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Immunosuppression by Endotoxin and its Lipoid A Component.* (31886)

H. Y. WHANG AND E. NETER
Departments of Bacteriology and Pediatrics, State University of New York at Buffalo Medical School; and Laboratories of Bacteriology, Children's Hospital and Roswell Park Memorial Institute, Buffalo, N. Y.

An unusual type of immunosuppression was identified on the basis of studies dealing with the seeming lack of immunogenicity of the common antigen (CA) present in cultures of enterobacteriaceae. This antigen was first recognized by Kunin *et al*(1,2) by means of the hemagglutination test and *E. coli* O14 antiserum. Although *Escherichia, Aerobacter, Salmonella, Shigella,* and *Proteus* all contain CA, only *E. coli* O14 upon intravenous injection into rabbits elicits the formation of CA antibodies in high titers. Otherwise, diagnostic antisera used for serotyping of enteric bacteria would contain CA antibodies in significant amounts, and they do not. The lack of immunogenicity of organisms other than *E. coli* O14 is not due to the fact that they produce less CA than the latter, for immunization with equivalent amounts of CA results in the formation of antibodies only with *E. coli* O14(3). Nor can the lack of immunogenicity of the bacteria be explained on the basis of its absence on the cell surface, for CA has been detected there by immunofluorescence(4). Ca antibodies in moderate titers can be produced if supernates of cultures of enteric bacteria are injected together with Freund's adjuvant or in the cell-attached rather than in the soluble state(3,5,6). Subsequent investigations in our laboratories have revealed that CA, after separation from the simultaneously present O antigen, is highly immunogenic(7). The surprising observation was then made that the injection of mixtures of CA and O antigen (lipopolysaccharide) failed to result in the formation of CA antibodies in high titer(8). Lipoid A produced the same immunosuppressive effect(9). This effect does not take place, if the inhibitors and CA are injected separately, although simultaneously(8). Even though both these inhibitors interfere with the production of circulating CA antibodies, they do not abolish immunologic priming, for animals thus immunized respond with the rapid formation of CA anibodies in significant titers upon the injection of subeffective doses of isolated CA (10). It would be surprising, indeed, if this type of immunosuppression by lipopolysaccharide and its lipoid A component were operative only with a single antigen. The present investigation has revealed that both lipopolysaccharide and lipoid A also interfere with the formation of circulating antibodies against a common antigen of gram-positive bacteria, first described by Rantz *et al*(11).

Materials and methods. Antigens were prepared as follows. Strains of *Staphylococcus aureus* (coagulase-positive; Difco) and *Bacillus subtilis* were grown on brain veal agar in Kolle flasks at 37°C for 18 hours. The growth was suspended in 25 ml of phosphate hemagglutination buffer (pH 7.3; Difco) per Kolle flask. The suspension was centrifuged at

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23,500 *g* for 20 minutes. The supernate was then mixed with ethanol to a final concentration of 85%. The mixture was kept at 20°C for 18 hours and then centrifuged at 23,500 *g* for 20 minutes. The sediment of the ethanol insoluble fraction was redissolved in phosphate hemagglutination buffer to the original volume. In accord with the findings of Chorpenning and Dood(12), the antigen described by Rantz was found to be present in this ethanol insoluble fraction. For control purposes supernates and the ethanol soluble fraction of *Salmonella typhimurium,* prepared according to the procedures previously described(7), were used.

Highly purified lipopolysaccharide from *S. dublin* and lipopid A from *E. coli* O8 were kindly supplied by Professor O. Westphal and Dr. O. Lüderitz, Max-Planck Institute, Freiburg, Germany. Both preparations were dissolved in phosphate buffer in a concentration of 1,000 μg/ml.

Mixtures of antigen and lipopolysaccharide or lipoid A were prepared as follows. Equal amounts of the ethanol insoluble fraction of *S. aureus* in a dilution of 1:10 and lipopolysaccharide or lipoid A in concentrations of 330 μg/ml and 200 μg/ml, respectively, were dissolved in 95% ethanol to a final ethanol concentration of 85%. The mixtures were kept at 20°C for 18 hours without centrifugation and then placed in open Petri dishes for evaporation of ethanol at 37°C. The dry material was redissolved in distilled water to the original volume. Antigen without inhibitor was treated in a like manner and served for control purposes.

Groups of 5 rabbits each (approximately 2 to 3 kg) were immunized intravenously according to schedules given below. Following the primary immunization and a rest period, the animals were challenged with supernate of *S. aureus* in a dilution of 1:100. This booster dose was chosen on the basis of experiments which showed that it produced a more rapid antibody response in primed than in non-primed animals.

