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Effect of Meningococcal Endotoxin on the Immune Response.* (22032)

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Administration of sublethal quantities of various preparations of Gram negative bacterial endotoxin produce fever, leucocytosis preceded by leucopenia, depletion of liver glycogen, alterations in blood sugar levels, vasomotor disturbances and hemorrhagic necrosis of rapidly growing tumor tissues(1,2). In addition to these systemic reactions endotoxins produce profound effects on the host's natural defense mechanisms(3-5). An intravenous injection of suitable endotoxin establishes a state of "preparation" for the generalized Shwartzman phenomenon(6), so that a subsequent intravenous injection of endotoxin results in widespread hemorrhagic necrosis in the visceral organs of rabbits. The rabbit in this "prepared state" exhibits impaired activity of the reticuloendothelial system, as measured by rate of clearance of colloidal gold or chromic phosphate from the systemic circulation(3,5). Moreover, rabbits receiving a single intravenous injection of a bacterial free meningococcal culture filtrate prior to production of a pneumococcal skin infection show a marked decrease in resistance to the infection (4). The demonstration that phagocytic activity and resistance to experimental infections can be significantly altered by Gram negative bacterial endotoxins suggested that these compounds might alter the immune response. Our study was undertaken to determine the effect of various doses of meningococcal endotoxin on antibody response of rabbits to a single, purified foreign serum protein, crystallized bovine serum albumin.

Materials and methods. Hybrid albino rabbits of both sexes, obtained from a single breeding stock and weighing 2 kilos were used. Rabbits were maintained on diet of Purina rabbit pellets and were free of disease. The endotoxin employed was derived from a strain of meningococcus (44-B) supplied by Dr. Gregory Shwartzman, Mount Sinai Hospital, New York. The method for preparing the toxin has been described in an earlier communication(7). The antigen used was crystallized (BSA) bovine plasma albumin, lot #67009, obtained from Armour and Co. All toxin dilutions and antigen solutions were made up in pyrogen free saline just prior to use. Solutions for injection were prepared so that the final desired dilution of toxin plus the antigen, 15 mg BSA, were contained in total volume of 2 cc. Control animals received 15 mg of BSA in 2 cc volume of pyrogen free saline. Injections were given by way of marginal ear vein. Eleven days after antigenic stimulation, at a time when circulating antibody was presumed to be at its peak level

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TABLE I. Effect of Massive Dose of Meningococcal Endotoxin on Immune Response.

Group	Procedure†	Animal No.	Antibody content of serum, γn/ml	t	P
I	Bovine serum albumin, 15 mg Toxin 1-10 dilution	1652	30		
		1654	80		
		1656	60		
		1651	60		
		1658	36		
		1659	28		
		1661	10		
		1663	60		
			Mean 45 ± 7.62*		
II	Controls, bovine serum albumin, 15 mg	1676	28		
		1677	10		
		1678	28		
		1679	48		
		1680	60		
		1681	32		
		1682	10		
		1683	20		
		1684	50		
			Mean 31 ± 5.55		
I and II				1.259	>.10

* Stand. error of mean.
† Each group received 2 ml I.V. of injection solution made in pyrogen free saline.

