Induction of Nonspecific Resistance by Endotoxin in Unresponsive Mice

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ABSTRACT

MARGHERITA, S. S. (Temple University School of Medicine, Philadelphia, Pa.), AND H. FRIEDMAN. Induction of nonspecific resistance by endotoxin in unresponsive mice. *J. Bacteriol.* **89**:277-280. 1965.—*Serratia marcescens* endotoxin was tested for protective effect in animals of varying immunological competence. A quantity of endotoxin capable of inducing resistance in mice to a standardized challenge of *Diplococcus pneumoniae* was equally effective in animals immunologically paralyzed with a high dose of homologous pneumococcal polysaccharide. Failure of the unresponsive state to influence the degree of resistance induced by endotoxin suggests that a specific humoral factor does not play a significant role in the nonspecific resistance.

Current theories regarding the mechanisms by which nonspecific resistance to infectious agents is induced by lipopolysaccharides of gram-negative bacteria or extracts of mycobacteria implicate strongly a stimulation of the reticuloendothelial system (Shilo, 1959). The observation that endotoxins may cause proliferation of lymphoid cells (Merritt and Johnson, 1963) suggests that these bacterial products may, in addition to their effect on the reticuloendothelial system, contribute to nonspecific resistance by causing accelerated proliferation of cells, present in low numbers, and producing "natural" antibody.

The direct evidence for the role of antibody in this phenomenon, however, is conflicting. Michael and Massell (1962) showed that endotoxin protected against hemolytic streptococci only in the presence of specific anti-M serum in concentrations too low for conferring passive protection alone, and suggested that a humoral component could not have been induced by endotoxin in that system. Cooper and Stuart (1962) were able to protect against pneumococci with endotoxin from gram-negative organisms, but did not find opsonins in the sera of such animals. The findings of these two groups of workers are at variance with those reports demonstrating the presence of humoral factors bactericidal for gram-negative bacteria in the sera of endotoxin-treated animals (Whitby et al., 1961).

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The experiments to be reported here describe attempts to clarify the role of antibody in nonspecific resistance to a gram-positive pathogen, *Diplococcus pneumoniae*, by comparing the activity of endotoxin in immunologically paralyzed and competent animals.

MATERIALS AND METHODS

Experimental animals. Male, Swiss-Webster mice (18 to 22 g) were obtained from Huntingdon Farms, Philadelphia, Pa. They were housed, usually ungrouped, for 4 to 7 days before use and were given free access to water and pellets throughout this period and during the course of the experiments.

Culture. The challenge organism, *D. pneumoniae*, was a type VIII encapsulated strain obtained from the American Type Culture Collection. It was passed once in mice after primary cultivation on blood-agar. The isolate from heart blood was grown in Brain Heart Infusion (BHI, Difco) broth for 18 hr, and portions were lyophilized. The LD₅₀ of the isolate, for the intraperitoneal route, was determined by the Reed and Muench method (Rivers, 1952) and was found to be approximately 23 organisms.

Endotoxin and pneumococcal polysaccharide. Type VIII pneumococcal polysaccharide (SVIII) and *Serratia marcescens* endotoxin were kindly provided by M. Heidelberger and A. Nowotny, respectively. The polysaccharide was a phenol-extracted preparation, and gave a single precipitin band in the Ouchterlony double-diffusion test with specific hyperimmune rabbit antiserum. The endotoxin, a crude trichloroacetic acid preparation, had an LD₅₀ for mice of approximately 700 µg when injected intraperitoneally. For injection

purposes, endotoxin and polysaccharides were dissolved in 0.15 M saline, buffered with 0.005 M phosphate at pH 7.2.

Challenge procedure. The source of the challenge inoculum was an 18- to 24-hr culture of *D. pneumoniae* in BHI. The number of viable cells was determined by spreading 0.2 ml of dilutions onto each of five blood-agar plates and determining the number of α -hemolytic colonies appearing after incubation at 37 C for 24 hr. A check of viability immediately before injection showed the count to remain fairly constant during the period of the experiment. Challenged animals were observed for deaths daily. Randomly selected dead animals were autopsied, and heart blood was cultured for pneumococci.

