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Inhibition by Lipopolysaccharide (Endotoxin) of Antibody Response of Rabbit to Common Antigen of Enterobacteriaceae.* (29698)

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An antigen common to numerous, though not all, enteric, gram-negative bacilli was discovered by Kunin *et al*(1) by means of the hemagglutination test with *Escherichia coli* O14 antiserum. This common antigen (CA) readily attaches to erythrocytes, and the modified red blood cells are then specifically agglutinated in the presence of the corresponding antibody(1,2). Surprisingly, the antibody does not cause bacterial agglutination, although the common antigen is present on the surface of the cells, nor does it produce precipitation of soluble antigen, obtained from heated cultures. Antibodies against CA are readily engendered after intravenous injection of *E. coli* O14, but numerous other serogroups of *E. coli* and many species of enteric bacteria are not immunogenic(1). It was shown recently that injection of the antigen from bacteria other than *E. coli* O14, together with Freund's adjuvant, into the footpad of rabbits stimulates antibodies against CA in high titer(3). In contrast to the soluble antigen, CA attached *in vitro* to autologous or heterologous erythrocytes, L cells, or to lymphocytes from patients with leukemia, upon intravenous injection into rabbits, also elicits antibody formation(4,5). Suzuki *et al*(6) recently observed that CA from enteric bacteria other than *E. coli* O14 is soluble in 85% ethanol and that intravenous injection of this fraction readily engenders CA antibodies in the rabbit. Furthermore, injection of a mixture of the ethanol soluble and insoluble fractions results in a decreased or absent CA antibody formation(6). These observations suggested the

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possibility that it is the O antigen (endotoxin), present in the insoluble fraction, that interferes with the formation of CA antibodies. The experiments reported here have revealed that highly purified lipopolysaccharides, when injected together with CA, interfere with the CA antibody response of the rabbit.

Materials and methods. Smooth strains of *S. typhimurium, S. enteritidis,* and *E. coli* O111, O86 and O14 were grown on brain veal agar in Kolle flasks for 18 hours at 37°C. The bacteria were suspended in 25 ml per flask of hemagglutination buffer (pH 7.3; Difco). The suspension was heated in boiling water for 1 hour and centrifuged at 23,500 *g* for 20 minutes. The supernate was then mixed with ethanol to a final ethanol concentration of 85%; the mixture was kept at room temperature for 18 hours and then centrifuged at 23,500 *g*. The supernate was evaporated at 37°C and redissolved in distilled water to the original volume. This ethanol soluble fraction contains most of the CA and only small amounts of O antigen. Highly purified lipopolysaccharides from *S. miami* and *S. isangi* and lipoid A from *E. coli* O111 were kindly supplied by Professor O. Westphal, Max-Planck Institute, Freiburg, Germany. The lipopolysaccharides were dissolved in phosphate buffer in a concentration of 1,000 μg/ml. For immunization of rabbit mixtures of CA and lipopolysaccharides were prepared in buffer (referred to as aqueous mixture). For additional experiments, equal amounts of CA (undiluted ethanol soluble fraction) and lipopolysaccharides were dissolved in 95% ethanol to a final ethanol concentration of 85%; the mixture was kept at 22°C for 18 hours; without prior centrifugation, ethanol was evaporated at 37°C and the dried material was redissolved in distilled water to the original volume (referred to as ethanol mixture).

Groups of 3 to 4 albino rabbits, weighing between 2 and 3 kg, were injected intravenously with 1 ml amounts daily; number of injections and amounts of CA and lipopolysaccharides are given below.

Blood was obtained prior to, and at weekly intervals during and after, immunization. Serum specimens were kept frozen at —20°C. Antibodies against common antigen and O antigens were titrated by means of the hemagglutination and hemagglutination inhibition tests, as described previously(6). Briefly, serum in 2-fold serial dilutions (0.2 ml) was mixed with rabbit erythrocytes (0.2 ml) that had been modified with antigen from an enteric microorganism (*e.g. E. coli* O86) unrelated to that used for immunization (*e.g. S. typhimurium*). After incubation at 37°C and centrifugation at 1300 *g* for 2 minutes, the resulting hemagglutination was read grossly. The specificity was documented by inhibition of hemagglutination by antigen from a third microorganism (*e.g. E. coli* O14). All of these microorganisms produce CA but different O antigens. Antibodies against O antigen were titrated by means of hemagglutination tests with erythrocytes modified with the corresponding O antigen, and the specificity was ascertained by the failure of an unrelated O antigen to inhibit the reaction.

Results. In the first series of experiments the effect of highly purified *S. miami* (group D) lipopolysaccharide on the antibody response of rabbits to common antigen (CA) from *S. enteritidis* (group D) was investigated. Groups of 3 rabbits were immunized by 8 daily injections of 1 ml each of either ethanol soluble fraction, or of mixtures of ethanol soluble fraction and lipopolysaccharide in 2 different concentrations, or of mixture of ethanol soluble and insoluble fractions. The dilutions of ethanol soluble and insoluble fractions and number of injections were as follows: 1:50, twice; 1:20, twice; and 1:10, 4 times. The corresponding amounts of lipopolysaccharide were 12.5 μg or 1.25 μg (twice); 31.6 or 3.1 μg (twice); and 63 or 6.3 μg, respectively (4 times). The total amounts were 0.54 ml of ethanol soluble or insoluble fraction, 336 μg and 33.6 μg, respectively, of lipopolysaccharide. The results are presented in Table I.

