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Strain-Related Differences in Immunosuppressive Effects of *Enterobacteriaceae* and Their Lipopolysaccharides on Production in Rabbits of Antibody to Enterobacterial Common Antigen

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Certain polysaccharides have been shown to inhibit the antibody response of rabbits to the common enterobacterial antigen (CA). The present investigation revealed that striking differences exist in the immunosuppressive effects of enteric bacteria and their lipopolysaccharides (LPS), depending upon CA production by the strains. Mixtures of immunogenic strains (*Escherichia coli* F2378 [R4], *E. coli* F470 [R1], or *Shigella boydii* F3140 [R1]) and non-immunogenic CA-producing strains, such as *E. coli* O1, *E. coli* O113, *Salmonella montevideo*, and *S. minnesota*, as well as the R mutants *E. coli* F614 (R1), *E. coli* F757 (R1), and *S. typhimurium his* 642 (Ra), failed to elicit CA antibodies. In contrast, mixtures of the immunogen and CA-negative strains *S. typhimurium his* 386 (Ra) and *S. minnesota* P595 (Re) or R555 (Ra) yielded CA antibodies in titers similar to those elicited by the immunogen alone. Further, LPS of CA-positive but not of CA-negative strains exerted this immunosuppressive effect. Quantitative studies revealed that LPS of *S. minnesota* in amounts of 100 μg/ml was strongly immunosuppressive, in amounts of 20 μg/ml slightly effective, and in amounts of 4 μg/ml ineffective. It is postulated that hitherto unknown differences exist, either in composition or in configuration, between LPS obtained from different microorganisms to account for the strain-related differences in immunosuppressive effects and, further, that the immunosuppressive LPS interacts with immunogenic CA.

After the detection of the common enterobacterial antigen (CA) by Kunin et al. (4), it was found that only a few enterobacterial strains readily engender CA antibody production in rabbits on intravenous injection of heat-killed bacterial suspensions. In particular, *Escherichia coli* O14, which was recently characterized as an encapsulated R mutant (17), and certain other R mutants of *E. coli* and *Shigella* (9, 22) elicit CA antibodies in high titers. In contrast, smooth strains of *Enterobacteriaceae* fail to engender CA antibodies in appreciable amounts, although they contain the CA determinant and are able to specifically prime the animals for an accelerated immune response (13). (For the sake of brevity, this form of CA is referred to as "non-immunogenic" CA.) However, CA preparations isolated from these strains and separated from LPS by treatment with 85% aqueous ethanol are highly immunogenic (19). Recombination of CA and lipopolysaccharide (LPS) in turn markedly reduces CA immunogenicity, provided the two materials are mixed in vitro before intravenous injection (19). It has been assumed that LPS interacts with CA and possibly causes its deaggregation (21).

Recently, CA-negative mutants of *Salmonella* became available (7, 8), and it was deemed of interest to learn whether LPS of these mutants likewise suppress CA antibody formation. The experiments described here compare the immunosuppressive effects of whole cells and of isolated LPS preparations from both CA-positive and CA-negative enterobacterial strains. In addition, strains belonging to families other than the family *Enterobacteriaceae* were included in the study.

MATERIALS AND METHODS

The smooth (S) strains and the rough (R) mutants of enteric microorganisms were from the collection of the Max-Planck-Institut für Immunbiologie, Freiburg, Germany; other strains were obtained from the Center for Disease Control, Atlanta, Ga. They are listed in Table 1 together with appropriate references. They are grouped in three categories: strains producing immunogenic CA, strains producing non-

TABLE 1. *Smooth (S) and rough (R) strains used in the study: their S or R type and their respective LPS mutation site (rf gene loci)*

