations of these investigators have been confirmed and extended in various parts of the world, including the Scandinavian countries by Kauffmann and Dupont (11), the Netherlands by Beeuwkes, Hodenpijl, and ten Seldam (12), Germany by Braun (13), and the United States by Modica, Ferguson, and Ducey (14), Neter and Shumway (15), Neter and Webb (16), Neter, Webb, Shumway, and Murdock (17). Very recently, Orskov (18) reported on the occurrence of *E. coli* belonging to O-group 26 in cases of infantile diarrhea and in white scours of newborn calves. Thus it appears likely that groups other than O111 and O55 may be associated with diarrheal disease of infants.

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It is an established fact that certain bacterial antigens may be adsorbed on red blood cells, thus rendering these erythrocytes specifically agglutinable by the homologous bacterial antiserum. Recently, it was shown by Neter, Bertram, and Arbesman (19) that the group-specific antigens of *E. coli* O111 and O55 can be demonstrated by means of such a hemagglutination test. The experiments to be reported in this communication were undertaken to study this hemagglutination test in greater detail, to determine whether the antigens can be demonstrated also by means of a hemolysis test, to compare the sensitivity of bacterial agglutination, hemagglutination, and hemolysis tests for the demonstration of *E. coli* antibodies, and to ascertain whether the antigens can be assayed by means of hemagglutination and hemolysis inhibition tests.

Material and Methods

Several strains of *E. coli* of serogroups O55 and O111, isolated from infants suffering from diarrheal disease, were used. The microorganisms were maintained on brain-veal-agar and transferred weekly. Each subculture was checked by means of group-specific antisera. For the experiments proper the strains were grown in brain-heart infusion and on brain-veal-agar (Kolle flasks). The broth cultures were incubated for 3 to 5 days at 37°C. and passed through a Seitz (EK No. 5) filter after the pH had been adjusted to 7. The filtrates were tested for sterility and kept in the refrigerator. The microorganisms grown on brain-veal-agar in a Kolle flask for 24 hours at 37°C. were suspended in 25 ml. of physiological saline solution. Unless otherwise indicated, both broth culture filtrates and agar suspensions were boiled for 2 hours.

Separation of bacterial cells from the surrounding fluid was accomplished by means of a refrigerated centrifuge (International, model P R-1; r.c.f. at tip of tube calibrated to be 24,000; centrifugalization for from 10 to 90 minutes). The sediment was resuspended in an equal amount of physiological saline solution.

Red blood cell suspensions (2.5 per cent) in physiological saline solution were prepared from oxalated or defibrinated blood of man and various animal species. The blood was washed in physiological saline solution prior to use.

Red blood cells were modified in the following manner. To the sediment of the red blood cell suspension was added *E. coli* antigen to make a 2.5 per cent suspension. The mixtures were kept in 15 ml. centrifuge tubes at 37°C. for 1 hour and mixed repeatedly. The cells were then washed three times in physiological saline solution and made up to a 2.5 per cent suspension.

The following *E. coli* antisera were employed: (*a*) serum from rabbits immunized with both boiled and unheated suspensions of the two serogroups; such sera contain both O and B antibodies; (*b*) serum from rabbits immunized with boiled suspensions; these antisera contain O antibodies; (*c*) for control purposes, antisera containing both O and B antibodies, made available through the kindness of Dr. William H. Ewing of the Communicable Disease Center, Chamblee, Ga. All sera were tested for specificity in the bacterial agglutination test.

The modified cells (volume 0.2 ml.) were then added to 0.2 ml. of various *E. coli* antisera in serial dilutions. The mixtures were incubated at 37°C. and agglutination was read in the gross at intervals up to 2 hours. Following the last reading the tubes were centrifuged at 2,000 R.P.M. for 1 minute and again read. In the hemolysis experiments complement (0.1 ml. of a 1:10 diluted guinea pig serum) was added to the antiserum prior to the addition of the

modified red cells. The complement was procured from Carworth Farms, Inc., New City, N. Y. Hemolysis was read in the gross after incubation at 37°C. at intervals up to 2 hours.

