Inhibition by Lipoid A of Formation of Antibodies Against Common Antigen of Enterobacteriaceae.* (30540)

H. Y. WHANG,† O. LÜDERITZ, O. WESTPHAL AND E. NETER
Departments of Pediatrics and Bacteriology, State University of New York at Buffalo Medical School; Laboratory of Bacteriology, Children's Hospital, Buffalo, N. Y.; and Max-Planck Institut für Immunbiologie, Frieburg, Germany

Recently it was shown that highly purified lipopolysaccharides (endotoxins) under certain conditions inhibit the formation of antibodies against the common antigen (CA) shared by many enteric bacteria(1). This observation was all the more surprising, because endotoxins act as adjuvants in enhancing antibody production against proteins. The inhibitory effect of the endotoxins was observed only when antigen and lipopolysaccharide were injected as mixtures, but not when the two substances were administered separately into different veins, although simultaneously. Further, it was shown that mixtures prepared in ethanol and then dissolved in phosphate buffer engender antibodies to an even lesser degree than mixtures prepared in buffer. On the basis of these findings it was concluded that the inhibitory effect on antibody formation is due to interaction of antigen and lipopolysaccharide rather than to an effect of the latter on the host. It was of interest to determine whether the lipoid A component of the lipopolysaccharide molecule exerts a similar effect, particularly since the role of lipoid A in the biologic effects of lipopolysaccharides has not yet been fully elucidated. The results are reported here.

Materials and methods. A smooth strain of *S. typhimurium* was grown on brain veal agar in Kolle flasks for 18 hours at 37°C. The resulting growth was suspended in 25 ml of phosphate hemagglutination buffer (Difco; pH 7.3) per Kolle flask. The suspension was heated in boiling water for 1 hour and centrifuged at 23,500 *g* for 20 minutes. The clear supernate contains both CA and O antigen. CA, first described by Kunin *et al*(2), was separated from the simultaneously present O antigen by means of 85% ethanol, as reported by Suzuki *et al*(3). After evaporation of ethanol, it was dissolved in distilled water in the original concentration. Lipoid A preparations were obtained from *E. coli* O8, *E. coli* O111, and *S. abortus equi* according to methods briefly described by Westphal *et al*(4) and in detail in 2 patents (5,6). These preparations were dissolved in phosphate buffer, their solutions being facilitated by continuous shaking of the tubes for several minutes in a waterbath at 56°C. Mixtures of CA and lipoid A were prepared either in ethanol (85%) or in phosphate buffer. The ethanol mixtures were kept at 20°C for 18 hours, the ethanol was evaporated from open Petri dishes kept at 37°C for 18 hours, and the powder was then redissolved in distilled water.

Albino rabbits, weighing approximately 2 kg, were immunized intravenously either with CA alone or with mixtures of CA and lipoid A, according to schedules given below. Whenever mixtures prepared in ethanol were used, the control preparation of CA, injected alone, was treated a second time with ethanol. In other experiments the two compounds were injected intravenously into different ear veins, although simultaneously, or administered subcutaneously into the footpads.

Serum specimens were obtained prior to, and at weekly intervals after initiation of, immunization. Antibodies against CA were titrated by means of the hemagglutination procedure described in detail previously(7). Briefly, rabbit erythrocytes (2.5% suspension) were treated with CA obtained from *E. coli* O14 (supernate of culture in dilution of 1:10), washed 3 times in phosphate buffer to remove unattached antigen, and mixed with equal amounts of serum in 2-fold serial dilutions (0.2 ml). The mixtures were incubated in a waterbath at 37°C for 30 minutes, and

* Study aided by Research Grant AI 00658, Nat. Inst. of Allergy and Infect. Dis., U.S.P.H.S.

† United Health Foundation Research Training Fellow.

TABLE I. Effect of Lipoid A on Antibody Response of Rabbit to Common Antigen (CA) Injected Intravenously.

Materials for immunization		Mean CA hemagglutinin titers (reciprocal) A†	B	C	D
CA of *S. typhimurium*	a*	<10	480	2240	3250
	b	11	160	960	960
Mixtures of CA of *S. typhimurium* and lipoid A‡ (100 μg/ml)					
Lipoid A M-2	a	<10	<20	23	38
	b	15	20	<10	13
" M-7	a	13	128	760	600
	b	<10	<20	<10	<10
" CO-3232	a	13	73	1700	2560
	b	13	13	10	30
" CO-3060	a	<10	125	1600	1600
	b	<10	<10	13	13
" AE-Lip	a	<10	125	800	640
	b	<10	13	43	57

* a = Aqueous mixtures. b = Ethanol mixtures.
† A = Pre-immunization. B to D = Weekly intervals after initiation of immunization.
‡ Lipoid A preparations = *E. coli* O8 (M-2), *E. coli* O8 (M-7), *E. coli* O111 (CO-3232), *E. coli* O111 (CO-3060), *S. abortus equi* (AE-Lip).

the resulting hemagglutination was read grossly after centrifugation at 1300 *g* for 2 minutes.