(8), blood was withdrawn by cardiac puncture and serum separated, quick frozen at −70°C and kept in storage at −30°C until antibody content was determined. The quantitative precipitin technic for determining serum antibody content was a modification of the method described by Heidelberger and Kendall(9). The frozen serum samples were thawed 10 minutes at 37°C, then centrifuged at 3000 rpm at 4°C for one hour. The antibody content was determined at two separate times, a preliminary precipitation using ½ ml portions of serum and final determination using 1 ml of serum. Fresh standard antigen solutions were prepared for each precipitation. Preliminary tests were made to determine antigen concentrations that would precipitate more than ⅓ of the antibody and approach the equivalence point. The serum sample from each animal was precipitated with 4 different concentrations of antigen, incubated at 37°C for 1 hour and placed at 4°C for 7 days. The precipitates were broken up daily by gently swirling contents of each precipitin tube. At end of incubation period the tubes were centrifuged at 2500 rpm for one hour at 4°C, the supernate decanted and precipitates washed with 0.145 molar saline in the cold. This operation was repeated twice. Total nitrogen of precipitate was determined colorimetrically using Heidelberger-MacPherson modification of the Folin-Ciocalteu color reaction(10). Optical density was read at 650 μ on a Coleman Universal Spectrophotometer Model 14. Optical density was converted to nitrogen content by reading a standard calibration curve prepared with a highly purified preparation of human gamma globulin generously supplied by Dr. John T. Edsall, Harvard University. Supernatant analysis for antigen excess was performed in capillary tubes using a hyperimmune anti BSA serum. Capillary tubes were incubated at 37°C for 1 hour, placed at 4°C for 36 hours, then read for presence or absence of a precipitate. Final determinations were performed in identical manner using instead 1 ml serum samples. Antigen solutions for precipitations were made in 0.145 molar nitrogen free sodium chloride solutions. Nitrogen content of standard antigen solution was determined by the micro Kjeldahl method using an all-glass modification of the Markham distillation apparatus. Dilutions of a culture free meningococcal filtrate ranging from 1-10 to 1-10000 were selected to determine their effect on response of rabbits to a standard antigenic stimulation with crystallized bovine serum albumin.

TABLE II. Enhancement of Antibody Production by Meningococcal Endotoxin.

Group	Procedure†	Animal No.	Antibody content of serum, γn/ml	t	P
I	Bovine serum albumin, 15 mg Toxin 1-500 dilution	1013	96		
		1014	163		
		1015	44		
		1016	10		
		1017	50		
		1018	131		
		1019	240		
		1020	35		
			Mean 96 ± 25.74*		
II	Bovine serum albumin, 15 mg Toxin 1-100 dilution	1037	39		
		1038	99		
		1039	139		
		1040	67		
		1042	63		
		1043	49		
		1044	48		
			Mean 70 ± 12.13		
III	Controls, bovine serum albumin, 15 mg	1676	28		
		1677	10		
		1678	28		
		1679	48		
		1680	60		
		1681	32		
		1682	10		
		1683	20		
		1684	50		
			Mean 31 ± 5.55		
I and III				2.378	<.05
II and III				2.95	<.05

* Stand. error of mean.
† Each group received 2 ml I.V. of injection solution made in pyrogen free saline.

Results. Effects of Large Dose of Endotoxin on Immune Response. Intravenous injection of 2 cc of the toxin dilution (1-10) produced death within 24 hours after administration in 50% of treated animals. From these effects it was assumed that this dosage of endotoxin is the maximum feasible amount which can be employed to study the effect of meningococcal endotoxin on immune response. As shown in Table I, antibody levels of toxin-treated rabbits, although more variable and slightly higher than those of control rabbits, do not differ significantly from those of the latter group. No evidence of interference with antibody production by this amount of endotoxin was observed. In contrast, as shown in Table II, a smaller amount of endotoxin produced significant enhancement of antibody production.

Optimal Dosage of Meningococcal Endotoxin for Enhancement of Antibody Production. The effect of varying quantities of endotoxin on immune response was investigated. Dosages of endotoxin ranging from large amounts capable of producing death or severe prostration in a high percentage of rabbits (2 cc of a 1-10 dilution) through the range capable of inducing profound circulatory changes, preparation for generalized Shwartzman reaction, and provocation of local Shwartzman reaction, (2 cc of 1-100 and 1-500 dilution) to small dosages capable of inducing a pyrogenic effect but little other evidence of intoxication (2 cc of 1-1000 to 1-10000 dilution) were employed. Results are summarized in Fig. 1. Here it may be seen that while neither the large doses nor the smallest doses of endotoxin used produced significant enhancement of antibody production, increased antibody formation occurred when an intermediate dosage was employed. Optimum dilution of meningococcal endotoxin for this effect lies somewhere between 1-100 and 1-500. This is the amount of toxin

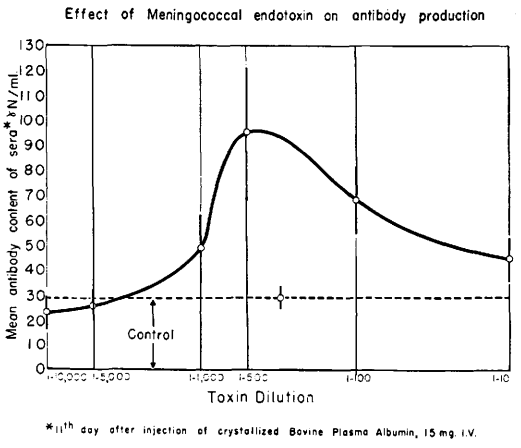

FIG. 1.

which produces profound circulatory changes, preparation for the generalized Shwartzman reaction and provocation of the local Shwartzman reaction in rabbits.