In the hemagglutination and hemolysis inhibition tests *E. coli* antiserum was mixed with *E. coli* antigens in various concentrations and incubated for 30 minutes at 37°C.; then, modified red blood cells were added. The mixtures were incubated and read as described above. In the hemolysis inhibition tests complement was either present during the first phase of the experiment or added just prior to the admixture of the *E. coli*-treated red blood cells.

These hemagglutination, hemolysis, hemagglutination inhibition, and hemolysis inhibition tests are based on the techniques developed during studies with other bacterial antigens by Keogh and associates (20, 21), Middlebrook and Dubos (22), Hayes and Stanley (23), Hayes (24), as well as Wright and Feinberg (25).

TABLE I

Agglutination of Red Blood Cells of Various Species Treated with E. coli O111 Antigen

Anti-*E. coli* serum		Man	Dog	Rabbit	Sheep	Guinea pig	Rat	Chicken
O111	1:100	4	4	4	4	4	4	4
	1:200	4	4	3	4	4	4	4
	1:400	3	4	3	4	3	3	3
	1:800	—	—	±	—	—	—	±
	1:1600	—	—	—	—	—	—	—
	0	—	—	—	—	—	—	—
O55*	1:100	—	—	—	—	—	—	—

— = no agglutination.

1 to 4 = various degrees of agglutination.

* This serum agglutinated in dilutions up to 1:800 red blood cells treated with *E. coli* O55 antigen.

RESULTS

Modification of Red Blood Cells of Different Species by E. coli Antigens.—It was previously shown (19) that red blood cells of both man and rabbit adsorb *E. coli* O111 and O55 antigens and thus become agglutinable by the homologous group-specific antisera; only the boiled, but not the unheated, *E. coli* suspensions and broth culture filtrates were found to be highly active. It was considered of interest to determine whether red blood cells of other species could be modified in like manner. To this end, washed blood cells of dog, guinea pig, sheep, chicken, and rat, in addition to cells from man and rabbit, were utilized. The results of a representative experiment with *E. coli* O111 antigen (boiled broth culture filtrate) after incubation for 1 hour at 37°C. and centrifugalization are summarized in Table I. It can be seen that blood cells from all species adsorbed the antigen and that the titer of the antiserum was roughly identical with all modified red blood cell suspensions. Antisera containing either O111 antibodies or both O111 and B antibodies

were effective. The specificity of this hemagglutination reaction is evident from the fact that *E. coli* O55 antiserum in like dilutions failed to agglutinate O111-treated red blood cells, although this serum strongly agglutinated red blood cells treated with *E. coli* O55 antigen.

Titration of E. coli Antigen and the Effect of Time on Its Adsorption by Red Blood Cells.—Numerous experiments were carried out in order to determine quantitatively the activity of various *E. coli* antigens in modifying red blood

TABLE II

Titration of E. coli Antigen and the Effect of Time on the Adsorption of This Antigen on Red Blood Cells

Anti-*E. coli* serum		*E. coli* O111 antigen diluted								
		1:5			1:25			1:50		
		and acting on red blood cells for								
		5 min.	15 min.	30 min.	5 min.	15 min.	30 min.	5 min.	15 min.	30 min.
O111	1:100	4	4	4	1	3	4	—	—	1
	1:200	4	4	4	—	1	3	—	—	—
	1:400	4	4	4	—	—	—	—	—	—
	1:800	1	1	3	—	—	—	—	—	—
	1:1600	—	±	1	—	—	—	—	—	—
	1:3200	—	—	—	—	—	—	—	—	—
	0	—	—	—	—	—	—	—	—	—
O55*	1:100	—	—	—	—	—	—	—	—	—

— = no agglutination.

1 to 4 = various degrees of agglutination.

* This serum agglutinated in dilutions up to 1:800 red blood cells treated with *E. coli* O55 antigen.

cells and to ascertain the effects of time on their adsorption. A typical experiment is presented in Table II.