Results. In the first series of experiments the effect of 5 lipoid A preparations on antibody response to common antigen (CA) obtained from *S. typhimurium* was investigated. Equal mixtures (1 ml) of lipoid A (100 μg/ml) and of the ethanol soluble CA (1:5), prepared in aqueous and ethanol solutions, were used for immunization. A total of 4 injections was given at 2-day intervals. The data obtained are summarized in Table I. It is evident that all 5 preparations of lipoid A effectively suppressed the antibody response to CA when mixtures prepared in ethanol were used for immunization. When mixtures prepared in aqueous solution were employed, one of the preparations (M-2) almost completely suppressed CA antibody response; the other preparations had only a slight effect, notably during the early immune response.

Quantitative experiments with lipoid A preparation M-2 from *E. coli* O8 revealed that, when injected together with CA from *S. typhimurium* (ethanol mixture), 20 μg/ml reduced antibody formation approximately 80%, and 4 μg/ml was ineffective. Aqueous mixtures of lipoid A (4 and 20 μg/ml) and CA were fully immunogenic. Additional experiments revealed that separate, although simultaneous, intravenous injection of lipoid A and CA resulted in an undiminished immune response.

It was shown previously(8) that injection of supernates of agar grown cultures of enteric bacteria containing CA into the footpad of rabbits, with or without Freund's adjuvant, resulted in antibody formation against CA. Experiments were undertaken, therefore, to determine the effect of lipoid A on antibody response to mixtures of CA and lipoid A given by this route. As in the previous experiments, mixtures of CA (1:5) and either of 2 lipoid A preparations (100 μg/ml) were prepared in ethanol. Equal parts of this mixture and complete Freund's adjuvant (total volume of 0.2 ml) were injected at weekly intervals into each footpad for a total of 4 injections. The results are shown in Table II and indicate that antibody formation was completely suppressed by both lipoid A preparations.

In contrast to the soluble crude CA (supernates of agar grown cultures), antigenically modified autologous erythrocytes engender CA antibodies(9). Experiments were undertaken, therefore, to determine the effect of lipoid A on the immunogenicity of cell-attached CA. To this end, mixtures (1 ml) of CA (1:5) and lipoid A (100 μg/ml) prepared in ethanol and redissolved in water,

TABLE II. Effect of Lipoid A on Antibody Response of Rabbit to Common Antigen (CA) Injected into Footpad with Freund's Adjuvant.

Materials for immunization (ethanol mixtures with complete Freund's adjuvant)	Mean CA hemagglutinin titers (reciprocal)				
	A*	B	C	D	E
CA of *S. typhimurium*	15	18	178	505	2585
CA of *S. typhimurium* and lipoid A (*E. coli* O8)	12	22	<10	<10	24
CA of *S. typhimurium* and lipoid A (*E. coli* O111)	<10	17	12	12	16

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

as described above, were mixed with 2 ml of autologous washed erythrocytes (50%), and the incubated suspension was used for immunization (5 intravenous injections, 2 days apart). Mixture of antigen and red blood cells was used for control purposes. These experiments, too, revealed suppression of the immune response to CA by lipoid A.

From these observations it may be concluded that lipoid A, like lipopolysaccharide, interferes with the antibody response to CA after the 2 materials were injected as mixtures prepared in ethanol, and that only one of the 5 lipoid A preparations, in the amounts used, markedly inhibited the antibody response when mixtures prepared in aqueous solution were employed. Separate injections of lipoid A and CA resulted in undiminished CA antibody response. It is likely, therefore, that lipoid A interacts with CA and thus effects the immunogenicity of CA in the rabbit. These results could be readily explained if lipoid A were to destroy the antigenic determinant of CA. This is not the case, for repeated *in vitro* experiments revealed that lipoid A in amounts of 60 to 500 μg/ml failed to affect the antibody neutralizing capacity of the antigen, and in amounts of 250 μg/ml it did not interfere with red cell attachment of CA and subsequent hemagglutination.

Discussion. The present investigation has revealed that 5 preparations of lipoid A, obtained from highly purified lipopolysaccharides (endotoxins), when injected together with the common antigen (CA) of *S. typhimurium,* interfere with the antibody response of the rabbit to this antigen. This inhibition was demonstrated with all preparations used in a concentration of 100 μg/ml, provided that the mixture of inhibitor and CA was prepared in ethanol. Only one preparation exerted a marked effect when the mixture was prepared in water. Quantitative experiments revealed that as little as 20 μg/ml of lipoid A reduced the antibody response; 4 μg/ml had no effect. This inhibitory effect was demonstrated after intravenous injection, administration with Freund's adjuvant into the footpad, and injection together with erythrocytes. This inhibitory effect parallels that observed with lipopolysaccharides(1). The observation that inhibition of antibody formation takes place only when mixtures of antigen and lipoid A or lipopolysaccharide are used for immunization and not when the two materials are injected into different veins, although simultaneously, strongly suggests that a reaction takes place *in vitro* between antigen and lipoid A, which in turn is responsible for the decreased or absent antibody stimulation.