Blood specimens were obtained prior to, during, and after immunization and challenge. Serum specimens were kept frozen at —20°C. Antibodies against the common antigen of gram-positive bacteria were titrated as follows. Rabbit erythrocytes (2.5%) were washed three times in phosphate hemagglutination buffer. To the sediment was added antigen from *S. aureus* or *B. subtilis* in a dilution of 1:10 and in a volume to restore the original erythrocyte concentration. The mixtures were incubated in a waterbath at 37°C for 30 minutes. The erythrocytes were then washed 3 times, in order to remove unattached antigen. Serum in 2-fold serial dilutions (0.2 ml) was then mixed with an equal volume of the antigenically modified erythrocytes. The mixtures were incubated in a waterbath at 37°C for 30 minutes, and hemagglutination was read after centrifugation at 1,300 *g* for 2 minutes. The specificity of the antibody response was documented by using antigen from *B. subtilis* for the antibody titration of sera of animals immunized with antigen from *S. aureus*. In addition, hemagglutination inhibition tests were carried out as follows. Serum in serial 2-fold dilutions (0.2 ml) from animals immunized with staphylococcal antigen was mixed with antigen from *B. subtilis* (0.2 ml); the mixtures were incubated in a waterbath at 37°C for 30 minutes; rabbit erythrocytes (0.2 ml) that had been modified with staphylococcal antigen were then added, and the hemagglutination test was completed as described above.

Results. The first series of experiments were carried out to determine whether lipopolysaccharide affects the antibody response of the rabbit to the common antigen of gram-positive bacteria in a manner similar to that previously observed with the common antigen of gram-negative bacteria. To this end, groups of 5 rabbits each were immunized as follows. Groups 1 and 2 received the mixture of the ethanol insoluble staphylococcal antigen and lipopolysaccharide. These materials contained antigen in a dilution of 1:10 and *S. dublin* lipopolysaccharide in a concentration of 333 μg/ml. They were injected intravenously daily in a volume of 2 ml, in dilutions of 1:50 (2 injections); 1:20 (3 injections); 1:10 (4 injections); and 1:5 (2 injections), for a total of 11 injections. This schedule of multiple injections had to be used because of the toxicity

of the lipopolysaccharide. Group 1, after a rest period of one week following the last injection, received staphylococcal antigen (supernate) in a dilution of 1:100. The animals of Group 2 served as controls, receiving antigen-lipopolysaccharide mixture as above but no secondary immunization. The rabbits of Group 3 were given the ethanol insoluble antigen alone, followed by a booster injection. Animals of Group 4 received a single injection of antigen in the dose used for booster purposes. The results of this experiment are summarized in Table I.

It is evident that the ethanol insoluble fraction of the staphylococcal culture engenders antibodies in significant titers (Group 3). In contrast, animals receiving the same antigen together with lipopolysaccharide failed to produce circulating antibodies (Groups 1 and 2). That the failure of response to the primary immunization is not due to delayed antibody production is evident from the fact that rabbits injected with the antigen-lipopolysaccharide mixture without secondary immunization did not develop circulating antibodies over the entire period of observation (Group 2). Nonetheless, these animals were immunologically primed, since they responded to the secondary injection of antigen with an increase in antibody titer as early as the third day (Group 1). In nonprimed rabbits (Group 4) the booster dose of antigen elicited demonstrable antibody formation only after the fourth day. It is evident from these results that lipopolysaccharide, when injected together with the common antigen of gram-positive bacteria, strikingly suppresses the production of circulating antibodies without interference with immunologic priming.

The effect of the lipoid A component of endotoxin on the immune response of the rabbit was explored next. Lipoid A was used in a concentration of 200 μg/ml and the ethanol insoluble fraction of the staphylococcal antigen in a dilution of 1:10. The materials in a dilution of 1:5 were given intravenously in amounts of 2 ml, for a total of 2 injections, 3 days apart. Group 1 received the mixture of antigen and lipoid A; Group 2 was given antigen and lipoid A sepa-

TABLE I. Effect of *S. dublin* Lipopolysaccharide on Hemagglutinin Response of Rabbit to Common Antigen of Staphylococcus.