A suspension in physiological saline solution of *E. coli* O111, harvested from brain-veal-agar and boiled for 2 hours, served as antigen. This antigen was used in dilutions of 1:5, 1:25, and 1:50 for treatment of human red blood cells for 5, 15, and 30 minutes, respectively. The agglutination of the modified red blood cells by the homologous *E. coli* antiserum in serial dilutions after incubation for 1 hour at 37°C. and centrifugalization is recorded in Table II.

Perusal of this table shows that the modification of the red blood cells by the more concentrated antigen occurs rather rapidly and increases but little following incubation for more than 5 minutes. On the other hand, red cells treated with the more diluted antigen are rendered more agglutinable following prolonged treatment. Thus, it is evident that the antigen modification

of red blood cells is a function of the activity or concentration of the antigen and of time.

The Relationship between Concentration of E. coli Antigen and Number of Red Cells, and the Adsorption of the Antigen.—It seemed to be of interest to determine whether the adsorption of *E. coli* antigen and the resulting hemagglutination by *E. coli* antiserum depend also upon the number of red blood cells treated. To this end, the following experiment was carried out.

TABLE III

The Relationship between Concentration of E. coli Antigen and Number of Red Blood Cells, and the Adsorption of the Antigen and Resulting Hemagglutination

E. coli O111 antiserum	Red blood cells from whole blood, *ml.*					
	0.2	0.2	0.2	0.2	0.6	1:8
	treated with *E. coli* O111 antigen (2 ml.) in dilutions of					
	1:5	1:15	1:45	1:135	1:5	1:5
1:100	4	4	4	–	4	–
1:200	4	4	4	–	4	–
1:400	4	4	1	–	4	–
1:800	4	4	–	–	1	–
1:1600	3	3	–	–	–	–
1:3200	1	–	–	–	–	–
1:6400	–	–	–	–	–	–
0	–	–	–	–	–	–

E. coli antigen (a suspension procured from brain-veal-agar and boiled for 2 hours) in dilutions of 1:5, 1:15, 1:45, and 1:135 (volume 2 ml.) was used for the treatment of red blood cells from 0.2 ml. of oxalated human blood. In addition, the antigen in a dilution of 1:5 (volume 2 ml.) was used for the treatment of red blood cells from 0.6 ml. and 1.8 ml. of the same blood specimen. The red blood cells were treated with this antigen for 1 hour at 37°C., washed three times in physiological saline solution, and suspended to give a 2.5 per cent red blood cell suspension. These blood cell suspensions were then used as modified antigen and mixed with *E. coli* O111 antiserum in serial dilutions and O55 antiserum as control. The resulting hemagglutination, read after incubation at 37°C. for 1 hour and centrifugalization, is recorded in Table III.

A perusal of this table shows that the more red cells were treated with the antigen in identical concentration the less agglutination occurred with the homologous *E. coli* antiserum. It is thus evident that the antigenic modification of red blood cells by *E. coli* antigen and the resulting hemagglutination by the homologous *E. coli* antiserum depend upon the concentration of the red blood cells and, as shown above, upon the concentration of the antigen and the time of treatment.

Simultaneous Adsorption of Two E. coli Antigens by Red Blood Cells.—In

order to ascertain whether red blood cells adsorb the two different *E. coli* antigens under consideration, the following experiment was carried out.

Human red blood cells were treated for 1 hour at 37°C. with (*a*) 2 ml. of a 1:5 diluted *E. coli* O111 agar suspension which had been boiled for 2 hours, (*b*) 2 ml. of the corresponding O55 *E. coli* antigen, and (*c*) a mixture of both antigens in equal amounts. These antigen-modified cells were then tested with both *E. coli* O111 and *E. coli* O55 antisera in serial dilutions. The results of this experiment after incubation for 1 hour at 37°C. and centrifugalization are presented in Table IV.

