STUDIES ON THE O ANTIGEN OF SALMONELLA TYPHOSA

V. Enhancement of Antibody Response to Protein Antigens by the Purified Lipopolysaccharide*

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Investigations of factors affecting the immunological state of the host constitute a voluminous literature, most of which has been reviewed in a comprehensive treatise by Perla and Marmorston (1). However, of the great array of substances investigated, only a few have proved to be of consequence in that they provided new and productive approaches to improvement in the antibody response of the host.

The contributions of major importance in this respect are: (*a*) the observations of Ramon (2) and of Glenny *et al.* (3) on the augmentation of antibody response to antigens adsorbed on particulate carriers; (*b*) the studies of Freund (4–6) which established the enhancement and prolongation of antibody response to antigens administered in water-in-oil emulsions; and (*c*) the finding that certain bacterial vaccines, notably pertussis and typhoid, incorporated in combined prophylactics, exert a synergistic effect on the antigenicity of toxoids (7).

Study of the factors responsible for the elevated antibody levels following combined immunization has been limited, and the mechanisms by which these vaccines augment antitoxin formation are unknown. The effect of bacterial components on antibody response to antigenic stimulus has received less attention. Staphylotoxin has been reported to raise the level of antibody developed in response to weakly antigenic substances such as ragweed pollen and crystalline beef lens, when administered combined with, or several hours prior to these antigens (8, 9). This synergistic action of staphylotoxin has been used to advantage by Hecht *et al.* in producing specific antiskin antibodies in rabbits (10). In addition, the type A toxoid of *Clostridium*

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botulinum was found to improve the immunizing properties of type B toxoid in mice (11). Streptococci have been reported to induce an increased reactivity in rabbits to cutaneous injection of horse serum. This increased reaction was attributed to an alteration in the functional activity of the reticulo-endothelial system (12). The antibody-enhancing capacity of the tubercle bacillus has been well established since the discovery by Lewis and Loomis (13) of an increased antibody formation when antigen was introduced into a site previously injected with tubercle bacilli. Isolated fractions of this organism have now been shown to effectively replace the intact cell as an adjuvant in the production of experimental allergic encephalomyelitis (14).

Immunization with typhoid vaccine has been reported to increase antibody formation to unrelated antigens. Thus, Herrman (15) observed that typhoid vaccine stimulated production of specific antistreptococcic agglutinin in rabbits which had failed to respond with antibody when cultures of streptococci alone were used. Immunization of patients against typhoid fever or cholera has been noted to cause a rise in the titer of *Shigella dysenteriae* agglutinins (16). In addition, antibody formation to a poorly antigenic blood group substance has been reported to occur to a much greater degree following non-specific stimulation with typhoid vaccine (17).

Recently, studies in these laboratories led to isolation and purification of the somatic O antigen of the O-901 strain of *Salmonella typhosa* (18). The purified product was characterized as a phosphorylated lipopolysaccharide free of protein, exhibiting the immunological (19) and toxic (20) attributes of the organism from which it was derived. The availability of this relatively well characterized bacterial component made possible a quantitative study of its role in the capacity of the typhoid organism to augment the resistance of the host against unrelated disease entities. This report records the marked enhancement of antibody formation to purified protein antigens provoked by the *typhosa* lipopolysaccharide. In addition, certain aspects of the mechanisms concerned in this enhancement were investigated and are discussed.

Materials and Methods

Salmonella typhosa Endotoxin.—This lipopolysaccharide was derived from *Salmonella typhosa* O-901 by the isolation and purification scheme of Webster and collaborators (18). It was shown to contain 0.6 per cent nitrogen (of which 0.4 per cent is attributable to hexosamine), 2 per cent phosphorus, and is best characterized as a phosphorylated lipopolysaccharide. Approximately two-thirds of the molecule is comprised of polysaccharide, with rhamnose, mannose, glucose, and galactose as constituent sugars. The remaining one-third of the molecule is lipide. A second preparation of endotoxin of somewhat higher nitrogen concentration (1.4 per cent) was used in several experiments and found to possess similar properties. Saline solutions of the dry lipopolysaccharide were kept in the frozen state until use. Dilutions of these solutions in appropriate volume were added to saline solutions of the antigen, and the mixture injected into the marginal ear vein of rabbits, unless otherwise indicated.

Endotoxins Derived from Other Bacterial Genera.—Preparations of endotoxin from *Proteus vulgaris* (Lot PV-27) and *Pseudomonas aeruginosa* (Lot PC9) were generously supplied by Travenol Laboratories, Morton Grove, Illinois. A purified endotoxin from *Serratia marcescens* (Lot P25) was kindly made available by Dr. M. Shear of the National Cancer Institute,

Bethesda. A trichloroacetic acid extract of *Hemophilus pertussis* was prepared by the Department of Biochemistry, Army Medical Service Graduate School. A preparation of endotoxin from *Brucella melitensis* (Lot 472) was furnished by Dr. W. Spink, University of Minnesota.

Quantitative Precipitin Reaction.—The quantitative precipitin test as applied to determination of either antigen or antibody was essentially that of Heidelberger and Kendall as described by Kabat and Mayer (21). To determine the antibody content of an unknown serum, three equal samples of antiserum were precipitated by a quantity of antigen ranging from that required at the equivalence point to slight antigen excess. Incubation was carried out for 2 hours at 37°C. followed by 2 days at 4°C., while sera of low antibody content were allowed to remain for 1 week at the latter temperature. Following centrifugation, the supernatant fluids were digested by a sulfuric acid–selenium mixture, and analyses for total nitrogen were carried out by a micro Kjeldahl procedure (22). The highest of the three values for antibody nitrogen after subtraction of the antigen nitrogen added and correction for volume, was taken as the antibody titer of the serum. No attempt was made to remove complement from any of the sera.

In order to analyze serum samples quantitatively for antigen, a calibration curve was first made on the basis of the addition of increasing amounts of antigen to a constant amount, 1 ml., of antiserum. Saline was added to a total volume of 3 ml. The micrograms of nitrogen in unknown antigen samples were determined by interpolation on the calibration curve after determination of the total nitrogen precipitated in antibody excess by the micro Kjeldahl technique. Appropriate antiserum and antigen controls were run throughout and modifications for samples of 10 to 100 μg. total nitrogen were made.

Rabbits.—New Zealand white rabbits, ranging from 2 to 3 kg., were used throughout. All rabbits in individual experiments were of one sex, either male or female.