Little information is available on the chemical composition of CA. It is known that this antigen is present in numerous, but not all, Gram-negative bacteria; further, it can be separated from the O antigen by means of 85% ethanol, CA being soluble and the O antigen being insoluble. Attempts at identification of CA have been only partially successful, for the purified material obtained by Kunin(10) had lost several of its activities, including its ability to become attached to erythrocytes. The nature of the reaction between lipoid A and CA must await further clarification of the chemical characteristics of the latter. It remains for future investigations to determine whether lipopolysaccharides and lipoid A may react with other antigens and whether this interaction, too, results in decreased immunogenicity. That lipopolysaccharides, in fact, do react with a variety of substances has been established, for it has been shown that certain serum proteins, lecithin, protamine, and polymyxin B, all inter-

act with lipopolysaccharides, thus interfering with their attachment to erythrocytes(11-15).

The present investigation and our previous report(1) suggest that lipopolysaccharides (endotoxins) and their lipoid A component render a certain antigen non-immunogenic; these mixtures, nevertheless, react well with CA antibodies *in vitro*. Thus, these findings are the reverse of those of haptens, for the latter become immunogenic upon attachment to certain foreign proteins. Further investigation to determine the precise mechanism of this inhibitory effect on immunogenicity of lipopolysaccharides and their lipoid A component may yield information on general aspects of the antibody response.

Summary. Five preparations of lipoid A, obtained from highly purified lipopolysaccharides (endotoxins), were shown to inhibit the antibody response to the common antigen (CA) of enteric bacteria. This inhibition occurred when mixtures of antigen and lipoid A (100 μg per injection) were prepared in ethanol *in vitro* and then redissolved in water for immunization. Only one of these 5 preparations was markedly effective as inhibitor when mixtures of lipoid A and antigen were prepared *in vitro* in aqueous solution. Quantitation of one of the preparations showed that as little as 20 μg per injection of lipoid A decreased, but did not abolish, the antibody response. Simultaneous injection of antigen and lipoid A into different veins failed to interfere with CA antibody formation. Lipoid A also interferes with the antibody response, when mixtures prepared in ethanol were injected into the footpad with Freund's adjuvant or in the presence of autologous erythrocytes. It is concluded that lipoid A interacts with CA and that antibody formation is suppressed as a result of this interaction.

1. Suzuki, T., Whang, H. Y., Gorzynski, E. A., Neter, E., Proc. Soc. Exp. Biol. and Med., 1964, v117, 785.

2. Kunin, C. M., Beard, M. V., Halmagyi, N. E., ibid., 1962, v111, 160.

3. Suzuki, T., Gorzynski, E. A., Neter, E., J. Bacteriol., 1964, v88, 1240.

4. Westphal, O., Nowotny, A., Lüderitz, O., Hurni, H., Eichenberger, E., Schönholzer, G., Pharm. Acta Helv., 1958, v33, 401.

5. Folkers, K., U. S. Patent Office, 3,089,821, May 14, 1963.

6. German Patents 1073150, 1958 and 1175389, 1958.

7. Whang, H. Y., Neter, E., J. Bacteriol., 1962, v84, 1245.

8. Gorzynski, E. A., Whang, H. Y., Suzuki, T., Neter, E., Proc. Soc. Exp. Biol. and Med., 1963, v114, 700.

9. Neter, E., Whang, H. Y., Suzuki, T., Gorzynski, E. A., Immunology, 1964, v7, 657.

10. Kunin, C. M., J. Exp. Med., 1963, v118, 565.

11. Neter, E., Zak, D. A., Zalewski, N. J., Bertram, L. F., Proc. Soc. Exp. Biol. and Med., 1952, v80, 607.

12. Neter, E., Zalewski, N. J., Zak, D. A., J. Immunol., 1953, v71, 145.

13. Neter, E., Gorzynski, E. A., Westphal, O., Lüderitz, O., ibid., 1958, v80, 66.

14. Lüderitz, O., Westphal, O., Eichenberger, E., Neter, E., Biochem. Z., 1958, v330, 21.

15. Rudbach, J. A., Johnson, A. G., Nature, 1964, v202, 811.

Received July 15, 1965. P.S.E.B.M., 1965, v120.

Thymidylate Dephosphorylation in Granulocytes.* (30541)

IRA D. STEIN AND JOSEPH R. RUBINI (Introduced by W. J. Harrington)
Department of Medicine, University of Miami Medical School, Miami, and V. A. Hospital, Coral Gables, Fla.

Normal granulocytes of human beings and most other mammals contain an alkaline phosphatase (AP) capable of hydrolyzing a variety of natural and synthetic phosphate monoesters. This enzyme has been extensively studied by other workers both biochemically using leukocyte homogenates and cytochemically in blood smears. Most of

* This investigation was supported by USPHS Research Grant CA08063-01.