Serotype	Strain no.	S or R type	Mutation at *rf* site	Reference
Strains producing immunogenic CA				
Escherichia coli O14:K7	F2378	*coli* R4	*rfb*	17
E. coli O8⁻:K27⁻	F470	*coli* R1	*rfb*	16
Shigella boydii type 3⁻	F3140	*coli* R1	*rfb*	10
Strains producing non-immunogenic CA				
E. coli O1		S		
E. coli O113		S		
E. coli O8⁻:K27⁻	F614	*coli* R1	*rfaL*	9
E. coli O9⁻:K31⁻	F757	*coli* R1	*rfaL*	
Salmonella minnesota	S1114	S		8
S. montevideo	SH94	S		7
Arizona O9a⁻:9c⁻	F628	Ra	*rfb*	
S. typhimurium	*his* 642	Ra	*rfb*^{del}	14
Aeromonas shigelloides	CDC 1383-69	S		20
Yersinia enterocolitica	CDC 495-70	S		
S. minnesota	R1000	Re	*rfa*	5
Strains failing to produce CA				
S. minnesota	R595	Re	*rfb*, *rfe*	5, 7
S. minnesota	R555	Ra/Rb	*rfa*(?), *rfe*	5
S. typhimurium	*his* 386	Ra	*rfb*^{del}, *rfe*	14
S. typhimurium	*his* 1448	Ra	*rfb*^{del}, *rfe*	14
Aeromonas hydrophila	CDC 3331-68	S		20

immunogenic CA, and strains that fail to produce CA.

In addition to the above microorganisms, nonenteric bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, which fail to produce CA, were obtained from the collection of Children's Hospital, Buffalo, N.Y., and included in the study.

The strains were grown in Kolle flasks on brain veal agar for 18 h at 37 C. The resulting growth was suspended in 25 ml of phosphate hemagglutination buffer (Difco; pH 7.3) per flask.

LPS obtained from some of the above strains were prepared by the phenol-water method or by the phenol-chloroform-petroleum ether method of Galanos et al. (3) unless indicated otherwise. An additional phenol-water preparation from *E. coli* O113 was kindly supplied by Jon A. Rudbach, University of Montana, Missoula. These preparations were dissolved in phosphate hemagglutination buffer at a concentration of 1,000 μg/ml and stored at −20 C until used.

Demonstration of CA produced in vitro. For the demonstration of CA produced in vitro by the strains under investigation, the suspension, grown on agar and suspended in phosphate buffer, was heated for 1 h at 100 C. The supernatant obtained after centrifugation at 23,500 × *g* for 20 min was used in the previously described hemagglutination, hemolysis, and hemagglutination inhibition tests (23, 24). Briefly, washed rabbit erythrocytes (2.5% suspension) were mixed with the supernatant, incubated at 37 C for 30 min, and washed to remove excess antigen. CA antiserum, obtained by immunization of rabbits with *E. coli* O14, was mixed in twofold serial dilutions (0.2 ml) with equal amounts of the antigenically modified erythrocytes. The mixtures were incubated for 30 min at 37 C, and the resulting hemagglutination was read after centrifugation at 1,300 × *g* for 2 min. In the passive hemolysis test, 0.1 ml of 1:20 diluted guinea pig complement (Carworth Laboratories, Inc., New York City, N.Y.) was added to the mixture of antiserum and antigenically modified sheep erythrocytes. The resulting hemolysis was read grossly after incubation at 37 C for 30 min. Absence of agglutination and of hemolysis by a high-titered (1:1,280 to 1:5,120) CA antiserum was considered evidence for the absence of CA. The presence of the CA determinant in supernatants of heated bacterial suspensions was further confirmed by a hemagglutination inhibition test, as described previously (24). Briefly, the supernatant fluid (0.2 ml) was added to 0.2 ml of 2 hemagglutinin units of CA antiserum. The mixtures were incubated in a water bath at 37 C for 30 min. Erythrocytes modified with CA of *Salmonella typhimurium* (0.2 ml) were then added. The mixtures were reincubated at 37 C for another 30 min, and the resulting hemagglutination was read grossly after centrifugation at 1,300 × *g* for 2 min. Hemagglutination inhibition was considered evidence for the presence of the CA determinant.

Immunogenicity of CA. To determine the immunosupppressive effect of enteric bacteria or the corresponding LPS, the following immunization experiments were carried out. One part of the suspension, containing about 10^9 cells/ml of enteric bacteria producing immunogenic CA, either undiluted or in a dilution of 1:10, was mixed with nine parts of the undiluted bacterial suspension of organisms that either produce nonimmunogenic CA or fail to syn-