Antigens.—A twice crystallized preparation of *ovalbumin*, (Lot A521), was obtained from Worthington Laboratories, Freehold, New Jersey. Appropriate quantities of this preparation were weighed and dissolved in 0.85 per cent NaCl solution at the initiation of each experiment. Concentrated *diphtheria toxoid* (Lot Pt-50) containing approximately 2500 Lf/ml. was supplied by the Biologic Laboratories, Institute of Laboratories, Boston. *Plague capsular protein* (Lot PC7-0.2 Na) was isolated from *Pasteurella pestis*, strain TJW, and purified according to the method of Amies (23) by Dr. M. E. Webster, Army Medical Service Graduate School. *Bovine serum albumin*, fraction V, (Lot L11604) was obtained from Armour and Company. *Vi antigen* (Lot 217 ED) was isolated and purified from *Escherichia coli*, strain 5396/38 by Webster *et al.* (24). A *Pasteurella tularensis* strain Schu-A vaccine was furnished by Dr. Paul Nicholes, University of Utah, Salt Lake City. A *Diplococcus pneumoniae* type III vaccine was prepared from a virulent encapsulated strain of this organism grown in beef heart infusion broth for 8 hours. The organisms were killed with formalin and the vaccine standardized to contain 10^9 organisms/ml.

Determination of Rate of Clearance of Radioisotope-Labelled Human Serum Albumin.—New Zealand white rabbits, ranging in weight from 2.2 to 2.6 kg., were selected for this experiment. In order to insure rapid excretion of isotopically labelled non-protein bound iodine, 1.5 mg. potassium iodide was added to the drinking water of rabbits for 1 week prior to injection of the radioisotope-labelled protein. Radioiodinated (I^{131}) human serum albumin (Risa, Lot 888-219-16) was obtained from Abbott Laboratories, North Chicago, as a sterile solution which assayed 790 μc./ml. at the time of preparation. Dilutions of the original concentration were injected intravenously into rabbits as described in the section on Results.

Bacterial Agglutination Test.—An alcohol-killed and washed suspension of *S. typhosa* 0-901, preserved with phenol and adjusted to a concentration of 10^9 organisms/ml. was employed as agglutination reagent. Tests were incubated at 52°C. for 16 hours. A reference typhoid 0 antiserum was included in each test.

RESULTS

Enhancement of Antibody Response to Ovalbumin by Typhoid Endotoxin

Effect of Dose, Route, and Number of Injections of Endotoxin.—

Initial experiments generally characterizing the enhancing effect of this lipopolysaccharide on antibody response were conducted principally with crystalline ovalbumin, since anti-ovalbumin can be measured with precision. The test conditions employed, such as quantity of antigen, and number and route of injections, were chosen to evoke minimal antibody response, in order that the effect of an enhancing agent would be demonstrated more readily. In determining the enhancing activity of endotoxin in rabbits, quantities of this product cover-

TABLE I

Enhancement of Antibody Response to Ovalbumin by Typhoid Endotoxin

No. of rabbits	Immunization: 3 injections i.v. at 3-day intervals	Antibody nitrogen/ml. serum	
		7 days after last injection	geometric mean
		μg.	μg.
8	2 mg. ovalbumin	27 27 21 36 1 5 10 8	11
4	2 mg. ovalbumin + 0.1 μg. endotoxin	32 21 56 34	34
4	2 mg. ovalbumin + 5 μg. endotoxin	135 264 249 239	215
4	2 mg. ovalbumin + 10 μg. endotoxin	146 478 141 79	167

ing a 100-fold range were administered intravenously, together with crystalline ovalbumin, 3 times at 3-day intervals. The results of a typical experiment are given in Table I. Within each group the usual animal variation was encountered, and for this reason the individual values are given in addition to the geometric mean antibody responses.

Quantities of endotoxin as small as 1 μg. resulted in a perceptible increase, while the response to 5 μg. was pronounced, being approximately 20 times that of control rabbits receiving only the antigen. To determine whether antibody attained maximum levels relatively early in those rabbits treated with endotoxin, serum samples were obtained 3, 5, 7, 9, 12, and 14 days after the last injection. Individual rabbits varied considerably; nevertheless the trend of antibody response was generally to rise to a maximum by the 7th day with relatively little fluctuation from the 7th to the 14th day. On the other hand,

those rabbits receiving 3 injections of ovalbumin alone responded poorly, with little or no antibody detectable in their serum during this period.

The enhancing action of endotoxin apparent in the results of the foregoing experiments was obtained with a course of 3 injections at 3-day intervals. It was desirable therefore, to determine whether the action of endotoxin could be attributable solely to an effect on the primary response of the host. The results of an experiment designed to determine whether endotoxin would enhance the response to a single intravenous injection of ovalbumin are given in Table II. As would be expected, the total amount of antibody was less than that developed by a series of 3 injections; however, significant concentrations

TABLE II

Enhancement of Antibody Response to Ovalbumin by Typhoid Endotoxin

Response to a single injection of ovalbumin

No. of rabbits	Immunization: single injection i.v.	Antibody nitrogen/ml. serum		
		7 days after last injection		geometric mean
		μg.		μg.
4	10 mg. ovalbumin	—	—	—
		—	—	
4	10 mg. ovalbumin	19	18	22
	+			
	1 μg. endotoxin	58	11	
3	10 mg. ovalbumin	21	58	49
	+			
	10 μg. endotoxin	85		

of antiovalbumin occurred uniformly in those rabbits receiving endotoxin, in contrast to the absence of precipitins in control animals.

Since the lipopolysaccharide derived from *Salmonella typhosa* is itself an extremely active antigen in the rabbit (quantities as small as 0.001 μg. are sufficient to elicit agglutinins in rabbits initially free of O antibody (19)), these animals produced typhoid O agglutinins to rather high titers. However, these titers were not related to the levels of antiovalbumin developed by the individual rabbits. Appropriate controls were included in these experiments in order to eliminate the possibility of any cross-reaction between ovalbumin and normal serum, or serum containing antibody to the endotoxin, and these were negative in all instances.

Endotoxic activity, *per se*, at the dosage levels employed in this and in subsequent experiments was reflected in pyrogenic responses of 3°F. or greater, accompanied by dyspnea and diarrhea, and it seemed reasonable to assume that the toxic syndrome induced by the lipo-

polysaccharide in its role as an endotoxin was implicated in its effect on antibody production. Since the characteristic toxicity of the endotoxin is most pronounced when injected intravenously, this was the route of choice. However, it was desirable to determine whether the augmenting effect could also be obtained when endotoxin was administered subcutaneously with ovalbumin.