Quantities of toxin capable of producing a vigorous febrile response as well as characteristic alterations of granulocytes (1-5000 to 1-10000) do not act as an adjuvant to antibody production. Slight enhancement of antibody production was noted when 2 cc of the 1-1000 dilution were administered.

Discussion. Increased serum levels of antibody to bovine albumin are produced when a single injection of meningococcal endotoxin is given with it. Maximal enhancement of antibody production was demonstrated with quantities of toxin which are effective in preparing for generalized Shwartzman phenomenon. Massive doses of the toxin did not inhibit immune response as might have been expected from the demonstrated interference of endotoxin with reticuloendothelial function. Instead a slight statistically insignificant enhancement of antibody production was observed. Small quantities of endotoxin which consistently produce a pyrogenic effect and certain other reactions characteristic of this group of substances had no demonstrable effect on immune response of rabbits to the antigen.

Enhancement of immune response with quantities of meningococcal endotoxin employed was an unexpected finding. Cornwell and Good(3) studying the effect of substances such as cortisone, thorotrast and Gram negative bacterial endotoxins on phagocytic activity of reticuloendothelial system found that meningococcal endotoxin, in quantities which this study has shown to enhance antibody production, was most effective in decreasing the rate of clearance of radio colloidal gold from systemic circulation of rats and rabbits. Furthermore, it has been demonstrated that this same quantity of endotoxin results in a marked enhancement of susceptibility of rabbits to experimental infections with pyogenic microorganisms(4). On the basis of these observations it was predicted that a single injection of this dose of endotoxin would result in diminished production of antibody against an unrelated antigen administered simultaneously. Instead of diminishing antibody production the adjuvant effect herein described was observed. The basis for enhancement of antibody production by meningococcal endotoxin is not clear. It is possible that this action is related to the effect of endotoxins on phagocytic capacity of reticuloendothelial system previously mentioned. In support of this concept is the early demonstration by Lewis and Loomis(11) that certain dosages of trypan blue increased production of hemolysin against sheep erythrocytes. Other investigations, however, employing different blockading agents and even different dosages of trypan blue in association with varying immunization procedures, have demonstrated decreased rather than increased antibody production to result from "blockade" of the R. E. system(12,13).

Alterations in distribution of antigen based on changes in circulatory pattern in key organs or even alterations in vascular permeability might be responsible for the enhancement of antibody production herein reported. It seems possible that endotoxins might in some way activate the multipotent reticulum, the differentiation of which appears to be responsible for antibody formation in the rabbit (14). Unfortunately, discussion of the mechanism involved in reactions produced by Gram-negative bacterial endotoxins is hampered by lack of direct information concerning initial site of their action. Indeed, the biologic basis for the toxic properties of these sub-

stances has thus far not been elucidated. In general, however, it has been found that reactions produced by endotoxins derived from one group of Gram-negative bacteria are qualitatively indistinguishable from those produced by preparations from other unrelated Gram-negative bacteria(1,2,15). The phenomenon of enhanced antibody production appears to be no exception. In a recent communication, Johnson *et al.*(16) reported a marked enhancement of antibody production against egg albumin when this antigen was given in conjunction with a highly purified lipopolysaccharide antigen (endotoxin) derived from *Salmonella typhosa*.

The recent observations of Stetson(17) that reactions to Gram-negative endotoxins are strikingly similar to reactions produced with tuberculin in specifically sensitized rabbits are provocative and may represent a step toward better understanding of reactions to endotoxins from Gram-negative bacilli. These studies combined with the early demonstration by Lewis and Loomis(18) that guinea pigs infected with tubercle bacilli produce more antibody than do control uninfected animals suggest that perhaps a similar process is responsible for enhancement of antibody production in the two circumstances. Whether tuberculin in appropriately sensitized animals would act as does endotoxin in enhancing production of circulating antibodies is being investigated in our laboratory.