thesize this antigenic determinant. In additional experiments, one part of the suspensions of immunogenic strains was mixed with nine parts of LPS in amounts given below. Unless specified otherwise, the mixtures were heated for 1 h at 100 C and then stored at 4 C until used. Each component alone, in equivalent concentration, was used for control purposes. For immunization, the materials were diluted 1:100 and injected intravenously in 1-ml amounts into albino rabbits weighing between 2 and 3 kg. Injections were given on days 0, 3, 7, and 10. Serum specimens for antibody titration were obtained on days 0, 7, 10, and 14. To determine whether immunological priming resulted from immunization, animals that had failed to respond with a significant increase in CA antibody titers were given a single intravenous booster injection of minimally effective immunogenic CA (1 ml of the ethanol-soluble fraction of *S. typhimurium*) on day 14, as described previously (13). For control purposes, nonimmunized rabbits were given the same amount of antigen, and other immunized rabbits without the booster injection were followed in parallel. Sera were obtained 3, 4 and 7 days after the booster injection. The above-described passive hemagglutination was used to titrate CA antibodies. To this end, the serum was titrated in serial twofold dilutions ranging from 1:10 to 1:5,120; rabbit erythrocytes modified with ethanol-soluble CA obtained from *S. typhimurium* were used as indicator antigen. The mean CA antibody titers of the sera of three rabbits per group are recorded.

RESULTS

In the first series of experiments, a comparison was made of the immunosuppressive activity of strains producing CA and others that do not produce this antigen and certain corresponding LPS (100 μg/ml) (Table 2). As immunogen, the immunogenic F3140 (R1) strain of *S. boydii* was used.

It is evident that CA antibody production was inhibited by all CA-producing strains and their LPS but not by the CA-negative strains and their LPS preparations. Nor did the cultures of nonenteric organisms, such as *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Aeromonas hydrophila* (20), which do not synthesize CA, inhibit the antibody response. Essentially identical results were obtained with two other highly immunogenic strains as immunogen, namely, *E. coli* F2378 (R4) and *E. coli* F470 (R1). Experiments not included in Table 2 revealed that all other non-immunogenic, CA-positive strains listed in Table 1 were immunosuppressive, in contrast to the CA-negative strains. These results agree with those summarized in Table 2.

In a second series of experiments, the quantitative aspects of the immunosuppressive effects of LPS were investigated. Using the R1 strain of *Shigella boydii* F3140 as immunogen, LPS from *Salmonella minnesota* (S1114), when used in amounts of 100 μg/ml, completely inhibited CA antibody production; when used in amounts of 20 μg/ml only slight inhibition was observed; and with 4 μg/ml none was observed (Table 3). Table 3 also shows that LPS (100 μg/ml) obtained from the CA-negative mutant *S. minnesota* R595 failed to exert immunosuppressive effects. Neither of the LPS preparations alone induced CA antibody production.

In additional experiments, it was shown that the presence of the CA determinant co-extracted with LPS was not the responsible factor for the observed immunosuppression. Two LPS preparations from *Salmonella montevideo* SH94 were used; one, CA positive, was obtained by the phenol-water extraction procedure and the other, CA negative, was obtained by phenol-water extraction followed by the Galanos method (3). These two LPS preparations, in amounts of 100 μg/ml, inhibited completely the CA antibody response to the highly immunogenic strain *S. boydii* F3140 (R1). These findings, together with the previous results dealing with LPS from smooth as well as rough strains of enteric bacteria, strongly suggest that the major inhibitory substance responsible for the observed immunosuppressive effects is not part of CA or the O antigenic determinants; rather, it is part of the LPS core and possibly of its lipid A moiety.

The question then arises whether or not, in the experiments using suspensions of immunogenic and inhibitory strains, the suppression of immunogenicity is due to cell-to-cell contact. Since LPS can serve as an inhibitor of the CA antibody response, it is conceivable that LPS released from the inhibitory cells may account, at least in part, for the immunosuppressive effect. So far as the immunogenic CA is concerned, experiments with washed cells have revealed that the cells themselves engender CA antibodies. In one such experiment, cells of the R1 mutant of *E. coli* O8 (F470) washed twice induced CA antibodies in a titer of 1:2,560 on day 14 of immunization, and the unwashed suspension induced antibodies in a titer of 1:1,280. It is important to note that the last wash fluid did not engender CA antibodies. Hence, it appears that the CA immunogen was located on the bacterial cell (LPS-CA complex, 11). Additional experiments showed that, when washed cells, consisting of the R1 mutant F470 and the smooth strain *E. coli* O1, were used for immunization, antibody formation was almost completely suppressed, the maximum titer being 1:27 versus 1:2,560 for the control. The precise mode of action of LPS and of other inhibitors on the CA determinant present on the surface of