It was observed that rabbits receiving 10 μg. of endotoxin with each of 3 subcutaneous injections of 10 mg. ovalbumin, developed twice as much antibody as did animals receiving the ovalbumin alone (geometric mean, μg. antibody nitrogen/ml. serum: 69 and 38, respectively), indicating that enhancement also occurs following administration of endotoxin by the subcutaneous route.

Effect of Separate Administration of Endotoxin and Antigen.—The antigen and lipopolysaccharide were administered together as a solution of the two products in each of the aforementioned experiments. In order to determine whether this admixture was essential for enhancement, three experiments were performed in which antigen and endotoxin were administered separately.

The first of these employed a single dose of endotoxin and/or antigen, and resulted in little' if any, enhancement when endotoxin was administered 6 hours before or 5 minutes after the ovalbumin. A more extended schedule of injections involving pretreatment of rabbits with endotoxin 16 hours before each of three injections (spaced at 3-day intervals) of ovalbumin had no effect on the subsequent antibody response. However, endotoxin given intravenously in a different site (opposite ear vein) 15 minutes after each of three injections of ovalbumin, produced an enhancement of antibody response which was comparable to that obtained when endotoxin was administered together with the antigen (Table III).

It is therefore evident that *in vitro* admixture of antigen and endotoxin is not essential for antibody enhancement to occur.

Failure of Endotoxin to Evoke a Non-Specific Anamnestic Response.—The non-specific elevation of antibody levels following administration of typhoid vaccine has been observed by a number of workers (1). To determine whether the antibody enhancement described in this report was a result of the action of endotoxin in stimulating antibody-containing cells (either directly or indirectly) to release immune globulin from a preformed cellular reservoir, the following experiment was performed. Ten rabbits previously immunized with ovalbumin were injected intravenously with 10 μg. of the lipopolysaccharide alone at a time when antiovalbumin had declined to low levels. No rise in antiovalbumin was detectable 24 hours later, indicating that endotoxin does not provoke an anamnestic reaction. Further evidence for this was found in a subsequent experiment using bovine albumin as antigen.

Failure of Typhoid Vaccine to Enhance Antibody Levels.—It was of interest to determine whether the augmentation of antibody response by this lipopolysaccharide component could be duplicated in experiments of comparable design employing typhoid bacilli. Accordingly, the effect on antibody response of

rabbits to ovalbumin was studied with two heat-killed, phenol-preserved typhoid vaccines, a monovalent vaccine prepared from *S. typhosa*, V-58, and a triple typhoid vaccine, containing *Salmonella paratyphi* A, and *Salmonella schottmülleri* in addition to *Salmonella typhosa*. The quantity of endotoxic material contained in the volume of organisms injected as the monovalent vaccine was estimated to be approximately 3 μg., while that in the triple vaccine was

TABLE III

Effect of Separate Administration of Endotoxin and Ovalbumin

No. of rabbits	Immunization: 3 injections i.v. 3-day intervals	Antibody nitrogen/ml. serum		
		7 days post injection		Geometric mean
		μg.		μg.
3	2 mg. ovalbumin	—	—	—
		—		
5	2 mg. ovalbumin	147	45	94
	+			
	5 μg. endotoxin	77		
	Given together	81	180	
4	5 μg. endotoxin given in opposite ear vein 15 min. after	35	43	70
	2 mg. ovalbumin	77	201	

considered to be 10 μg. Neither vaccine, in the dosage level employed, evoked levels of antiovalbumin greater than those of the control rabbits.

Enhancement of Antigenicity of Proteins Other Than Ovalbumin by Typhoid Endotoxin

In order to establish the scope of the antibody-enhancing capacity of typhoid endotoxin with respect to the variety and types of antigens affected, the study was extended to include the following antigens:—

Diphtheria Toxoid.—

One of the major components of combined prophylactics in use at present is diphtheria toxoid, and it was therefore of interest to determine the enhancing effect of endotoxin on this antigen. Preliminary experiments, employing 50 or 10 Lf of diphtheria toxoid in the immunization schedule of 3 injections at 3-day intervals, resulted in a 10-fold increase in antitoxin titers in those rabbits receiving 10 μg. endotoxin in conjunction with toxoid.

The marked augmentation by the lipopolysaccharide of both primary and secondary antibody response to diphtheria toxoid is shown in Table IV. The

quantity of antibody developed 10 days following the last of three injections of 5 μg. endotoxin in conjunction with 20 Lf diphtheria toxoid was approximately 30-fold greater than that following immunization with diphtheria toxoid alone. 29 days later when antitoxin had declined to low levels, a single booster injection of 5 Lf was given to control rabbits, while the other group again received lipopolysaccharide in addition to the toxoid. The data given in this table show that the anticipated secondary response was observed 7 days later in the control animals, while endotoxin-treated rabbits responded with

TABLE IV

Effect of Typhoid Endotoxin on Antibody Response to Diphtheria Toxoid

Rabbit No.	Primary immunization 3 injections i.v. at 3-day intervals	Antibody response*						
		10 days Post-immunization		29 days (Pre-booster injection)		Booster injection	7 days Postbooster injection	
		Antitoxin units	Geometric mean	Antitoxin units	Geometric mean		Antitoxin units	Geometric mean
1	20 Lf *C. diphtheriae*	0.20		0.46		5 Lf *C. diphtheriae*	3.50	
2	toxoid	0.08	0.065	0.0093	0.013	toxoid	0.60	1.0
3		0.03		0.0025			0.46	
4		0.03		0.0025			0.10	
5	20 Lf *C. diphtheriae*	1.10		0.20		5 Lf *C. diphtheriae*	6.20	
6	toxoid + 5 μg.	0.46		0.20		toxoid + 5 μg.	19.5	
7	endotoxin	0.46		0.46		endotoxin	83.0	
8		2.60	1.9	1.10	0.145		(died)	32.0
9		6.30		2.60			83.0	
10		0.46		1.10			15.0	
11		1.10		2.60			83.0	

* Determined by rabbit intradermal neutralization test.

far higher levels of antitoxin. However, the level of antitoxin present at the time of booster dose may be a determinant of the height attained in the secondary response, and inasmuch as the endotoxin-treated animals at this time exhibited approximately 10 times the antitoxin level present in controls, the magnitude of the secondary response may not be solely attributable to the booster injection of endotoxin.

Pasteurella pestis Capsular Protein.—

Plague capsular protein is a relatively poor antigen in rabbits (25). The antigenicity of this bacterial protein was enhanced more than 20-fold when it was given in conjunction with 5 μg. of typhoid endotoxin (Table V).