Group	Material for immunization: Primary	Material for immunization: Secondary	Mean hemagglutinin titers (reciprocal) and ranges: Prior to challenge: A†	B	C	D	E	Days after challenge: 1 F	2 G	3 H	4 I	7 J
1	Mixture of staphylococcus (EI)* & *S. dublin* lipopolysaccharide	Staphylococcus (supernate, 1:100)	<10	<10	<10	<10	<10	<10	18 (<10–20)	123 (20–640)	518 (20–1280)	690 (80–2560)
2	*Idem*	—	<10	<10	<10	<10	12	12	<10	<10	<10	<10	<10
3	Mixture of staphylococcus (EI) & buffer	Staphylococcus (supernate, 1:100)	13 (<10–20)	170 (<10–640)	408 (<10–1280)	500 (40–1280)	375 (20–1280)	415 (20–1280)	420 (40–1280)	450 (40–1280)	760 (160–1280)	800 (320–1280)
4	—	*Idem*	11	—	—	—	—	—	11 (<10–20)	15 (<10–40)	16 (<10–40)	116 (<10–1280)

* (EI) = Ethanol insoluble fraction. † A = Pre-immunization. B-E = During and after primary immunization. F-J = After secondary immunization.

rately, although simultaneously. Animals of Group 3 served for control purposes, receiving the antigen alone. After a rest period of 2 weeks all animals of Groups 1, 2, and 3 received staphylococcal antigen (1:100) for booster purposes. Rabbits of Group 4 were injected with the mixture of staphylococcal antigen and lipoid A, but without a booster dose. The results are summarized in Table II.

The Table shows that lipoid A, when injected together with the antigen, suppresses the antibody response to primary immunization without impairing immunologic priming (Groups 1 and 4). It is also evident that separate injection of antigen and lipoid A is not associated with immnosuppression (Group 2). Injection of staphylococcal antigen alone (Group 3) resulted in a primary as well as a secondary response. From these results it is evident that both lipopolysaccharide and its lipoid A component interfere with the production of circulating antibodies against the common antigen of Gram-positive bacteria and that this interference takes place only when inhibitor and antigen are injected as a mixture.

The specificity of the immune responses in the above animals was documented by the study of selected sera in both hemagglutination and hemagglutination inhibition tests, in which antigen from *B. subtilis* was utilized. It was shown that sera of animals immunized with either the ethanol insoluble fraction or supernate of staphylococcal cultures agglutinated rabbit erythrocytes that had been modified with *B. subtilis* antigen. In addition, *B. subtilis* antigen completely neutralized the antibodies of these sera in hemagglutination tests utilizing staphylococcal antigen as indicator.

The specificity of immunologic priming by mixtures of antigen and lipopolysaccharide or lipoid A was documented by the observations that the common antigen of Gram-negative bacteria neither primed rabbits for a specific antibody response to the antigen of Gram-positive bacteria, nor did it serve as a booster antigen in rabbits immunized with antigen from Gram-positive bacteria.

The effects of lipopolysaccharide and its

TABLE II. Effect of *E. coli* O8 Lipoid A on Hemagglutinin Response of Rabbit to Common Antigen of Staphylococcus.

Group	Material for immunization: Primary	Material for immunization: Secondary	Mean hemagglutinin titers (reciprocal) and ranges: Prior to challenge: A†	B	C	Days after challenge: 1 D	2 E	3 F	4 G	7 H
1	Mixture of staphylococcus (EI)* and lipoid A	Staphylococcus (supernate, 1:100)	<10	<10	<10	13 (<10–20)	20 (10–40)	317 (20–1280)	1600 (320–5120)	2773 (640–5120)
2	Staphylococcus (EI) and lipoid A (separately)	"	<10	387 (80–640)	207 (20–640)	313 (20–1280)	353 (40–1280)	1373 (40–5120)	2153 (40–5120)	3430 (20–5120)
3	Mixture of staphylococcus (EI) and buffer	"	12 (<10–20)	324 (20–640)	168 (20–640)	296 (20–1280)	350 (20–1280)	432 (80–1280)	846 (320–1280)	3840 (1280–5120)
4	Mixture of staphylococcus (EI) and lipoid A	—	<10	<10	<10	—	—	—	—	<10

* (EI) = Ethanol insoluble fraction. † A = Pre-immunization. B, C = After primary immunization. D-H = After secondary immunization.

lipoid A component on the immune response of the rabbit to common antigen of Gram-positive bacteria could be readily explained if these inhibitors affected the antigenic determinant. This was shown not to be the case, for antigen in the presence of the inhibitors in concentrations used in the immunization experiments neutralized antibodies as effectively as antigen alone.

It may be concluded, then, that lipopolysaccharide and its lipoid A component, when injected together with the common antigen of Gram-positive bacteria, interfere with the production of circulating antibodies without preventing immunologic priming.