It can be seen that *S. miami* in amounts of 336 μg when injected together with CA almost completely prevented the formation of antibodies against the latter, and, when used in smaller amounts, interfered with the early CA antibody response. In accord with previ-

TABLE I. Influence of *S. miami* Lipopolysaccharide and of *S. enteritidis* Ethanol Insoluble Fraction on CA Antibody Response of Rabbits to *S. enteritidis* Ethanol Soluble Fraction.

Materials for immunization	Mean CA antibody titers (reciprocal)				
	A	B	C	D	E
S. enteritidis ethanol soluble fraction	<10	320	1066	213	120
Mixture of *S. enteritidis* ethanol soluble fraction and *S. miami* lipopolysaccharide (336 μg)	<10	<10	20	20	20
Mixture of *S. enteritidis* ethanol soluble fraction and *S. miami* lipopolysaccharide (33 μg)	<10	33	640	106	60
Mixture of *S. enteritidis* ethanol soluble fraction and *S. enteritidis* ethanol insoluble fraction	<10	<10	60	16	13

A = Prior to immunization. B-E = Weekly intervals after initiation of immunization.

ous observations, the ethanol insoluble fraction also depressed the formation of CA antibodies. Similar results were obtained in additional experiments.

In view of the facts that the crude soluble antigen from enteric bacteria other than the *E. coli* O14 fails to engender CA antibodies upon intravenous injection into rabbits and that the removal of the ethanol insoluble fraction renders the soluble fraction immunogenic, it was postulated that a complex of CA and lipopolysaccharide may be present in the supernate of bacterial suspensions. With these considerations in mind, the immunizing capacity of mixtures of CA and lipopolysaccharide prepared in water or ethanol was compared. It should be emphasized that, as mentioned above, ethanol was removed from the mixture and the material redissolved in water for injection. Also, in this experiment lipopolysaccharide and CA were obtained from antigenically unrelated serogroups. Groups of 3 to 4 rabbits were given 12 daily intravenous injections of 1 ml each of either ethanol soluble fraction, or of aqueous or ethanol mixtures of ethanol soluble fraction and lipopolysaccharide, or of lipopolysaccharide alone. The dilutions and number of injections of the ethanol soluble fraction were as follows: 1:125, 5 times; 1:25, once; 1:10, twice; and 1:5, 4 times. The corresponding amounts of lipopolysaccharide were 5 μg (5 times), 25 μg (once), 62 μg (twice), and 125 μg (4 times). Total amounts of 0.8 ml of CA from *S. typhimurium* (group B) and 675 μg of *S. isangi* (group C) lipopolysaccharide were injected. The results are summarized in Table II. Lipopolysaccharide interfered with CA antibody formation, and this effect was greater with the mixture of CA and lipopolysaccharide prepared in ethanol rather than in water. The lipopolysaccharide itself failed to stimulate CA antibodies. Control experiments revealed that a second ethanol treatment of CA does not materially affect its immunogenic potency. The moderate or poor antibody response to the O antigen of group B *Salmonella* is due to the small amounts of this antigen in the ethanol soluble fraction. In additional experiments it was shown that *S. miami* lipopolysaccharide in total amounts as small as 35 μg given in 8 to 11 injections together with CA interfered with CA antibody formation, when the mixtures were prepared in ethanol rather than in water.

Experiments were then carried out to determine whether lipopolysaccharide also inhibits CA antibody formation when injected separately from, but simultaneously with, CA into different veins. Repeated experiments using lipopolysaccharides in total doses ranging from 15 μg to 360 μg for 5 to 11 injections have failed to show an unequivocal inhibitory effect. It is conceivable, however, that other schedules and doses may be effective, particularly since separate injection of ethanol soluble and insoluble fractions resulted in lower CA antibody titers than injection of the former alone.

The above described effect of lipopolysaccharides on antibody formation against CA would be readily explained if the antigenic determinant of CA were altered. Therefore, the hemagglutinin neutralizing capacity of CA and of mixtures of CA and lipopolysaccharides was determined. Lipopolysaccha-

TABLE II. Effect of *S. isangi* Lipopolysaccharide on Antibody Response to Ethanol Soluble Common Antigen of *S. typhimurium*.