Measurement of antibody by the quantitative precipitin test showed that those rabbits receiving the plague antigen alone contained no precipitable anti-

body 7 or 14 days after the last immunizing injection, while the endotoxin-treated animals yielded values ranging from 48 to 130 μg. antibody nitrogen/ml. serum.

Bovine Serum Albumin.—Another example of pronounced augmentation of antigenicity by endotoxin was obtained in experiments with bovine serum

TABLE V

Enhancement of Antigenicity of P. pestis Capsular Protein by Typhoid Endotoxin

No. of rabbits	Immunization: 3 injections i.v. at 3-day intervals	Reciprocal hemagglutination titer*					
		7 days post immunization		Geometric mean	14 days post immunization		Geometric mean
4	250 μg. plague capsular protein	20 40	40 80	40	20 160	160 40	87
5	250 μg. plague capsular protein + 5 μg. endotoxin	1280 640 1280	2560 1280	1280	1280 1280 1280	2560 1280	1470

* Measured by a hemagglutination test for plague antibody (28).

TABLE VI

Enhancement of Antibody Response to Bovine Serum Albumin by Typhoid Endotoxin

No. of rabbits	Immunization: 3 injections i.v. at 3-day intervals	Antibody nitrogen/ml. serum			
		7 days after last injection			Geometric mean
		μg.			μg.
9	10 mg. bovine albumin	—*	—*	42	
		61	—*	—*	4
		—*	—*	225†	
9	10 mg. bovine albumin + 10 μg. endotoxin	147	471	119	
		477	217	117	162
		139	98	56	

* Antigen (bovine albumin) was shown to be present in these sera by a qualitative precipitin test.

‡ During the course of the experiment this animal became sick and subsequently died after ear bleeding. Consequently this value may not represent a typical response to this injection schedule of bovine albumin.

albumin. Incorporation of 10 μg. endotoxin into the schedule of injections previously employed, resulted in high antibody nitrogen values, in marked contrast to those seen in control animals receiving the antigen alone (Table VI). It is noteworthy that bovine albumin persisted in the serum of rabbits receiving this antigen alone. The significance of this observation will be discussed in a subsequent experiment.

The inability of endotoxin to evoke a non-specific anamnestic reaction, which had been demonstrated in experiments with ovalbumin, was confirmed in the bovine albumin system. The injection of 10 μg. endotoxin alone, at a time when antibody concentration had diminished to a low level did not increase the amount of circulating antibody when measured 1, 2, 4, or 7 days later.

Failure of Typhoid Endotoxin to Enhance Antigenicity of Polysaccharides

Since polysaccharides constitute an important class of antigenic substances chemically distinct from proteins, experiments were designed to determine whether the typhoid endotoxin exerted an effect on antibody response to polysaccharide antigens.[1] The results of these experiments offer evidence that the antibody response to polysaccharide antigens may not be affected by endotoxin under conditions of test shown to augment the antigenicity of proteins.

Purified Vi Antigen.—A group of four rabbits receiving 5 μg. of endotoxin together with 50 μg. of this purified bacterial polysaccharide in a series of three injections spaced at 3-day intervals, developed a mean Vi antibody titer (hemagglutination (26)) of 1:70 11 days after the last injection. The mean titer in rabbits receiving Vi antigen alone was 1:60, indicating that no enhancement of antibody response was produced. In a second experiment employing a lower dosage of Vi antigen (10 μg.), endotoxin again failed to elevate anti-Vi titers, the mean titer of 1:120 being essentially similar to that of 1:100 in control animals.

Pasteurella tularensis Vaccine.—Preliminary observations by Nicholes and Bubel (27), indicated that immunization with *P. tularensis* vaccine in rabbits subjected to a general systemic stress such as is afforded by injection of a bacterial pyrogen, resulted in levels of antibody higher than those in rabbits receiving the vaccine alone. Consequently, an attempt was made to enhance the antigenicity of their *tularensis* vaccine in a group of eight rabbits by injection of the highly pyrogenic typhoid endotoxin together with this polysaccharide encapsulated organism. The resultant mean antibody titers in endotoxin-treated and control rabbits (determined by a hemagglutination procedure employing the tularensis polysaccharide (28)) 7 days following the last of 3 injections were 1:2000 for both groups. It is therefore apparent that under these conditions the typhosa endotoxin did not augment the antigenicity of this organism.

Diplococcus pneumoniae, Type III. Since this organism also possesses a capsular polysaccharide, it was of value to determine in a similar experiment whether its antigenicity could be enhanced by endotoxin. Antibody levels (hemagglutinin (29)) in those rabbits receiving 10^9 *D. pneumoniae*, type III, alone and those in animals administered 5 μg. endotoxin in conjunction with this organism were similar, and thus provided evidence that the antigenicity of the pneumococcus was not affected by the endotoxin.

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[1] It was appreciated that the limited number of antigenic polysaccharides would markedly restrict the scope of such a study. Of the available polysaccharide antigens, the majority have been isolated from Gram-negative bacilli and possess endotoxic attributes. Consequently, their use in these experiments was precluded.

Enhancement of Antibody Response to Ovalbumin by Endotoxins of Varied Generic Origin

The somatic antigens of many Gram-negative bacilli possess endotoxic characteristics comparable to those exhibited by the lipopolysaccharide derived from *S. typhosa*. Consequently, endotoxins from a number of Gram-negative bacilli, *Proteus vulgaris*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Hemophilus pertussis* and *Brucella melitensis* were examined for activity as antibody-enhancing agents. The precipitin titers in rabbits receiving 3 injec-

TABLE VII

Enhancement of Antibody Response to Ovalbumin by Endotoxins of Varied Generic Origin

No. of rabbits	Endotoxin derived from:	Immunization: 3 injections i.v. at 3-day intervals	Antibody nitrogen/ml. serum	
			7 days after last injection	Geometric mean
			μg.	μg.
4		2 mg. ovalbumin	33 17 7 20	17
6	*Proteus vulgaris*	2 mg. ovalbumin + 100 μg. endotoxin	66 100 151 19 40 29	55
4	*Pseudomonas aeruginosa*	2 mg. ovalbumin + 500 μg. endotoxin	100 105 234 146	138
5	*Serratia marcescens*	2 mg. ovalbumin + 10 μg. endotoxin	67 39 118 56 187	84
4	*Hemophilus pertussis*	2 mg. ovalbumin + 50 μg. endotoxin	42 140 21 31	44
4	*Brucella melitensis*	2 mg. ovalbumin + 50 μg. endotoxin	24 50 43 29	35

tions of ovalbumin plus these endotoxins of varied generic origin are compared with those receiving ovalbumin alone in Table VII. All the endotoxins included in this study were effective in significantly increasing antibody to ovalbumin. However, only a single concentration of each of these products was tested, and these results are not construed as representing a quantitative comparison of the antibody-enhancing ability of endotoxins from these bacterial genera.