It can be noted that the red blood cells treated with the two antigens became agglutinable by both antisera. It should be pointed out that each an-

TABLE IV

Simultaneous Adsorption by Red Blood Cells of Two Different E. coli Antigens

E. coli antisera		Agglutination by *E. coli* antisera of red blood cells treated with *E. coli*		
		O55 antigen	O111 antigen	O55 and O111 antigens
O111	1:100	—	4	4
	1:200	—	4	4
	1:400	—	4	4
	1:800	—	4	—
	1:1600	—	—	—
	0	—	—	—
O55	1:100	4	—	4
	1:200	3	—	4
	1:400	—	—	—
	1:800	—	—	—

— = no agglutination.
1 to 4 = various degrees of agglutination.

tiserum caused complete hemagglutination, as all red cells became agglutinated in one solid clump and unagglutinated red cells were not present. This observation, which was made in repeated experiments, suggests that all the red cells had adsorbed both antigens rather than that half of the red cells had adsorbed one antigen and the other half the other. Proof that red cells are capable, indeed, of adsorbing both *E. coli* antigens is furnished by the results of the following experiment. Human blood cells were treated with O111 antigen in one series, with O55 antigen in the second, and with a mixture of both antigens in the third. An equal mixture of O111-treated cells and O55-treated cells was then prepared. All cell suspensions were tested with O111 and O55 antisera. It was found that both antisera produced only partial agglutination of the mixture of O111- and O55-treated cells but agglutinated completely the red cells treated either with the homologous anti-

gen or a mixture of both antigens. The same results were obtained when O111-treated cells were mixed with untreated cells and then tested with O111 antiserum. It is concluded, therefore, that each red cell adsorbs both antigens.

The Effects of Subsequent Treatments of Red Blood Cells with Different E. coli Antigens.—It is conceivable that the adsorption of one *E. coli* antigen renders the blood cells incapable of subsequently adsorbing another *E. coli* antigen. To test this hypothesis, a large number of experiments were carried

TABLE V

The Effects of Subsequent Treatments of Red Blood Cells with Different E. coli Antigens on the Adsorption of These Antigens

E. coli antisera		Agglutination of red blood cells by *E. coli* antisera after treatment with *E. coli* antigens			
		O55	O111	O111 followed by O55	O55 followed by O111
O111	1:100	–	4	4	4
	1:200	–	4	4	4
	1:400	–	4	4	4
	1:800	–	3	3	4
	1:1600	–	2	±	2
	1:3200	–	±	–	–
	0	–	–	–	–
O55	1:100	4	–	4	4
	1:200	2	–	4	3
	1:400	2	–	1	2
	1:800	–	–	–	–
	1:1600	–	–	–	–

out in which the two *E. coli* antigens under consideration were used consecutively rather than simultaneously.

Red cells were treated with one *E. coli* antigen, washed three times, and then treated with the other, followed by washing. For control purposes red cells were treated with one antigen alone and incubated and washed in like fashion. The results of a typical experiment are given in Table V.

It can be noted that red cells treated with O111 antigen later adsorb O55 antigen and *vice versa*. This consecutive treatment with two antigens resulted in hemagglutination by both antisera in essentially identical dilutions. It is conceivable that under different conditions blocking of one antigen may result from previous treatment with another. Thus far, this had not been observed. In this connection it can be noted that human red cells which had adsorbed either one or both *E. coli* antigens were agglutinated readily by blood group-specific and Rh-specific antibodies, indicating that the *E.*

coli antigens did not block the A, B, or Rh antigens of red blood cells and prevent agglutination by the homologous blood group-specific antibodies.

Hemolysis by E. coli Antisera.—Experiments were carried out to determine whether red blood cells modified by *E. coli* antigens can be lysed in the presence of *E. coli* antibodies and complement. Numerous experiments yielded identical results.

Human red blood cells (blood group O) and sheep cells were treated for 1 hour at 37°C. with a 1:5 diluted *E. coli* O111 brain-veal-agar suspension, which had been previously boiled for 2 hours. The washed red blood cell suspensions were then mixed with four different *E. coli* antisera (sera containing either O antibodies only or O and B antibodies) and normal rabbit serum in serial dilutions, in the absence of complement in one series and in the presence of complement (1:10 dilution) in the other. The same antisera were also tested for antibodies producing bacterial agglutination; the identical boiled bacterial suspensions, which were used for the modification of the red blood cells, were employed. The hemagglutination and hemolysis tests were read after incubation at 37°C. for 1 hour; the bacterial agglutination after 18 hours at 56°C. The results are presented in Table VI.