RESULTS

The results of experiments designed to locate the immunizing and paralyzing dose ranges of SVIII are summarized in Tables 1 and 2. Different responses to challenge were observed in groups previously given varying amounts of polysaccharide (Table 1). Thus, injection of 20 LD₅₀ doses of viable pneumococci into animals given 0.1 μ g of SVIII resulted in 60% survival; 100 μ g of the polysaccharide protected none of the animals similarly challenged. Table 2 illustrates the results of a supplementary experiment confirming the paralyzing effect of high doses of SVIII. Mice injected intraperitoneally with doses ranging from 10 to 1,000 μ g showed varying degrees of paralysis, even when a second immunizing dose of SVIII was given 3 months later. Failure of the known immunizing dose to protect animals previously given excessive amounts of the same antigen satisfies the criterion for paralysis established by Felton (1949).

The capacity of endotoxin to protect against pneumococcal challenge was examined by giving mice varying doses of endotoxin and challenging

TABLE 1. Response to challenge* with viable type VIII pneumococci after injection of various doses of type VIII pneumococcal polysaccharide (SVIII)

Group	Dose (SVIII)	Total survivors	Survival
	μ g		%
A	0.01	2/5	40
B	0.1	3/5	60
C	1.0	2/5	40
D	10.0	1/5	20
E	100.0	0/5	0
F	—	0/4	0

* All groups challenged intraperitoneally 7 days after intraperitoneal injection of SVIII. Challenge dose = 20 LD₅₀ = 460 organisms.

TABLE 2. Failure of immunizing dose* to protect mice pretreated with high doses of SVIII against challenge with viable type VIII pneumococci†

Group	Primary SVIII dose	Survivors (of total injected)	Survival
	μ g		%
A	1,000	0/6	0
B	500	0/3	0
C	250	0/5	0
D	125	0/5	0
E	100	1/6	16.7
F	10	2/6	33.3
G	—	1/5	20.0

* SVIII (0.1 μ g) injected intraperitoneally into the animals of groups A to F, 3 months after primary injection.

† All groups challenged with 10 to 20 LD₅₀ doses 7 days after immunizing injection.

TABLE 3. Protection afforded by *Serratia marcescens* against challenge with 100 LD₅₀ doses of type VIII pneumococci*

Endotoxin dose	No. of animals dying on days				Mean survival time (days)
	1	2	3	4	
μ g					
10	0	3	1	1	2.6
50	0	0	7	10†	3.6
100	0	2	2	1	2.8
—	3	1	7	0	2.4

* Challenge dose given intraperitoneally 20 hr after intraperitoneal administration of endotoxin.

† One animal survived challenge.

with 100 LD₅₀ doses of viable pneumococci within 24 hr. The results demonstrating nonspecific protection are summarized in Table 3. Resistance was measured as the increase in time to death after challenge. A 50- μ g amount produced the greatest degree of protection; doses higher and lower than this amount were less effective in this respect, owing perhaps to suboptimal concentration in the former case, and to a toxic effect in the latter.

Experiments to test for the effect of specific immunity and paralysis on the capacity of endotoxin to protect nonspecifically were designed in the following manner. Mice were given an initial intraperitoneal injection of SVIII; this was followed 7 days later with a protective dose of endotoxin, also by this route. Animals were challenged with 2 to 3 LD₅₀ doses of viable pneumococci 20 hr later. The results of a typical experiment are summarized in Table 4. The data

revealed that (i) 50 μ g of *Serratia* endotoxin produced approximately a five-fold increase in survival for animals not injected with SVIII (comparison of group E with F), and (ii) pre-establishment of an unresponsive state did not diminish the protective effect of endotoxin. In addition, the data suggest that endotoxin may have increased the resistance of animals given the lower SVIII dose; however, the level of the specific immunity induced by the polysaccharide was higher than that required to show such an effect conclusively. Survival time of animals succumbing was longer in groups given endotoxin than in those given the respective initial injection only. Differences in time to death, though not strictly comparable in magnitude with percentage of survivors in the various groups, paralleled the results obtained with that response measurement.