Possible Mechanisms of the Enhancing Action of Salmonella typhosa Endotoxin

Enhancing Capacity of Components of the Lipopolysaccharide.—The purified lipopolysaccharide used in this study has been shown to be a phosphorylated

complex, two-thirds of which is composed of polysaccharide and one-third lipide (18). It was desired to determine whether there is a causal relationship between the antibody-enhancing property of endotoxin and its bound lipide moiety, or whether this biological action is due to the polysaccharide fraction, or both. The following experiments indicate that the intact lipopolysaccharide, as isolated, may not be essential for augmentation of antibody response.

1. Endotoxin Subjected to Weak Alkaline Hydrolysis.—Exposure of typhoid endotoxin to weak alkaline hydrolysis (*i.e.* 0.02 N NaOH for 18 hours at room temperature) results in a product of reduced antigenicity, pyrogenicity, and lethality in rabbits. While it is appreciated that many unknown structural alterations in the endotoxin may occur as a result of such treatment, one significant change which was found was that it no longer yielded the usual large

TABLE VIII

Enhancement of Antibody Response to Ovalbumin by Alkali-Degraded Endotoxin

No. of rabbits	Immunization: 3 injections i.v. at 3-day intervals	Antibody nitrogen/ml. serum	
		7 days post immunization	Geometric mean
		μg.	μg.
4	2 mg. ovalbumin	— — — —	—
4	2 mg. ovalbumin + 20 μg. alkali-degraded endotoxin	41 34 15 —	18
3	2 mg. ovalbumin + 200 μg. alkali-degraded endotoxin	51 46 15	33
3	2 mg. ovalbumin + 5 μg. unaltered endotoxin	58 30 —	21

quantity of lipide following acid hydrolysis (30). Nevertheless, this product retained some ability to enhance antibody formation to ovalbumin (Table VIII). Incorporation of alkali-degraded endotoxin at levels of 20 and 200 μg. (concentrations able to evoke toxic manifestations) resulted in enhancement of antibody comparable to that observed with 5 μg. of the unaltered endotoxin.

2. Effect of Lipide on antibody-enhancement to ovalbumin.—Following hydrolysis of the endotoxin with 0.1 N acetic acid, the lipide component may be completely recovered by chloroform extraction. One hundred μg. of this lipide when suspended in an aqueous menstruum by means of a small amount of span 20, was administered intravenously to rabbits with each of 3 injections of ovalbumin. Antiovalbumin was evoked in 2 of 4 rabbits, while 4 rabbits receiving only the antigen failed to develop demonstrable antibody. In a second experiment, 20 μg. of lipide incorporated with ovalbumin, produced a geometric mean of 47 μg. antibody nitrogen/ml. serum while control rabbits developed a mean of 13 μg. of antibody nitrogen/ml. serum.

Host Susceptibility to Endotoxin as a Requirement for Antibody Enhancement.—Host susceptibility to endotoxin varies with different species of animals.

Thus, the rabbit, dog, and horse represent species possessing a high degree of susceptibility to endotoxin, as determined by pyrogenic response and lethal action. On the other hand, the mouse, guinea pig, and chick are much less susceptible to endotoxic activity (20). Consequently, a factor of considerable interest was whether host responsiveness to the toxic syndrome induced by the lipopolysaccharide was a necessary prerequisite for antibody enhancement. With this in mind, experiments were carried out to determine the antibody-enhancing capacity of endotoxin in mice, in guinea pigs, and in rabbits rendered tolerant to endotoxic action.

1. Mice.—Groups of twenty Bagg strain albino mice were given 2 injections of 1 Lf diphtheria toxoid separated by an interval of 14 days. Quantities of endotoxin extending over a

TABLE IX

Effect of Typhoid Endotoxin on Antibody Enhancement in Mice

No. of mice	Immunization: 2 Injections intraperitoneally at 14-day intervals	Antiserum titer* 14 days	Antitoxin units intradermal neutralization test 14 days
20	1 Lf diphtheria toxoid	205	<.001
20	1 Lf diphtheria toxoid + 1.0 μg. endotoxin	142	>.01 <.03
20	1 Lf diphtheria toxoid + 1.0 μg. endotoxin	205	>.03 <.01
20	1 Lf diphtheria toxoid + 10 μg. endotoxin	713	>1.0 <3.0
20	1 Lf diphtheria toxoid + 100 μg. endotoxin	1580	>1.0 <3.0

* Expressed as reciprocal of the hemagglutination titer (31).

range of 1000-fold were administered intraperitoneally in conjunction with this antigen. Table IX shows that enhancement of antitoxin levels did not become apparent until 10 μg. of endotoxin (a relatively high dose based on the weight of the animal) was administered. These levels were further increased by the addition of 10 times this amount of endotoxin.

2. Guinea pigs.—Attempts to enhance antibody formation to 5 Lf of diphtheria toxoid in guinea pigs were carried out employing both the intracardial and subcutaneous route of inoculation. Endotoxin (10 μg.) did not significantly affect the levels of antitoxin when given by either of these routes, as was evidenced by a similarity in hemagglutinin titers (31) 14 days following immunization in both control and endotoxin-treated groups of animals. A booster injection of 5 μg. endotoxin and 1 Lf toxoid 2 weeks later also failed to enhance antibody titers.

To determine whether an increase in the quantity of endotoxin would elevate antibody levels, doses of the lipopolysaccharide ranging by 2-fold increments from 25 to 200 μg. were given to groups of 4 guinea pigs in conjunction with a single injection of 5 Lf diphtheria toxoid. Antibody titers in guinea pigs which had received endotoxin were no greater 14 days later than those in controls, regardless of the quantity of endotoxin administered. One month

later, these groups of animals received a second injection consisting of 1 Lf toxoid, and a quantity of endotoxin the same as that given in the primary injection. Once again, antibody levels as measured 10 days later were unaffected by the endotoxin.