TABLE VI

Hemagglutination and Hemolysis by E. coli O111 Antiserum of Human and Sheep Red Blood Cells Treated with E. coli O111 Antigen

Antisera containing antibodies to antigens	Titer of *E. coli* antisera causing				
	Hemagglutination of cells treated with O111 antigen		Hemolysis of cells treated with O111 antigen		Bacterial agglutination of boiled bacterial suspension
	Human cells	Sheep cells	Human cells	Sheep cells	
O111	1:1600	1:1600	1:400	1:51,200	1:1600
O111 + B	1:1600	1:1600	1:100	1:25,600	1:800
O55*	—	—	—	1:800	—
O55 + B*	—	—	—	1:200	—
Normal rabbit serum	—	—	—	—	—

* This serum caused hemagglutination and hemolysis of red blood cells treated with *E. coli* O55 antigen.

— = no reaction with serum in dilutions of 1:100 and higher.

It can be noted that both *E. coli* O111 antisera produced hemagglutination and hemolysis of the modified red cells and agglutination of the bacteria. It is also evident that the titer of these antisera producing sheep cell hemolysis is greater by far than the titer producing sheep and human cell hemagglutination and bacterial agglutination. It can also be seen that the heterologous *E. coli* antisera failed to produce agglutination of the bacteria, hemagglutination and hemolysis of human cells, and hemagglutination of sheep cells; however, these sera in relatively low titer produced sheep cell hemolysis. This cross-reacting antibody, as demonstrated by sheep cell hemol-

ysis, represents only a small fraction of the titer of antibodies causing lysis of sheep cells treated with the homologous antigen. A poorly developed cross-reaction between *E. coli* O55 and O111 has been demonstrated previously by Kauffmann (1). This hemolysis test may offer a sensitive method for further studies on the cross-reaction between the two serogroups of *E. coli*.

Distribution of E. coli Antigens Operative in Hemagglutination by E. coli Antiserum between Bacterial Cells and Supernate.—The distribution of the *E. coli* antigens operative in hemagglutination between bacterial cells and supernate was investigated next.

TABLE VII

Distribution of E. coli Antigen Operative in Hemagglutination by E. coli Antiserum between Bacterial Cells and Supernate

E. coli O111 antiserum	Agglutination of red cells treated with *E. coli* O111 (suspension in physiological saline of organisms obtained from brain-veal-agar)				
	Suspension after 2 hrs. at 100°C.	Sediment of unheated suspension after 2 hrs. at 100°C.	Supernate of unheated suspension after 2 hrs. at 100°C.	Sediment of boiled suspension	Supernate of boiled suspension
1:100	4	4	—	3	4
1:200	4	4	—	2	4
1:400	4	3	—	1	4
1:800	3	3	—	—	3
1:1600	2	2	—	—	—
1:3200	1	1	—	—	—
0		—	—	—	—

— = no agglutination.
1 to 4 = various degrees of agglutination.

A suspension of *E. coli* O111 from brain-veal-agar in physiological saline solution was divided into two portions; one portion was separated in the refrigerated centrifuge into sediment and supernate, the other was first boiled for 2 hours and then centrifugalized. The sediments were resuspended in identical amounts of physiological saline solution. All unheated materials, which failed to modify red blood cells for hemagglutination, were boiled for 2 hours. These materials were then used together with the boiled, unseparated suspension for modification of human red blood cells. The results of this experiment are recorded in Table VII.

