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SEPARATION BY ETHANOL OF COMMON AND SOMATIC ANTIGENS OF ENTEROBACTERIACEAE

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ABSTRACT

SUZUKI, T. (Children's Hospital, Buffalo, N.Y.), E. A. GORZYNSKI, AND E. NETER. Separation by ethanol of common and somatic antigens of Enterobacteriaceae. J. Bacteriol. **88**:1240-1243. 1964.—Ethanol in a concentration of 85% permits separation from crude culture supernatant fluids of the common enterobacterial antigen and the O antigen, the former being ethanol-soluble, the latter being ethanol-insoluble. The evidence was obtained in hemagglutination, hemolysis, and hemagglutination-inhibition tests. In contrast to the crude antigens obtained from enteric bacteria other than *Escherichia coli* O14, the ethanol-soluble fraction is immunogenic in the rabbit upon intravenous injection, and antibodies against the common antigen are produced in relatively high titers. Aqueous mixtures of ethanol-soluble and -insoluble fractions engender antibodies against the common antigen in titers significantly lower than those stimulated by the soluble fraction alone. Ethanol-treated whole antigen fails to stimulate antibody formation against this antigen. These results suggest the presence of an inhibitor in the ethanol-insoluble fraction.

Several species of Enterobacteriaceae such as *Escherichia coli*, *Aerobacter aerogenes*, salmonellae, and shigellae produce, in addition to the well-known somatic or O antigen, another one common to all. This common antigen (CA) was first described by Kunin, Beard, and Halmagyi (1962). [This antigen was referred to previously as common hapten by Kunin et al. (1962) and as heterogenetic enterobacterial antigen (Kunin) by Whang and Neter (1962).] Kunin et al. (1962) considered the possibility that the CA determinant resides in the O antigen complex, which also functions as endotoxin. Subsequent studies by Whang and Neter (1962), however, failed to detect in highly purified lipopolysaccharides, with the exception of that from *Shigella sonnei*, more than a trace of this antigenic specificity. Lipopolysaccharides prepared by both phenol-water and aqueous-ether extraction methods were used. Attempts were made, therefore, to search for the common antigen in the discarded materials. Because the bacterial cells had been treated with ethanol and acetone for the preparation of some of these lipopolysaccharides, studies on the solubility of CA in these solvents were undertaken. It will be shown here that ethanol separates, although not completely, the common antigen from the O antigen, and that the ethanol-soluble fraction is far more immunogenic in the rabbit than in the crude material from which it is obtained.

MATERIALS AND METHODS

Smooth strains of various enteric bacteria were grown on brain veal agar (Difco) in Kolle flasks for 18 hr at 37 C. The resulting growth was suspended in phosphate Hemagglutination Buffer (pH 7.3; Difco). The suspension was heated in boiling water for 1 hr. The supernatant fluid, containing both somatic and common antigens, was obtained by centrifugation at 23,500 $\times$ *g* and was stored at −20 C.

These supernatant fluids were mixed with 95% ethanol in amounts to yield a final ethanol concentration of 85%. The mixture was kept at room temperature for 18 hr. For some of the experiments, this ethanol-extracted crude antigen was centrifuged at 23,500 $\times$ *g*, and the ethanol-soluble and -insoluble fractions were dried separately in open petri dishes in an incubator at 37 C. The powders thus obtained were scraped off the glass and dissolved in the original volume of, respectively, distilled water or phosphate buffer or as 1,000 μg/ml solutions, and are referred to as the ethanol-soluble and the ethanol-insoluble fractions. For additional experiments, crude antigen was treated with ethanol as described above, with the exception of centrifuga-

tion and consequent separation of the two fractions. The material thus obtained was dissolved in distilled water to the original concentration, and is referred to as the ethanol-treated antigen.

For the detection of the antigenic specificities, hemagglutination, hemolysis, and hemagglutination inhibition tests were employed. In the former, a 2.5% erythrocyte suspension was washed three times in large amounts of phosphate buffer; the antigen under study was added to the sediment to restore an erythrocyte concentration of 2.5%; the mixtures were incubated in a water bath at 37 C for 30 min; the red blood cells were again washed three times to remove excess antigen. Antiserum, containing antibodies against either common antigen or O antigen, in twofold serial dilutions (0.2 ml) was mixed with equal amounts of modified red blood cells. The mixtures were incubated in a water bath at 37 C for 30 min, and the resulting hemagglutination was read grossly after centrifugation at 1,300 × *g*. In the hemolysis test, 0.1 ml of guinea pig complement (1:20) (Carworth Laboratories, New City, N.Y.) was added to the mixtures of antiserum and modified cells. The resulting hemolysis was read grossly after incubation at 37 C for 30 min. In the hemagglutination-inhibition test, the antigen under investigation (0.2 ml) was added to antiserum of known specificity in twofold serial dilutions (0.2 ml). The mixtures were incubated in a water bath at 37 C for 30 min. Subsequently, appropriately modified erythrocytes (0.2 ml) were added. The resulting hemagglutination was read after incubation at 37 C for 30 min and centrifugation at 1,300 × *g*.