3. Rabbits Tolerant to Endotoxin.—A state of insusceptibility to the action of endotoxins can be induced in rabbits by a series of daily injections of sublethal amounts of the lipopolysaccharide (20). When conditioned in this manner, these animals do not respond to further injection of endotoxin with pyrexia, diarrhea, or other symptoms typical of those produced in normal rabbits. In order to obtain information on the antibody-enhancing capacity of the lipopolysaccharide in such a non-reactive host, eight rabbits were rendered tolerant to endotoxin by a series of 10 daily injections ranging from 1 to 20 μg. of the lipopolysaccharide. The initial dosage (1 μg.) was progressively increased by 5 μg. at 2-day intervals. On the 11th day, immediately preceding the beginning of immunization with ovalbumin, intravenous injection of 1 μg. of endotoxin failed to elicit a temperature response (average rise of 0.5°F.), indicating that these animals were in the tolerant state. This was in marked contrast to an average temperature rise of 4°F. exhibited by these animals following the initial injection of 1 μg. on the 1st day. These tolerant rabbits were randomly divided into two groups and injected with ovalbumin alone, or ovalbumin plus endotoxin. Control groups (*i.e.* non-tolerant rabbits) received ovalbumin alone, or this protein plus endotoxin. The results of this experiment are given in Table X and show that only a trace of antiovalbumin was evoked in all tolerant rabbits, whether or not they had received endotoxin; whereas the typical enhancement by endotoxin occurred in control rabbits known to be susceptible to endotoxic action.

TABLE X

Effect of Typhoid Endotoxin on Antibody Enhancement in Rabbits Tolerant to Endotoxin

No. of rabbits	Immunization: 3 injections at 3-day intervals i.v.	Antibody nitrogen/ml. serum	
		8 days after last injection	Geometric mean
		μg.	μg.
4	2 mg. ovalbumin	— —	1
		— 7	
3	2 mg. ovalbumin + 5 μg. endotoxin	50 80	79
		122	
4	*Tolerant* 2 mg. ovalbumin	15 7	3
		5 —	
4	*Tolerant* 2 mg. ovalbumin + 5 μg. endotoxin	7 —	1
		— 24	

Effect of Typhoid Endotoxin on Rate of Clearance of Antigen in Circulation of Rabbits.—During this investigation, it was noted repeatedly that sera from those rabbits receiving endotoxin with a protein antigen contained demonstrable antibody in samples taken at intervals of 1 or 2 weeks after immunization. In contrast, sera of control animals receiving the protein alone generally

were conspicuous by the presence of antigen and absence of antibody. These observations suggested that the lipopolysaccharide in some manner facilitated the removal of circulating antigen.

1. Quantitative Study of the Effect of Endotoxin on the Rate of Clearance of Bovine Serum Albumin.—A quantitative study of the influence of typhoid endotoxin on the rate of clearance of bovine serum albumin was conducted using the precipitin reaction for analysis of both circulating antigen and subsequent antibody levels. A comparison of the mean rates of clearance

TABLE XI

Effect of Typhoid Endotoxin on Clearance of Bovine Albumin from Circulation of Rabbits

Days after injection	μg. antigen nitrogen/ml serum*		
	Bovine albumin 50 mg.	50 mg. bovine albumin + 10 μg. endotoxin	50 mg. bovine albumin + 10 μg. endotoxin 4 days later
1	44	40	42
2	32.8	28.4	32.1
3	29	24	30
4	24.7	19	26
5	20	17.3	18.4
6	16.7	12	13.8
7	11	3	8
		——‡	
8	6.6	8	
9	4.4	26	
10	1.9	23	
	——‡		
12	10	31	
14	14	32	
18	25	41	
21	15	26	
25	5	12	

* Geometric mean values for groups of 5 rabbits.

‡ Figures below this line represent geometric mean values for antibody nitrogen/ml. serum.

of a single injection of 50 mg. bovine serum albumin in groups of five rabbits receiving this antigen alone, 10 μg. of endotoxin together with the albumin, or 10 μg. of endotoxin given 4 days after injection of the albumin, is illustrated in Table XI. A faster rate of clearance of antigen occurred in those rabbits receiving the endotoxin in conjunction with the bovine albumin. The subsequent antibody response was demonstrable earlier, and rose to approximately twice the level attained in rabbits receiving bovine albumin alone. It is noteworthy that the higher antibody response in the former group did not persist over an extended period of time, but diminished at the same rate as control animals.

The rate of clearance of antigen in rabbits receiving endotoxin 4 days following injection of the antigen was of the same order of magnitude as the control group. Although an increase in the rate of removal of circulating antigen appears to have occurred between the 4th and 5th day following injection of endotoxin this apparent fall was not significant when the data were

subjected to statistical analysis. Since two of these animals died on the 7th day, it was not considered justifiable to continue the mean clearance of this group.

2. Effect of Endotoxin on the Clearance Rate of a Radioactive-Labelled Protein Antigen.—The effect of endotoxin in accelerating the rate of clearance of antigen was documented further in experiments employing a radioactive-labelled protein antigen, human serum albumin. In this experiment, 30 New Zealand white rabbits were given a single intravenous injection of approximately 2 mg. human serum albumin, containing 80 to 90 μc. I^{131}. Thirteen rabbits received 10 μg. of the lipopolysaccharide together with this dose of antigen, while six were injected intravenously with endotoxin 4 days after the administration of the antigen, in order to determine whether this would cause an abrupt change in the rate of elimination of antigen. Following these injections, 15 minutes were allowed for equilibration between circulating antigen and body fluids, and a bleeding taken from all rabbits at this time represented the zero level

TABLE XII

*Essential Identity of Radioactivity Measurements of Plasma-Protein-Bound Antigen with Antigen in Whole Blood**

Day	Whole blood Per cent original antigen injected‡	Protein precipitate§ Per cent original antigen injected
0	100	100
1	50.7	50.4
5	23.8	23.4
6	19.2	19.4
7	16.8	17.1
8	14.4	14.7
11	10.0	10.0
12	8.7	8.8
13	7.2	7.1
14	6.6	6.5

‡ Blood sample taken 15 minutes after injection considered as 0 time value. Counted in scintillation counter.

§ Trichloroacetic acid precipitate counted following removal of supernatant fluid.

* Values represent arithmetic mean of 5 rabbits.

of circulating antigen. Subsequent bleedings (usually 1 to 3 ml.) were taken daily from the marginal ear vein, heparin added as an anticoagulant, and appropriate dilutions of whole blood[2] counted in a deep well scintillation counter. All counts were corrected for the normal rate of decay of the iodine isotope and are expressed as per cent of protein injected (*i.e.* 15 minute count). The circulating antigen (arithmetic mean per cent) in all groups at varying intervals is shown as a semilogarithmic plot in Fig. 1.