	Serum specimens*									
	A		B		C		D		E	
	Mean antibody titers (reciprocal) †									
Materials for immunization	CA	O	CA	O	CA	O	CA	O	CA	O
S. typhimurium ethanol soluble fraction	<10	<10	173	60	900	107	1280	133	533	93
Aqueous mixture of										
S. typhimurium ethanol soluble fraction and *S. isangi* lipopolysaccharide	10	<10	30	27	87	93	140	93	67	67
Ethanol mixture of										
S. typhimurium ethanol soluble fraction and *S. isangi* lipopolysaccharide	<10	<10	13	<10	40	20	13	25	<10	23
S. isangi lipopolysaccharide	<10	<10	<10	<10	<10	<10	<10	<10	<10	<10

* A = Prior to immunization. B-E = Weekly intervals after initiation of immunization.
† CA = Common antigen. O = Salmonella group B antigen.

rides in concentrations ranging from 62 to 500 μg/ml did not materially influence the reaction.

The question arises whether it is the lipopolysaccharide macromolecule or a component thereof that is responsible for the observed depressing effect on antibody formation. Preliminary experiments, with groups of 3 to 4 rabbits each, have revealed that ethanol mixture of CA from *S. typhimurium* and lipoid A from *E. coli* O111 (total amounts of 0.7 ml of the former and 500 μg of the latter), when administered by 5 daily intravenous injections, failed to elicit CA antibody formation, whereas immunization with CA alone resulted in a rise in titer from less than 1:10 to 1:560. Only minimal or questionable inhibition occurred when aqueous mixtures of lipoid A and CA were used for immunization. Similar results were obtained in an additional experiment in which 3 daily injections were given for a total of 0.3 ml of CA and 300 μg of lipoid A. Further studies with additional lipoid A preparations and with other fractions of lipopolysaccharides are needed.

Discussion. The present investigation has revealed that highly purified lipopolysaccharides (endotoxins) interfere with the antibody formation of the rabbit to the common antigen (CA) first described by Kunin *et al*(1). This observation is all the more surprising since endotoxins act as adjuvants, enhancing antibody formation (cf. 7). While the adjuvant effect of lipopolysaccharides is indirect in nature, affecting the host rather than the antigen used for immunization, the effects of these endotoxins on the CA antibody response may be due to their interaction with the antigen. This assumption is supported by the observation that, when mixtures of lipopolysaccharide and CA are prepared in ethanol and the ethanol later replaced by water, the inhibitory effect of lipopolysaccharide is even more marked than that in aqueous mixture, and that separate injections of CA and lipopolysaccharide resulted in undiminished or questionably depressed CA antibody formation. Interaction of lipopolysaccharides with CA would not be without parallel, for the former do react with certain serum proteins, lecithin, and some antibiotics, such as polymyxin B. With these latter materials, complexes presumably are formed, and the lipopolysaccharides are no longer attachable to erythrocytes, although the antibody combining capacity remains unimpaired; certain complexes, too, are less toxic(8-11). Clarification of the possible reaction between CA and lipopolysaccharides must await purification of fully immunogenic CA.

Since *E. coli* O14, in contrast to most other gram-negative bacteria containing CA, is immunogenic in the rabbit, it is conceivable that CA in the former is not bound to lipopolysaccharide or that the CA determinant is

part of a different molecule. Unpublished observations, indeed, indicate that there is a difference in CA from *E. coli* O14 and from other bacteria, for CA from the former is largely insoluble and that from the latter soluble in 85% ethanol.

The present observations of the inhibitory effect of lipopolysaccharides, and probably of lipoid A as well, on the CA antibody response of the rabbit appear to explain the poor immunogenicity of CA present in the bacterial cells and in crude supernates from organisms other than *E. coli* O14. Moreover, eventual elucidation of the mode of action of lipopolysaccharides as inhibitors of the CA antibody response may throw light on basic mechanisms of antibody formation.

Summary. A common antigen (CA) is present in many species and serogroups of enteric bacteria, but only the antigen from *E. coli* O14 engenders CA antibodies in the rabbit upon intravenous injection. The ethanol soluble fraction of the former, however, induces the formation of CA antibodies. The present study has revealed that lipopolysaccharides (endotoxins) inhibit this CA antibody response. This inhibition was observed regularly when CA and lipopolysaccharides were injected as mixtures, but not when administered separately, although simultaneously, into different veins. Injection of mixtures prepared in ethanol were even less immunogenic than those prepared in water. A single preparation of lipoid A also inhibited the CA antibody response. It is postulated that the poor immunogenicity of enteric bacteria, other than *E. coli* O14, is due to the inhibitory effect of the lipopolysaccharide on the fully immunogenic CA.

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Salivary Gland Atrophy in Rat Induced by Liquid Diet.* (29699)

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The role of the nervous system in maintenance of structure and function of innervated organs is not completely understood. It is known, particularly from investigation of skeletal muscle(1) and salivary glands(2), that denervation markedly affects the morphological and physiological status of these organs, causing atrophy. It is not, however, clear to what extent these changes can be attributed to reduced activity of the organ as distinct from trophic influences unrelated to activity. The present data suggest that the innervation influences structure and function of the salivary gland largely by control of organ activity. It has been found that substitution of liquid ration for solid lab chow caused marked atrophy of the salivary glands of rat. Since these atrophic changes occurred when the innervation to the glands was in-

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