DISCUSSION

It has been postulated that substances capable of inducing nonspecific resistance do so by causing a general enhancement of antibody response (Whitby et al., 1961). Elucidation of the mechanism by which endotoxin protects requires a basic knowledge of its biological activity. Endotoxin has been shown to decrease the lag period of antibody formation and to cause elevated levels of antibodies (Merritt and Johnson, 1963). These changes were associated with lymphoid tissue hyperplasia and suggested a proliferative effect of endotoxin. More direct and quantitative evidence has been provided by utilization of the in vitro plaque method of Jerne and Nordin (1963) for determining antibody-producing cells. Using this technique, Heuer and Pernis (1964) were able to show an increase in the number of lymphoid cells producing antibody against sheep erythrocytes in the spleens of animals injected only with endotoxin, prepared from *Escherichia coli*. Although the proliferation may have been due to secondary stimulation, caused by shared determinant groups present in sheep red blood cell stroma antigen and endotoxin, a mechanism for nonspecific resistance based on classical antibody was suggested by these findings.

The experiments reported here were designed to show whether or not endotoxin could also cause nonspecific production of antibodies against pneumococcal polysaccharide. The finding that pre-establishment of the tolerant state did not result in abolition of the protection afforded by endotoxin suggests that classical antibody does not play a significant role. This interpretation would lend support to the work of Cooper and Stuart (1962), who could not detect opsonins in the sera

TABLE 4. *Effect of endotoxin on mice given immunizing or paralyzing doses of type VIII pneumococcal polysaccharide (SVIII)**

Group	Dose		Avg time to death (days)	Survivors (of total injected)	Survival	P†
	SVIII	Endotoxin				
	μ g	μ g			%	
A	100	50	2.5	6/10	60	<0.01
B	100	—	2.3	0/9	0	
C	0.5	50	3.0	9/10	90	0.1
D	0.5	—	2.7	7/10	70	
E	—	50	3.0	5/9	55.6	<0.01
F	—	—	2.1	1/10	10	

* All injections given intraperitoneally. Schedule of injections as follows: SVIII, day 1; endotoxin, day 8; challenge, day 9, 20 hr after endotoxin injection. Challenge dose = 2 to 3 LD₅₀.

† P = probability calculated by χ^2 method. Comparisons are of groups given initial injection and endotoxin with those given only the corresponding primary treatment.

of animals protected against pneumococci by injection of endotoxin. The experiments demonstrating increased levels of bactericidins active against gram-negative bacteria in the sera of animals treated with reticuloendothelial system stimulants (Whitby et al., 1961) may have been due to greater sensitivity of that method. It is equally possible, however, that there exists a basic difference in the mechanisms of nonspecific resistance to gram-positive and gram-negative bacteria.

An alternative explanation, that of overcoming of the tolerant state, can also be invoked to explain the findings. Such termination of the unresponsive state has been shown by Weigle (1962) to be brought about in animals made tolerant to protein by challenging with homologous protein containing haptenic side groups, and by Neep and Seastone (1963) by injection of water-in-oil emulsion of pneumococcal polysaccharide in animals paralyzed by injection of large doses of this antigen. The experiments reported here could not provide direct evidence for such a response to endotoxin.

Nevertheless, the findings of Cohen, Crosby, and Talmage (1964), that endotoxin is capable of converting a state of immunological incompetence in sublethally irradiated mice given isologous spleen cells from immunized donors to a state of antibody formation, suggest that a similar effect of endotoxin may be operative in the experiments described here. It may be significant, however, that Neep and Seastone (1963) were not able to demonstrate overcoming of unrespon-

siveness to pneumococcal polysaccharide by injection of *E. coli* endotoxin.

Experiments to determine more precisely the changes occurring upon injection of endotoxin into animals rendered immune or unresponsive seem warranted. These are currently in progress.

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