[2] Several investigators (32, 33) have shown a correlation between antigen concentration values in plasma found by quantitative precipitin tests and by plasma-protein-bound radioactivity determinations, and it is now accepted that the latter is an accurate measure of blood antigen concentration. Data illustrating the fact that radioactivity measurements of whole blood following the injection of I-131-labelled human serum albumin are entirely comparable to those measured as plasma-protein bound, are given in Table XII. These unpublished data were generously supplied by Dr. Kingsley Stevens, Department of Biochemistry, Army Medical Service Graduate School, Washington, and the authors are grateful for permission to include them in this report.

The rate of clearance is similar in the 3 groups until the 6th day, when a pronounced acceleration occurred in those animals which had received endotoxin together with the antigen. When 4 days were allowed to elapse before the lipopolysaccharide was administered, the subsequent rate of elimination was essentially similar to that observed in control animals. The half-life of the protein antigen was found to be 3.5 days for those rabbits receiving endo-

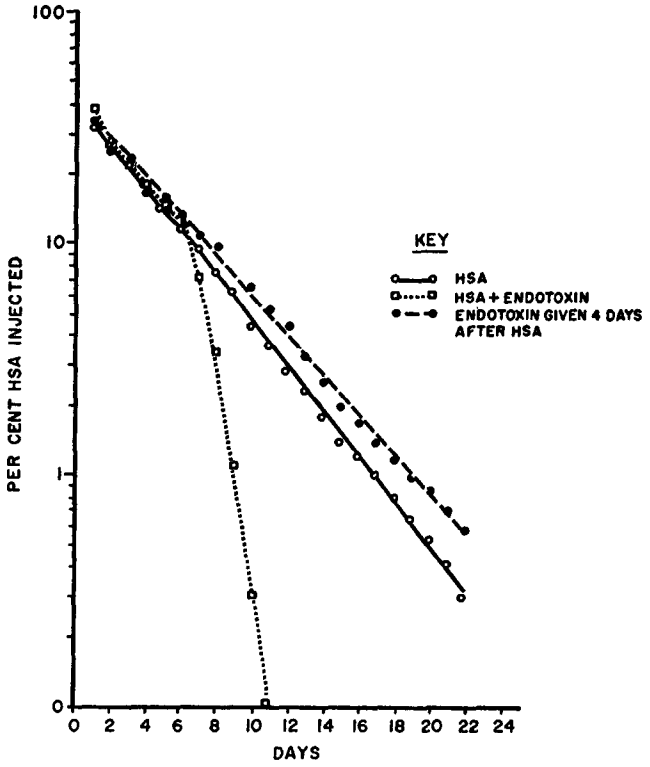

FIG. 1. Effect of *typhosa* endotoxin on rate of clearance of human serum albumin in circulation of rabbits.

toxin 4 days after human serum albumin; 3.0 days for the group receiving the antigen alone, and 0.8 days for the fast clearance portion of the curve for the group given endotoxin in conjunction with the protein.

Table XIII records the time in days required for clearance of antigen from the circulation of the individual rabbits in each group. A rather wide variation in clearance time was observed in rabbits receiving human serum albumin alone; approximately 50 per cent of these required 21 days or more to remove the antigen from their circulation. Similar variations occurred within that group in which 4 days were allowed to elapse before injection of the endotoxin.

In contrast, 100 per cent of the animals receiving endotoxin together with the antigen were able to clear the latter from their circulation in 13 days.

TABLE XIII

Effect of Typhoid Endotoxin on Clearance of HSA-I^{131} from Circulation of Rabbits

Products administered	No. of rabbits	Days required for clearance of antigen from circulation												
			9	10	11	12	13	14	16	22	25	27	28	29	30
2 mg. HSA*	11	No.	1		1	1	1		1	2	1	2		1	
		Per cent	9		18	27	36		45	64	73	91		100	
2 mg. HSA + 10 μg. endotoxin	13	No.	2	4	4	2	1								
		Per cent	15	46	77	92	100								
2 mg. HSA + 4 days later 10 μg. endotoxin	6	No.						1	1			2	1		1
		Per cent						17	33			67	83		100

* Human serum albumin.

DISCUSSION

The ability of bacterial endotoxins to enhance antibody formation to a variety of protein antigens has been well documented by the quantitative experimental results obtained in this investigation. Investigation of the mechanism of this enhancing action of endotoxin however, presents a formidable problem, inasmuch as little is known of the manner in which the physiological effects of endotoxins are engendered; furthermore, there is a paucity of information on the mechanisms involved in the synthesis of antibody. Nonetheless, the data presented provide some insight into the conditions necessary for endotoxin to bring about this powerful augmenting effect on antibody production.

The ability of the host to respond physiologically with the stress symptomatology which characterizes endotoxin appears to be essential in order for the lipopolysaccharide to exert its adjuvant action. This hypothesis is supported principally by the finding that rabbits rendered tolerant to endotoxin failed to develop elevated antibody levels after administration of endotoxin and antigen. This observation suggests that elicitation of the toxic syndrome is associated with ability to increase antibody production. Ancillary evidence for this hypothesis is provided by the following observations: (*a*) Endotoxin failed to augment antibody levels in the guinea pig. This species has been found to be highly resistant to the toxic and lethal action of the lipopolysaccharide, both in failure to exhibit a thermal response and in tolerating doses of endotoxin lethal for other species. The guinea pig LD_{50} is variable and ranges from 0.5 to 1.0 mg. This stands in marked contrast to an LD_{50} of 0.02 to 0.05 mg. for a susceptible species such as the rabbit (20); (*b*) Antibody enhancement does not occur with relatively low concentrations of endotoxin

in the mouse, a second species exhibiting a state of relative resistance to endotoxin. An elevated temperature response does not occur following injection of the lipopolysaccharide, and in this species the LD_{50} was approximately 0.3 mg. (20). However, as the dosage of endotoxin is increased and approaches a more toxic level, the mouse responds with an increase in antibody production; (*c*) Antibody enhancement in the rabbit appears to be dependent on the administration of a minimum toxic level of the lipopolysaccharide. Quantities of endotoxin which are insufficient to produce an enhancement of antibody formation are also noticeably less toxic to this species. However, the antibody-enhancing action of endotoxin is not associated only with its capacity to induce pyrexia, resulting in a non-specific rise in metabolic activity. This pyrogenic attribute of the lipopolysaccharide had been shown to lie in the 0.001 to 0.1 μg. range, with the latter dose eliciting a maximum increase of 3 to 5°F. in the temperature of rabbits (20). Yet, quantities of endotoxin less than 1 μg. failed to increase antibody formation even though they were considerably more than the quantity of this substance necessary to evoke a febrile response.

