FACTORS INVOLVED IN THE INDUCTION OF NON-SPECIFIC RESISTANCE TO STREPTOCOCCAL INFECTION IN MICE BY ENDOTOXIN*

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Development of non-specific resistance to infection following the administration of endotoxin has been repeatedly demonstrated with regard to infections caused by Gram-negative pathogens (1). It has been shown that endotoxin increases the activity of the reticuloendothelial system (2) and causes a temporary elevation of specific antibodies against many species of Gram-negative bacteria.

Although limited observations have been made on the induction of non-specific resistance to Gram-positive bacteria, such as staphylococci (3, 4), the factors involved in the development of this resistance have not been clarified.

In the present study, which involves experimental streptococcal infection, we have investigated the role of cellular and humoral factors in the induction of non-specific resistance.

Materials and Methods

Endotoxins.—Commercial preparations, made by the Boivin method, were obtained from Difco Company, Detroit. These endotoxins, derived from *Escherichia coli* 0127:B4, *Salmonella enteritidis*, and *Shigella flexneri*, were suspended in saline before use.

Bacteria.—*Salmonella typhosa*, Ty2 strain, was obtained from Dr. S. Gaines, Walter Reed Army Medical Center, Washington, D. C. Highly virulent strains of *Streptococcus pyogenes*, Group A, were furnished by Dr. Rebecca Lancefield, The Rockefeller Institute, New York. They were Strain B930 of Type 3, Strain S43 of Type 6, and Strain T1 of Type 1.

Cultures.—*Salmonella typhosa* was grown on brain-heart agar for 18 hours at 37°C. The growth was resuspended in sterile saline and transferred to fresh brain-heart agar and incubated for 3 hours. The 3-hour culture was then used for challenging mice.

Streptococcal strains were grown overnight at 37°C in Todd-Hewitt broth. In the morning a 3-hour culture was prepared by resuspending an aliquot of the overnight growth in fresh medium, which was then used for infecting the animals.

Animals.—6-week-old female white Swiss mice weighing about 16 to 20 gm were obtained from the Harvard Animal Farm. New Zealand rabbits weighing approximately 5 lb. each were obtained from the same source.

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Antisera.—These were furnished by Dr. Rebecca Lancefield and by the Communicable Disease Center, Public Health Service, Atlanta, Georgia. They were prepared by hyperimmunizing rabbits with heat-killed streptococci.

Bactericidal Tests with Rabbit Blood.—The method has been fully described by Rothbard (5). A 3-hour culture of a small inoculum of streptococci (100 to 400 chains in 0.1 ml) was mixed with 0.3 ml fresh heparinized rabbit blood and rotated at a speed of 6 RPM for 3 hours in an incubator at 37°C. In some instances blood was supplemented with 0.1 ml type-specific immune serum to enhance its phagocytic effect. Samples of 0.1 ml were removed from each tube after incubation, and the number of surviving streptococcal chains was determined by colony counts in poured plates.

TABLE I

Effect of Endotoxin Derived from Sh. Flexneri on Resistance of Mice to Virulent Streptococci and Salmonella Typhosa

Hours elapsed between injections of endotoxin and bacteria‡	Survivors/tested receiving challenge of 100 LD-50 of the organism*							
	Type 1 streptococcus		Type 3 streptococcus		Type 6 streptococcus		*Sal. typhosa* Ty 2	
	24 hours	1 week	24 hours	1 week	24 hours	1 week	24 hours	1 week
0	1/20	1/20	0/20	0/20	3/20	1/20	3/20	0/20
6	2/20	0/20	0/20	0/20	0/20	0/20	4/20	2/20
24	8/20	2/20	6/20	1/20	13/20	2/20	18/20	18/20
48	14/20	3/20	10/20	2/20	6/20	1/20	17/20	17/20
72	13/20	0/20	4/20	1/20	11/20	2/20	14/20	13/20
Control (no endotoxin)	12/20	1/20	8/20	0/20	10/20	0/20	2/20	1/20

* LD-50 for the test strains Type 1 streptococcus, 6×10^3; Type 3 streptococcus, 5×10^2; Type 6 streptococcus, 3×10^3; *Sal. typhosa*, 2×10^6.

‡ Endotoxin given ip in dose of 10 μg per animal.

RESULTS

Effect of Endotoxin.—

Salmonella typhosa, Strain Ty2, and streptococcal Strains B930 (Type 3), S-43 (Type 6), and T1 (Type 1) were given 6, 24, 48, and 72 hours after the intraperitoneal administration of 10 μg of endotoxin. All bacteria were injected intraperitoneally in a dose of 100 LD-50. Experiments were done with endotoxins derived from *Salmonella enteritidis*, *Shigella flexneri*, and *Escherichia coli* 0127:B4.

Table I shows the results obtained with Shigella endotoxin. As expected, within 24 hours after the injection of endotoxin, the mice developed a marked increased resistance to typhoid bacteria. However, no protective effect of endotoxin was observed against any one of the 3 types of the streptococcal pathogens. On the contrary, when streptococci were given less than 24 hours after the injection of endotoxin, animals succumbed more rapidly than the control group, and thus they seemed to have developed an increased suscepti-

bility to infection. The results obtained with the 2 other endotoxins (*Sal. enteritidis*, *E. coli*) did not differ significantly from the foregoing, and, therefore, they seemed to confirm the fact that endotoxins fail to induce non-specific resistance to streptococcal infection.