Many agents have been employed to augment production of circulating antibodies. Some of the more common include alum precipitation of antigen, tapioca, staphylococcus toxin and oil-in-water emulsions to which ground killed tubercle bacilli may be added (19,20). The mode of action of these various adjuvants has been generally attributed to production of prolonged inflammatory reactions associated with antigen retention at the site of injection(21-23). Halbert *et al.* (21) reported that with oleacious adjuvants appreciable quantities of antigen remain at the injection site for periods exceeding 22 weeks. However, it seems unlikely that the adjuvant property reported in our studies can be explained on the basis of prolonged retention of antigen at a local depot with consequent slow release and better antigenic stimulation. Rather, it seems likely to us that the ultimate explanation for the adjuvant effect of endotoxin lies in understanding the effect of endotoxin on the cellular system responsible for antibody production. This hypothesis has the advantage of permitting further investigation of the adjuvant action of endotoxin by morphological technics.

Summary. 1. The enhancement of antibody production against crystalline bovine serum albumin by the simultaneous injection of meningococcal endotoxin is reported. 2. An optimum dosage of endotoxin for production of the adjuvant effect is approximately the same as that required to prepare rabbits for the generalized Shwartzman reaction or to provoke the local Shwartzman phenomenon. 3. Failure of inhibition of antibody production by dosages of endotoxin capable of producing delayed uptake of radio active colloidal gold by the reticuloendothelial system is mentioned. 4. Possible mechanisms responsible for the adjuvant phenomenon are discussed.

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Binding of S^{35}-Labeled Sulfate in Serum of Rats as Studied by Filter-Paper Electrophoresis.* (22033)

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Filter paper electrophoresis of blood serum or plasma has been used to study the protein binding of radioactive substances(1-5). Dziewiatkowski(6) recently reported some observations on the association of sulfate sulfur with serum proteins. The present study was undertaken to extend our knowledge of sulfate binding by serum constituents. The results indicate that about 40% of the S^{35}-sulfate administered to Sprague-Dawley rats becomes bound to a substance or substances which migrate to the α_1-globulin region during electrophoresis on filter paper.

Materials and methods. Male Sprague-Dawley rats weighing 300-400 g were injected intravenously with 1200 μc of carrier-free $Na_2S^{35}O_4$. Blood was withdrawn from the heart 24 or 48 hours postinjection with a syringe containing 0.2 ml of mineral oil. The clot was spun down and the serum drawn off with a syringe. The S^{35} activity of the whole serum was determined by counting 100-μl samples in a gas flow proportional counter. Duplicate samples were spread on flat metal planchets and air dried for counting. The serum to be used for electrophoresis was stored at −30°C. Electrophoresis of 25 μl of blood serum placed on 3 by 29 cm strips of Whatman No. 1 chromatography filter paper was carried out in an apparatus similar to that described by Durrum(7). The samples were subjected to electrophoresis for 10 hours in a barbiturate buffer of ionic strength 0.05 and pH 8.6. Best results were obtained when the strips were immersed in the buffer and thoroughly blotted before the sample was applied. The current was maintained at 1.0 ma/strip, and the initial and final potentials were 300 and 150 volts, respectively. Following electrophoresis, the strips were dried at 60°C and developed in tetrabromophenolsulfonphthalein according to the procedure of Durrum(7). The dried and stained strips were scanned with a white light used in conjunction with a Photovolt photometer, and the optical density was recorded at 3-mm intervals. A standard serum electrophoresis pattern was obtained by plotting the optical densities against the distances from the origin. As an indication of the presence of carbohydrates, some of the strips were restained by the periodic acid-Schiff (PAS) routine according to the method of Köiw and Grönwall(8). The radioactivity associated with the serum proteins was determined by cutting the strips into 6 to 24 mm segments. Long or short segments were used where the activity was low or high, respectively. The segments were taped to planchets, and the S^{35} activity was determined in a gas flow proportional counter.

In view of the low S^{35} activity present on the segments of filter paper, it was necessary to

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