TABLE 2. *Immunosuppressive effect of CA-producing and non-CA-producing microorganisms*[a]

Inhibitors		CA production by HA, HL, HI[c]	Mean CA hemagglutinin titers (reciprocal)			
Microorganism	LPS[b] or culture		0[d]	7[e]	10[e]	14[e]
Control			<10	20	240	320
Enteric bacteria						
Escherichia coli O113	Culture	+	<10	<10	<10	<10
	LPS		<10	<10	<10	<10
Salmonella montevideo (SH94)	Culture	+	<10	<10	<10	<10
	LPS		<10	<10	<10	<10
S. typhimurium (*his* 642)	Culture	+	<10	<10	<10	13
	LPS		<10	<10	<10	<10
S. typhimurium (*his* 386)	Culture	−	<10	23	160	320
	LPS		<10	30	160	320
S. minnesota (R595)	Culture	−	<10	27	213	320
	LPS		<10	27	133	320
Nonenteric bacteria						
Pseudomonas aeruginosa	Culture	−	<10	33	160	320
Staphylococcus aureus	Culture	−	<10	20	160	320
Aeromonas hydrophila	Culture	−	<10	27	160	320

[a] The immunogen used in all experiments was *Shigella boydii* type 3⁻ (F3140-R1). Indicator antigen was CA *(S. typhimurium)*.
[b] LPS, Purified lipopolysaccharide (phenol-water extract).
[c] HA, Passive hemagglutination test; HL, passive hemolysis test; HI, hemagglutination inhibition test.
[d] Day pre-immunization.
[e] Day post-immunization.

TABLE 3. *Effect of LPS obtained from CA-producing or non-CA-producing strains of S. minnesota (S1114 or R595) on immune response of rabbits to CA*[a]

Immunogen	Inhibitor (LPS)[b] (amt in μg/ml)	Mean CA hemagglutinin titers (reciprocal)			
		0[c]	7[d]	10[d]	14[d]
S. boydii type 3⁻ (F3140-R1)	Buffer	<10	47	160	320
	S. minnesota (S)				
	(S1114) LPS (CA+) (100)	<10	<10	<10	<10
	(S1114) LPS (CA+) (20)	<10	27	93	133
	(S1114) LPS (CA+) (4)	<10	40	133	267
	S. minnesota (R)				
	(R595) LPS (CA−) (100)	<10	40	160	320
Buffer	*S. minnesota* (S)				
	(S1114) LPS (CA+) (100)	<10	<10	<10	<10
	S. minnesota (R)				
	(R595) LPS (CA−) (100)	<10	<10	<10	<10

[a] Indicator antigen was CA *(S. typhimurium)*.
[b] S, Smooth; R, rough; LPS, purified lipopolysaccharide (phenol-water extract).
[c] Day pre-immunization.
[d] Day post-immunization.

the bacterial cell remains to be elucidated.

In accord with previous observations (13), immunosuppression was observed after injection of the mixture of CA immunogen and inhibitor only if the two components were mixed in vitro before injection and not when they were injected separately, albeit simultaneously. In a representative experiment the results were as follows: the R1 mutant of *Shigella boydii* F3140 alone engendered CA antibodies on day 14 in a titer of 1:370; when the mixture of the immunogen and *Salmonella minnesota* R1000 (Re) was used, the antibody titer was <1:10; and when the two components were injected separately but simultaneously, the antibody titer was 1:320. This experiment also clearly indicates that antigenic competition does not account for the observed immunosuppression.

To determine whether animals immunized with mixtures of the immunogenic R1 mutant *E. coli* O8 F470 and the non-immunogenic strain of *E. coli* O1 were immunologically primed, they were challenged with a minimally effective dose of ethanol-soluble CA of *S. typhimurium* (Table 4).

It is evident that immunization with the mixture did prime the animals, for they responded with antibody production in an accelerated and enhanced manner to the booster dose. Essentially identical results were obtained in additional experiments with different strains.

From the above experiments it is evident that a striking difference exists in the immunosuppressive capacity of enteric bacteria and their LPS on the CA antibody response of rabbits, CA-producing, non-immunogenic strains and their LPS being effective in contrast to CA-negative mutants and their LPS.