It can be noted that separation of the boiled suspension yields both active sediment and supernate, although the supernate is somewhat more effective in modifying red blood cells than the sediment. In contrast, when the unboiled suspension was separated and the two materials were boiled separately, the sediment was highly active and the supernate completely inactive. It is evident, then, that boiling of the bacterial suspension results in the appearance of the modifying antigen in the supernate, either because of activation of the antigen itself or because of elimination of an inhibitor. It is interesting to

note that in the hemagglutination and hemolysis inhibition tests, described below, it was possible to demonstrate antigen in the supernate obtained from unheated suspensions. This finding indicates that either the antigen is capable of adsorbing the antibody without being able to modify red blood cells or that

TABLE VIII

Hemagglutination and Hemolysis Inhibition Tests

E. coli O111 antiserum (1:100) mixed with *E. coli* O111 antigen		Agglutination and hemolysis of sheep blood cells modified by *E. coli* O111 antigen by O111 antiserum treated with serial dilutions of *E. coli* O111 antigen			
		Hemagglutination		Hemolysis	
		(*a*)	(*b*)	(*a*)	(*b*)
Unheated	1:10	—	—	—	—
	1:20	—	—	—	—
	1:40	—	—	—	—
	1:80	—	—	—	1
	1:160	—	—	2	3
	1:320	—	—	3	3
	1:640	1	2	3	4
	1:1280	1	2	3	4
	0	2	4	4	4
Heated	1:10	—	—	—	—
	1:20	—	—	—	—
	1:40	—	—	—	—
	1:80	—	—	—	—
	1:160	—	—	2	3
	1:320	—	—	4	4
	1:640	—	3	4	4
	1:1280	—	3	4	4
	0	2	4	4	4

— = no agglutination or hemolysis.
1 to 4 = various degrees of agglutination or hemolysis.
(*a*) = read after 30 minutes at 37°C.
(*b*) = read after 1 hour at 37°C.

two different antigens are involved in hemagglutination and hemagglutination inhibition tests. Identical results were obtained in repeated experiments using both *E. coli* O111 and O55 suspensions.

Hemagglutination and Hemolysis Inhibition Tests.—Experiments undertaken to determine whether the *E. coli* antigens under discussion inhibit hemagglutination and hemolysis yielded interesting results. A typical experiment is described herewith.

E. coli O111 antigen from brain-veal-agar in both the unheated and the boiled state in

serial dilutions (volume 0.1 ml.) was mixed with *E. coli* O111 antiserum in a dilution of 1:100 (volume 0.2 ml.). These mixtures were prepared in duplicate; one set for hemagglutination and the other for hemolysis; to each tube of the second set was added 0.1 ml. of a 1:10 diluted guinea pig serum (complement). All mixtures were kept for 30 minutes at 37°C. Then, to all tubes 0.2 ml. of 2½ per cent sheep cell suspension previously treated with *E. coli* O111 antigen (boiled agar suspension, diluted 1:5) was added. The resulting hemagglutination (read after incubation for 1 hour at 37°C. and centrifugalization) and hemolysis (read after 1 hour at 37°C.) are recorded in Table VIII.

Perusal of this table indicates that both the unheated and boiled *E. coli* antigens prevented hemagglutination and hemolysis. That the degree of hemolysis inhibition is of a lower order of magnitude than inhibition of hemagglutination is readily explained by the fact that *E. coli* antiserum is active in far higher dilutions in producing sheep cell hemolysis than hemagglutination. It is of considerable interest to point out that the unheated *E. coli* suspensions, which do not modify red blood cells for hemagglutination, are highly active in the hemagglutination and hemolysis inhibition tests, although somewhat less so than the boiled antigens. These results were confirmed in repeated experiments; with some antigens the boiled suspensions were considerably more active than the unheated ones.

Red Blood Cells Modified by E. coli Antigen as Immunizing Agent.—The antigens of *E. coli* serogroups O55 and O111 adsorbed on red blood cells act as antigens *in vivo*, as shown in the following experiment.

Six rabbits, whose sera in a dilution of 1:10 failed to agglutinate modified rabbit blood cells used in the hemagglutination test, were injected intravenously with 5 ml. each of a 2½ per cent suspension of rabbit red blood cells which had been treated with a 1:5 diluted, boiled brain veal agar suspension of *E. coli* O111 and O55, respectively. Three such injections were given 3 days apart and the rabbits were bled 8 days after the last injection.