For the immunization of rabbits, antigen was injected intravenously at daily intervals for a series of five primary injections; after a resting period of 3 days, three additional intravenous injections were given. Blood specimens were obtained prior to, and at weekly intervals after, primary immunization, and the antibodies were titrated by means of the above-described hemagglutination test.

CA antiserum was prepared by intravenous immunization of rabbits with heat-killed suspensions of *E. coli* O14; O antisera were obtained from rabbits immunized with heated suspensions of enteric bacteria, or were procured on the open market from Lederle Laboratories, Pearl River N.Y.

CA specificity was proved when the serum of rabbits immunized with *E. coli* O14, for example, produced agglutination in high titer of erythrocytes treated with CA from *Salmonella typhimurium* or other enteric bacteria, and when this agglutination was inhibited specifically by CA obtained from an organism unrelated to those used for immunization or as indicator. O specificity was proved when hemagglutination was inhibited only by antigen obtained from strains of the identical O group, but not by antigens from unrelated bacteria.

RESULTS

In the first series of experiments, the ethanol-soluble and ethanol-insoluble fractions obtained from *S. typhimurium* and the corresponding crude supernatant fluid were tested by means of the hemagglutination test with CA and O antisera. The results of a representative experiment are shown in Table 1.

The erythrocytes modified by the ethanol-soluble fraction were agglutinated by CA antibodies, but not by O antibodies. In contrast, only O agglutination was obtained with red blood cells modified with the insoluble fraction. The original crude supernatant fluid modified red blood cells for agglutination by the two kinds of antibodies. Similar results were obtained with the two fractions prepared from *E. coli* O111 and

TABLE 1. *Hemagglutination of erythrocytes treated with ethanol-soluble and -insoluble fractions of Salmonella typhimurium**

Antiserum and dilution	Ethanol-soluble fraction (1,000 μg/ml)	Ethanol-insoluble fraction (1,000 μg/ml)	Crude antigen (1:10)
CA†			
1:200	4	—	3
1:400	3	—	3
1:800	2	—	1
1:1,600	1	—	—
O antigen			
1:200	—	3	3
1:400	—	3	3
1:800	—	1	1
1:1,600	—	—	—
0	—	—	—

* Various degrees of hemagglutination are indicated by numbers 1 to 4; a minus sign indicates no hemagglutination.

† Common antigen.

O55, *Salmonella choleraesuis* and *S. enteritidis*, and *Shigella flexneri*. Numerous experiments were carried out on the efficacy of ethanol in various final concentrations (50 to 90%); for near optimal results, 85% was selected.

Additional experiments revealed that the ethanol-soluble fraction of the above microorganisms modified sheep erythrocytes for lysis by CA antibodies and guinea pig complement. In accord with the above hemagglutination experiments, the ethanol-insoluble fraction proved to be operative only with O antibodies and complement.

Studies with these fractions were then carried out with the more sensitive hemagglutination-inhibition test. The results of a representative experiment are recorded in Table 2.

TABLE 2. *Hemagglutination-inhibitory capacity of ethanol-soluble and -insoluble fractions and of crude antigen of Salmonella typhimurium**

Antigen dilution	Ethanol-soluble fraction		Ethanol-insoluble fraction		Antigen dilution (reciprocal)	Crude antigen	
	CA†	O‡	CA	O		CA	O
μg							
2,000	—	—	3	—	1	—	—
1,000	—	2	3	—	2	—	—
500	—	3	3	—	4	—	—
250	—	3	3	—	8	—	—
125	—	3	3	—	16	—	—
62	—	3	3	—	32	2	—
30	2	3	4	—	64	3	—
15	3	3	3	—	128	3	—
7.5	3	3	3	—	256	3	—
3.5	3	3	3	—	512	3	—
1.7	3	3	3	1	1,024	3	1
0	3	3	3	3	0	3	3

* Various degrees of hemagglutination are indicated by numbers 1 to 4; a minus sign indicates no hemagglutination.

† CA, common antigen. For CA antigen titration, *Escherichia coli* O14 antiserum (1:500) and *E. coli* O86 modified erythrocytes.

‡ For O antigen titration, *Salmonella* group B antiserum (1:500) and *S. typhimurium* modified erythrocytes.