The ability of certain lipides to bind protein molecules is well known. Since approximately ⅓ of the *typhosa* lipopolysaccharide is comprised of lipide, the possibility was recognized that endotoxin might be entering into combination with the protein antigen, thereby resulting in changes in the physical state of the antigen. Such changes could conceivably be reflected in an increased order of antigenicity by directing the antigen into more, or different, antibody-producing cells, or by causing a retention of the antigen *in vivo* for longer periods of time. However, the administration of lipopolysaccharide and ovalbumin individually, separated by an interval of 15 minutes, still resulted in enhancement of antiovalbumin levels comparable to that achieved in animals receiving these products together. From this observation and the evidence previously discussed, it is concluded that the enhancing property of endotoxin is mediated through the host and is not a result of direct action on the antigen, *per se*.

Endotoxin given in conjunction with antigen (human serum albumin) has been shown in this study to result in a rapid clearance of the latter from the circulation. On the other hand, the lipopolysaccharide did not cause this effect when given 4 days after the antigen, indicating that the effect of endotoxin is exerted early, and may be limited to an induction phase of antibody formation.[3] This observation that the later injection of endotoxin did not result in an increase in antibody production (as would be reflected by an accelerated clearance rate of antigen) provides some evidence that perhaps only the basic

[3] This limitation has also been found to apply to the effect on antibody production produced by sublethal doses of x-irradiation (34). While this effect is inhibitory, in contrast to the enhancing action of endotoxin, neither stimulus acts when given several days after antigen. Thus, the lack of a positive effect when endotoxin is given 4 days after antigen may be considered as additional evidence for the existence of an induction phase in the formation of antibody.

level of antigen acquired within a cell during this induction period is necessary to fully activate its antibody-forming mechanism.

The proliferation of plasma cells is a major histological change manifested by the host following administration of endotoxin-bearing organisms (35). No histological examinations were made in this study, but it is a reasonable assumption that plasma cell proliferation was induced by the lipopolysaccharide. Such an increase in antibody-forming cells by endotoxin could possibly participate in elevating antibody levels by causing distribution of antigen among greater numbers of these cells. However, the absence of an accelerated phase of antigen clearance following any plasma cell proliferation induced by endotoxin administered 4 days after antigen, indicates that this may not be the major factor responsible for the increased antibody levels observed in this study.

On the basis of the aforementioned, it is postulated that the enhancing action of endotoxin is mediated through a cellular constituent concerned in the induction phase of antibody formation. Such a constituent logically could be considered a self-duplicating template for the production of antibody, the formation of which is genetically controlled (36). It is postulated that this template (or the gene responsible for its formation) in some unknown manner is activated by endotoxin, and in this activated state is more susceptible to the effects of antigen.[4]

The adjuvant characteristics of the lipopolysaccharide and the Freund-type water-in-oil emulsions have not been compared experimentally; nevertheless, the data in this report are sufficient to indicate that a major difference exists in the means by which these enhancing agents act. The prolongation of antibody response, one of the cardinal features of adjuvants such as water-in-oil emulsions and mineral carriers, was not found to be associated with the increased antibody responses produced by the lipopolysaccharide. In this respect, the tissue retention of antigen, thought to be a major factor in bringing about the persistence of an elevated antibody response by other adjuvants, does not appear likely to be a characteristic of endotoxin-induced enhancement. The rapid removal of antigen from the circulation of animals receiving endotoxin may be interpreted as indirect evidence for enhancement to occur without tissue retention of antigen. However, it is well to remember that the latter comparison involves different sites of initial antigen deposition.

The possibility is recognized that endotoxins may stimulate the production

[4] Varying degrees of template reactivity may occur following antigenic stimulus in the normal host (no endotoxin treatment) as is brought out by the data in Table XIII. When these antigen clearance rates of rabbits receiving human serum albumin alone were plotted individually, approximately 40 per cent of the animals exhibited an accelerated rate after the 6th day. On the other hand, endotoxin appeared to cause a uniformly high degree of reactivity of the postulated template for the antigen, the resultant antibody production occurred, and was associated with an accelerated removal of antigen.

of serum constituents other than antibody. Of interest in this respect are a number of immunologically reactive serum components known to occur in the serum of rabbits. Of these, two may be considered in association with any elevation in serum proteins following administration of bacterial endotoxins. One, Cx-reactive protein, is an acute phase substance associated with certain experimentally induced inflammatory conditions in rabbits. Recent studies by Wood (37), have provided evidence that certain protein antigens *per se* also are capable of evoking production of Cx-reactive protein. Furthermore, he found a correlation between the amount of this substance produced and the subsequent development of precipitin titers. A second constituent appearing in the serum of rabbits following injection of killed Gram-negative bacteria has been the subject of a series of studies by Coombs *et al.* (38) who termed it immunoconglutinin. They presented evidence that this serum factor functions as a specific antibody directed against complement. The stress condition produced in the host by the purified endotoxin may be comparable to that known to be required for both the experimental production of immunoconglutinin and for acute infections which result in host elaboration of Cx-reactive protein. Consequently, it is possible that endotoxin (lipopolysaccharide) may be a bacterial component in Gram-negative bacilli responsible for a general activation of protein synthesis by the host, which is represented only in part by an increased production of antibody.

SUMMARY

Quantitative studies have demonstrated that a purified lipopolysaccharide (endotoxin) derived from the O-901 strain of *Salmonella typhosa* markedly enhanced the antibody response of rabbits when given separately or in conjunction with protein antigens. The augmentation of antibody levels varied from 2- to 40-fold with the number of injections, the dosage of antigen and endotoxin, and the route of administration. This antibody-enhancing property was found to be common to a broad group of endotoxins from Gram-negative bacilli and was not restricted to the lipopolysaccharide derived from *S. typhosa*.

Factors affecting this enhancement were investigated, and data are presented which indicate that host susceptibility to endotoxin is a prerequisite for elevation of antibody levels; the intact lipopolysaccharide, as isolated, might not be essential for this activity; and the rate of clearance of antigen from the circulation of rabbits was accelerated when endotoxin was given in conjunction with protein.

The data obtained are discussed in relation to postulated mechanisms on the antibody-enhancing action of endotoxin.

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