The rabbits developed group-specific *E. coli* antibodies, which produced hemagglutination in titers of 1:160 and 1:320 of rabbit cells modified by the homologous antigen. Rabbit red cells treated with the heterologous antigen were not agglutinated in these dilutions of serum. The antibody response was of a low order; it is likely, however, that a larger number of injections may engender antibodies in higher titer. Essentially the same results were obtained in two additional rabbits following injection of rabbit blood cells modified by a boiled *E. coli* broth culture filtrate. The filtrate itself, following three intravenous injections, engendered agglutinins in high titer (1:1600 to 1:6400), as measured by the hemagglutination test.

DISCUSSION

It is an established fact that certain viruses and bacteria agglutinate red blood cells; this phenomenon may be referred to as direct bacterial or viral hemagglutination. Moreover, antigens of some microorganisms, including viruses, protozoa, and

bacteria, may be adsorbed onto red blood cells and thus render them agglutinable by the antimicrobial antibody, reactions, which may be referred to as indirect or conditioned (26) microbial hemagglutination and hemolysis.

Keogh, North, and Warburton (20, 21) were the first to demonstrate this indirect bacterial hemagglutination. Since then, the following species or types of bacteria have been found capable of modifying red blood cells for agglutination or hemolysis by antibacterial antibodies.

Cocci:—
- *Micrococcus pyogenes* (20, 24)
- *Streptococcus pyogenes* (20, 27)
- *Diplococcus pneumoniae* (20, 24)
- *Neisseria meningitidis* (20)

Bacilli:—
- *Hemophilus pertussis* (28, 29)
- *Hemophilus influenzae* type b (20, 30)
- salmonellae (20)
- *Salmonella typhosa* (Vi antigen) (31)
- *Shigella sonnei* (24)
- *Escherichia coli* (23, 24, 19)
- *Bacterium tularense* (25)
- *Pasteurella pestis* (32)
- *Corynebacterium diphtheriae* (mitis type) (24)
- *Mycobacterium tuberculosis* ((22) and others)

The experiments described in this communication revealed that the hemagglutination and hemolysis tests can be used successfully for the demonstration of antibodies active against *E. coli* O111 and O55 or the homologous antigens. The group-specific rabbit immune sera produced agglutination of red cells of a variety of animal species which had adsorbed the homologous *E. coli* antigen. The hemagglutinin titer of these antisera is of the same order of magnitude as that yielding bacterial agglutination. However, when sheep cells were treated with *E. coli* antigen, hemolysis occurred in the presence of complement and antiserum in titers far higher than those yielding agglutination of the bacteria or of the modified red blood cells. With these new procedures available it is now possible to reinvestigate the important question regarding the antibody response of infants suffering from diarrheal disease associated with the presence of these serogroups of *E. coli.* Such a study is of obvious interest, since in a fair number of such patients it was hitherto impossible by means of the bacterial agglutination test to demonstrate group-specific antibodies and since the finding of such antibodies in increasing titer during the course of the disease would add further evidence to the concept of the etiological role of these microorganisms in epidemic and sporadic diarrheal disease of infants. Indeed, preliminary observations indicate that it is possible with the aid of the indirect hemagglutination test to demonstrate antibodies

against *E. coli*, serogroups O111 and/or O55, in human sera, which fail to agglutinate the homologous bacterial suspensions (Neter and Gorzynski, unpublished data). It is now necessary to determine the titer of "normal" antibodies against *E. coli* O111 and O55 in human sera in relation to age, health, and past maladies of the individuals. It also remains to be determined whether the hemolysis test, which is extraordinarily sensitive with a system consisting of guinea pig complement and antibodies produced by rabbits, can be used or so modified as to be equally as sensitive for the demonstration of human antibodies. In this connection it may be noted that complement of different animal species is not uniformly effective in all hemolytic systems and that hemolysis depends, in addition to the specificity of antigen and antibody, upon the sources of red blood cells, complement, and antibody (*cf.* Rice (33)).

It has been shown in the experiments reported here that the antigens characteristic of *E. coli* O111 and O55 can be demonstrated by means of hemagglutination and hemolysis inhibition tests. These procedures may prove to be useful in studies on the isolation and purification of the group-specific *E. coli* antigens and in studies on the presence of soluble antigen in clinical material, such as fecal specimens.