Perusal of the table indicates that the ethanol-soluble fraction contains relatively large amounts of CA and small amounts of O antigen. The ethanol-insoluble fraction, on the contrary, contains only O antigen. As expected, the crude antigen exhibits both antigenic specificities. Similar results were obtained from fractions obtained from the other above-mentioned enteric bacteria. From these results, then, it is evident that ethanol in a concentration of 85% separates, although not completely, CA from O antigen present in heated supernatant fluids of various enteric bacteria.

Although CA is present in various enteric bacteria, only *E. coli* O14 and a few other serogroups are immunogenic in the rabbit upon intravenous injection (Kunin et al., 1962). Recent studies indicated that this striking difference in immunogenicity is not due to the fact that cultures of *E. coli* O14 contain more CA than do the nonimmunizing strains, because it was shown that, when immunization is carried out with equivalent amounts of CA as determined in hemagglutination-inhibition tests, only *E. coli* O14 engenders CA antibodies (Neter et al., Immunology, *in press*). It was of interest, therefore, to determine whether ethanol-soluble fraction is immunogenic upon intravenous injection into rabbits. The results of a representative experiment with fractions from *E. coli* O111 are presented in Table 3.

TABLE 3. *Antibody response of rabbit to ethanol-soluble and -insoluble fractions of Escherichia coli O111*

Antigen used for immunization	Common antigen		O antigen	
	Preimmune	Postimmune	Preimmune	Postimmune
Crude	13*	45	<10	3,840
Ethanol-soluble fraction	10	1,920	<10	66
Ethanol-insoluble fraction	10	<10	<10	1,280
Mixture of ethanol-soluble and -insoluble fractions	16	133	<10	2,133
Ethanol-treated crude antigen	<10	13	<10	1,706

* Figures indicate mean antibody titer (reciprocal).

Only a minimal CA antibody response followed the injection of the crude antigen, although O antibodies in high titer were elicited. In contrast, the ethanol-soluble fraction engendered CA antibodies in substantial titers, and the O antibody response was slight. The ethanol-insoluble fraction failed to stimulate the formation of CA antibodies, but did engender O antibodies in substantial titer. It is of particular interest to point out that the ethanol-treated whole supernatant fluid failed to stimulate the production of CA antibodies, and that injection of the aqueous mixture of the ethanol-soluble and -insoluble fractions led to the formation of CA antibodies in titers significantly lower than those stimulated by the administration of the ethanol-soluble fraction alone (Table 3). Additional experiments revealed that, when the two fractions were mixed in 85% ethanol instead of phosphate buffer, the resulting material redissolved in buffer failed to elicit CA antibody formation. These experiments suggest that crude supernatant fluids of enteric bacteria grown on Brain Veal Agar contain an inhibitor that interferes with CA antibody formation. Experiments are now in progress to determine whether it is the O antigen (lipopolysaccharide) itself that is responsible for this surprising effect.

DISCUSSION

The present investigation has revealed that ethanol in a concentration of 85% separates, although not completely, the common enterobacterial antigen (CA) described by Kunin et al. (1962) from the well-known somatic or O antigen of enteric bacteria other than *E. coli* O14. This conclusion is based on the results of hemagglutination, hemolysis, and hemagglutination-inhibition tests. Of particular interest is the observation that, in contrast to the crude supernatant fluids, the ethanol-soluble fraction obtained therefrom stimulates CA antibody formation in the rabbit upon intravenous injection. It is also evident from the results presented here that the crude supernatant fluid contains a substance or substances which interfere with CA antibody formation, because it was shown that ethanol treatment of the supernatant fluids without separation of the soluble and insoluble fractions fails to render the material immunogenic. Immunogenicity is maintained when the ethanol-soluble fraction is treated with ethanol a second time and is then dissolved in water. Aqueous mixtures of the ethanol-soluble and -insoluble fractions stimulate CA antibody formation to a lesser degree than does the ethanol-soluble fraction alone. It may be assumed that a reaction takes place between molecules with CA specificity in the ethanol-soluble fraction and the inhibitor present in the insoluble fraction, and that this reaction takes place more effectively in ethanol than in aqueous solution. It may be assumed, also, that crude culture supernatant fluids from enteric bacteria other than *E. coli* O14 contain CA-inhibitor complexes. Such an assumption is different from the theory proposed by Kunin et al. (1962), who postulated that CA is present as a hapten in bacteria other than *E. coli* O14. The question presents itself whether it is the O antigen (lipopolysaccharide) that acts as inhibitor. The identity of the inhibitor and its mode of action, as well as the differences of the molecules with CA specificity in immunogenic *E. coli* O14 and in other nonimmunogenic enteric bacteria, remain to be elucidated.

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