Discussion. The immune response to an antigenic stimulus can be suppressed by specific and nonspecific means; immunologic unresponsiveness or tolerance and the interference of pre-existing antibodies being examples of the former group, and X-irradiation and immunosuppressive drugs of the latter. The present investigation has revealed an unusual type of non-specific immunosuppression, for it was demonstrated that lipopolysaccharide (endotoxin) and its lipoid A component interfere with the production of circulating antibodies against a common antigen of Gram-positive bacteria without preventing specific immunologic priming. These findings strikingly parallel the observations made previously with the common antigen of Gram-negative bacteria(10). One of the unusual features of this type of immunosuppression is the fact that the inhibitors are effective only when injected together with the antigen, but not when injected separately, although simultaneously. This fact strongly suggests that the inhibitors do not directly affect the immunologic competence of the host through toxicity to the immunologic apparatus. Since the inhibitors do not interfere with the antibody-combining capacity of the antigen, the above observations cannot be explained by the simple mechanism of alteration of the immunologic determinant. Thus, the question arises whether binding of antigen and inhibitor into a complex occurs and whether the presence of the inhibitor together with the antigen or a derivative thereof in the immunologically competent cell affects the antibody response, by suppressing the proliferation of antibody-forming cells or by interference with the release of antibody molecules. In either case, it is evident that this inhibitory effect does not eliminate the establishment of immunologic memory. It is also clear that, for as yet unexplained reasons, lipopolysaccharides (endotoxins) and their lipoid A component may serve both as adjuvant in immunization with protein antigens and as inhibitor of the immune response, as described here. Suppression by endotoxin of the antibody response to actinophage, as observed by Bradley and Watson(13), may be due to cytotoxicity.

So far as the antigen common to many Gram-positive bacteria is concerned, recent studies by Chorpenning and Dodd(12) suggest that 2 different antigens may exist, one representing the antigen first described by Rantz *et al*(11) and the other present in several species of *Bacillus* and *S. aureus* but not in streptococci. Studies by Moskowitz (14) and by Jackson and Moskowitz(15) resulted in the chemical characterization of the antigen obtained from streptococci, indicating that it is made up of glycerophosphate as a polymer and D-alanine, thus being a member of the class of substances called teichoic acid. In the view of these authors, the former part of the molecule is responsible largely for its reactivity with antibodies, and the latter plays a critically important role in the attachment of the antigen to erythrocytes. That D-alanine also may be involved in serologic specificity is suggested by the observations of McCarty(16). Whether the D-alanine component is involved in the assumed complexing of the antigen used in this investigation with lipopolysaccharide and lipoid A remains to be determined.

The capacity of lipopolysaccharides and lipoid A to inhibit the production of circulating antibodies against common antigens of both Gram-positive and Gram-negative bacteria is not a generalized phenomenon, for these inhibitors do not interfere under similar conditions with the production of antibodies against the Vi antigen of enteric bacteria (unpublished observations). Whatever the mode of action of these inhibitors in the

immune response to certain antigens may be, it is clear from the observations reported here that they interfere non-specifically with the productive phase of antibody formation rather than with the establishment of immunologic memory.

Summary. Lipopolysaccharide and its lipoid A component, when injected together with the common antigen of Gram-positive bacteria obtained from *S. aureus,* inhibit the production of circulating antibodies detected by hemagglutination. Nonetheless, the animals so treated are immunologically primed, as evident from a more rapid development of antibodies following a booster injection of antigen than is observed in unprimed animals. The common antigen of Gram-negative bacteria neither primes the animals nor functions as a booster antigen for the production of antibodies against the staphylococcal antigen, indicating the specificity of priming. The specificity of the immune response to staphylococcal antigen was ascertained by the fact that the antibodies react with erythrocytes modified by *B. subtilis* antigen as well, and that the latter antigen inhibits completely the hemagglutination of red blood cells modified by staphylococcal antigen. It is postulated that lipopolysaccharide (endotoxin) and its lipoid A component interact with certain antigens *in vitro,* and that the inhibitor, when injected into rabbits together with the antigen, interferes with the production of circulating antibodies without eliminating the establishment of immunologic memory.

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Insulin Secretion *in vitro* by Pancreatic Tissue from Normal, Adrenalectomized, and Cortisol-Treated Rats.* (31887)

WILLY J. MALAISSE, FRANCINE MALAISSE-LAGAE, EDWARD F. MC CRAW, AND PETER H. WRIGHT
Department of Pharmacology, Indiana University School of Medicine, Indianapolis

Decreased tolerance to glucose is a classical feature of hyperadreno-corticism(1,2) and has been related to the antagonistic effects of glucocorticoid and insulin upon hepatic (3) and peripheral(4) glucose metabolism. The impaired tolerance to glucose occurs despite hyperplasia of the Islets of Langerhans (5,6,7) and elevated levels of circulating insulin(8,9,10). A possible explanation for

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