Regarding the question of the types of antigens operative in hemagglutination, hemolysis, and hemagglutination and hemolysis inhibition tests, the following facts should be kept in mind. It was established by Kauffmann (1) that these serogroups of *E. coli* contain, in addition to flagellar H antigens, heatstable, somatic O antigens, and heat-labile, envelope B antigens. Serogroup O111 contains the B4 antigen and sero-group O55 the B5 antigen. The B antigens are responsible for O inagglutinability of unheated bacterial suspensions; boiling inactivates the B antigen and thus makes O agglutination possible. However, the antibody-binding property of the B antigens is thermostable. Thus far strains of *E. coli* O111 and O55 lacking the B antigen have not been found, and it has not yet been possible to prepare pure B4 and B5 antisera (1). All the experiments reported above gave identical results with antisera containing O and B antibodies (sera obtained from rabbits immunized with unheated suspensions of bacteria) as well as with antisera which presumably contained only O antibodies (sera of rabbits immunized with boiled suspensions of organisms or boiled broth culture filtrates). Therefore it may be tentatively concluded that the above described tests are based on a reaction between the O antigen and the homologous antibody. Furthermore, it was shown that injection into rabbits of rabbit red blood cells treated with boiled cultures of either *E. coli* O111 or O55 elicited the formation of group-specific *E. coli* antibodies; if, indeed, the B antigen is thermolabile, as stated by Kauffmann (1), the antibodies thus produced cannot be B antibodies. When pure B4 and B5 antisera become available, it will be possible to determine whether these antigens, too, can be demonstrated by means of hemaggluti-

nation and hemolysis tests. It is of interest to note that only boiled antigens were found to be highly active in modifying red blood cells for agglutination by the group-specific *E. coli* antisera. On the other hand, the unheated materials do contain antigen which specifically inhibits hemagglutination and hemolysis. It remains to be elucidated whether boiling renders the O antigen capable of adsorption on red blood cells through the inactivation of the B antigen or by means of other mechanisms.

SUMMARY

A study on hemagglutination and hemolysis by *Escherichia coli* O111 and O55 (rabbit) antisera and on hemagglutination and hemolysis inhibition by *E. coli* O111 and O55 antigens revealed the following facts.

1. Red blood cells of man, dog, rabbit, guinea pig, sheep, rat, and chicken adsorb *E. coli* O111 and O55 antigens and thus become specifically agglutinable by the homologous *E. coli* antisera.

2. The adsorption of these *E. coli* antigens is a function of the concentration of the antigen, the time (from 5 minutes to 2 hours) of treatment of the red blood cells with the antigen, and the concentration of the red blood cells used.

3. Red blood cells of man and sheep adsorb simultaneously both antigens, as indicated by the fact that both antisera give agglutination of all red blood cells. Complete agglutination does not occur when a mixture of red blood cells treated separately with the two antigens is added to one or the other of the two antisera.

4. Treatment of red blood cells of man with one of the antigens does not block the adsorption of the second antigen. Human cells treated with either or both antigens are still agglutinated by the homologous blood group (A, B, and Rh)-specific antibodies.

5. In the presence of guinea pig complement, *E. coli* O111 and O55 antisera produce hemolysis of modified human red blood cells in titers of the same order of magnitude as those giving hemagglutination and bacterial agglutination. The same antisera produce hemolysis of sheep cells treated with the identical antigens in titers exceeding by far those giving agglutination of modified human or sheep red blood cells.

6. Both sediment and supernate of a boiled *E. coli* suspension are capable of modifying red blood cells for *E. coli* hemagglutination; in contrast, the supernate obtained from an unboiled suspension and then heated does not modify red blood cells for hemagglutination, although it contains the antigen which can specifically adsorb *E. coli* antibodies, as shown by means of the hemagglutination and hemolysis inhibition tests.

7. Both the unheated and the boiled suspensions of *E. coli* O111 and O55 inhibit hemagglutination and hemolysis specifically.

8. Rabbit red blood cells modified by either *E. coli* O111 or O55 antigens, upon intravenous injection into rabbits, engender specific *E. coli* antibodies. The significance of the results is discussed.

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