DISCUSSION

The original studies of Kunin et al. (4) resulted in the discovery of a hitherto overlooked antigen common to enteric bacteria (CA). Equally important, these authors observed that only a few strains are able to elicit CA antibodies, notably *E. coli* O14, which was recently characterized as an encapsulated R mutant (17), and, as shown recently (9, 22), certain R mutants. CA immunogenicity of whole cells seems to be dependent on a genetically determined association between CA and certain LPS cores of these mutants. Such a close association of LPS core and CA is not observed in the non-immunogenic, CA-producing strains (11; unpublished results). The lack of immunogenicity of the CA determinant in these strains appears to be due to the simultaneous presence of immunosuppressive LPS, for separation of the two antigens by ethanol results in a highly immunogenic CA preparation. In turn, the immunogenicity of the ethanol-soluble preparation can be significantly reduced by LPS as well as by certain other substances (1, 13, 19, 25). Since it was shown that immunogenic CA preparations and LPS must be mixed in vitro before immunization to obtain this immunosuppressive effect, it appears that the inhibitor interacts with CA to decrease its immunogenicity, although its antibody-binding capacity remains unchanged. It is conceivable that deaggregation of CA is the mechanism, since heat causes a decrease in the immunogenicity of CA and repeated freezing and thawing restores immunogenicity to a large extent (21). The experiments reported here have revealed that CA-producing, non-immunogenic strains of microorganisms and their LPS, when mixed with highly immunogenic strains in vitro before immunization, suppress CA antibody production to a significant degree, in contrast to CA-negative strains and their LPS. It remains to be seen whether it is the chemical composition or the particular configuration of the LPS preparations that accounts for these differences. It is noteworthy that the common characteristic of those enterobacterial strains that do not suppress the CA antibody response is a defect in the *rfe* gene region (Table 1). The function of genes of the *rfe* cluster is required for the synthesis of CA (7, 8), of T_1 (18), and, in certain *Salmonella* O groups, of O chains (6). It is conceivable that, in addition, hitherto unrecognized functions are determined by this gene region that confer to LPS the immunosuppressive properties described above.

The nature of the interaction between LPS and immunogenic CA, either in the form of bacterial cells or of the ethanol-soluble preparation, requires further study. Certainly, LPS is not a specific inhibitor of immunogenic CA, for other substances, such as chlorphenesin and certain lipids, also are effective immunosuppressants in this system (1, 25). LPS as an immunosuppressant is remarkable, since it is a well-known adjuvant. However, LPS can serve,

TABLE 4. *Immunological priming to CA induced by immunization with mixture of bacterial cells of E. coli F470 (R1) and E. coli O1*[a]

Immunogen	Inhibitor	Mean CA hemagglutinin titers (reciprocal)						
		0[b]	7[c]	10[c]	14[c]	17[d]	18[d]	21[d]
E. coli 08⁻:K27⁻ (F470-R1) suspension	Buffer	<10	320	853	1,280			
	E. coli O1 (S) (NCDC) suspension	<10	13	17	13	187	427	853
Nonprimed		<10				17	33	27

[a] Indicator antigen was CA *(S. typhimurium)*.
[b] Day pre-immunization.
[c] Day before booster injection.
[d] Day after booster injection.

under specified conditions, both as an adjuvant and as an immunosuppressant of other antigens, such as erythrocytes (12). Related to this problem is the unresolved question of why subcellular fractions obtained by treatment of bacterial cells in a French pressure cell yield immunogenic CA, although the whole cells themselves are not immunogenic (2). Future studies will be greatly facilitated by the recent isolation of pure CA and its partial chemical characterization as a heteropolymer of amino sugars (D-glucosamine and D-mannosamine uronic acid), partly esterified by plamitic acid (D. Mannel and H. Mayer, in preparation).

A brief comment of historical interest may be added. F. Kauffmann, Copenhagen, Denmark, who established the *E. coli* typing schema and classified the first 25 serogroups, informed one of us on 2 February 1973 that during these initial investigations he hesitated identifying *E. coli* O14 as a smooth strain, since it had features of R mutants. Without the inclusion of *E. coli* O14 in the original serological schema, however, CA may have escaped detection for even longer than 1962.

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