Mouse Protection Tests.—Antibodies to M proteins of streptococci are known to confer immunity upon animals by means of passive protection.

We determined the minimal amount of this kind of type-specific antibody that would protect mice against 100 LD-50 of a virulent strain of type 3 streptococcus (B930). This was done by first injecting groups of animals intraperitoneally each with a tenfold dilution of homologous rabbit antiserum in saline and 6 hours later challenging the animals by the same route with the streptococci.

The results, shown in Table II, indicate that 0.5 ml of 1 to 1000 dilution of this immune serum prevented death in most animals, whereas 0.5 ml of 1 to 10,000 dilution of the same antiserum was completely ineffective.

TABLE II

Protective Effect of Type-Specific Rabbit Antiserum against Type 3 Streptococcal Infection in Mice

Dilution of antiserum (0.5 ml/mouse)*	Survivors/tested, 1 week-after challenge with 100 LD-50 of bacteria
1:10	20/20
1:100	19/20
1:1000	18/20
1:10,000	0/20
Control (no serum)	1/20

* Injected ip 6 hours before bacterial challenge.

The Combined Effect of Endotoxin and Antiserum.—We have previously shown that the induction by endotoxin of non-specific resistance to Gram-negative bacteria is produced through both a change in RES activity and an increase in level of bactericidal antibodies in the serum.

To establish whether the same factors will function in streptococcal infections, mice were first injected intraperitoneally with endotoxin (*Sal. enteritidis*), 10 μg per animal, and, later with immune serum (anti-M protein, rabbit antiserum) in doses which previously were found to be either partially protective (dilution 1/1000) or ineffective (dilution 1/10,000). The animals were challenged 6 hours after the injection of serum with a dose of 100 LD-50 of virulent streptococci (type 3).

The results are shown in Table III and can be summarized as follows: The administration of endotoxin a short time before the injection of immune serum (up to 6 hours) cancels the protection which is obtained by the immune serum alone. However, when a relatively longer period of time, more than 24 hours,

is allowed between the injections of endotoxin and that of immune antibody, a remarkable degree of resistance to infection is achieved. This resistance is obtained in the presence of an amount of antibody (dilution 1/10,000) which by itself is inadequate to confer protection. Thus, under these conditions, the combination of endotoxin and immune serum, each ineffective alone, markedly increase the resistance of the mice to streptococcal infections.

The Bactericidal Test with Rabbit Blood.—Endotoxin was shown to increase the phagocytic capacity of RES, including that of the leucocytes in the blood circulation (2).

To test whether the combined protective effect of endotoxin and small amounts of immune serum is a result of an improved phagocytic activity of the leucocytes, the bactericidal effect of fresh rabbit blood on streptococci was determined. Five rabbits were treated with a non-

TABLE III

Combined Effect of Endotoxin and Specific Antiserum in Experimental Type 3 Streptococcal Infection in Mice

Dilution of antiserum* (0.5 ml/mouse)	Survivors/tested 1 week after challenge with 100 LD-50 of bacteria					
	Controls (no endotoxin)	Hours elapsed between endotoxin administration and injection of bacteria‡				
		0	6	24	48	72
1:1000	15/20	0/20	0/20	17/20	19/20	16/20
1:10,000	1/20	1/20	2/20	18/20	20/20	12/20
No antiserum	2/20	0/20	0/20	1/20	0/20	1/20

* Injected ip 6 hours before bacterial challenge.

‡ Endotoxin derived from *Sal. enteritidis* and given ip in dose of 10 μg per animal.

lethal but highly pyrogenic dose of endotoxin (5 μg. iv per animal) and were then bled repeatedly, 6, 18, and 48 hours later. Samples of these blood specimens were tested for their bactericidal activity in the manner previously described. Type 3 streptococci, Strain 1930, were used in these tests, and all tests were done with and without the presence of homologous rabbit antiserum. The bactericidal effect that was observed reflected the phagocytic activity of the leucocytes present in the fresh rabbit blood. The number of survivors was determined by counting colonies in poured plates. It should be noted that, whereas normal human blood is considered to be extremely effective in killing virulent streptococci, rabbit blood is generally found to be quite inferior.

Data shown in Table IV compare the bactericidal activity of "normal" human blood with normal rabbit blood and with blood from an animal after endotoxin treatment. "Normal" human blood, as expected, in the presence of immune antiserum caused a substantial decrease in the number of bacteria, while normal rabbit blood had only a very slight effect on the streptococci. However, blood from a rabbit injected with endotoxin 48 hours earlier produced

a bactericidal effect equal to that of the human blood. It is to be emphasized that none of the blood samples tested were bactericidal in the absence of the homologous antiserum. Experiments shown in Table IV were done with blood of one rabbit. Similar results were observed with the blood specimens obtained from the other four rabbits.

TABLE IV

Effect of Treatment with Endotoxin on Bactericidal Activity of Rabbit Blood against Type 3 Streptococcus

Source of blood	No. colonies in 0.1 ml of reaction mixture after 3 hours' incubation	
	Without antiserum	Rabbit antiserum, dilution 1/10
Human	>1000	15
Untreated rabbit	>1000	640
Rabbit, 6 hrs. after endotoxin injection	>1000	>1000
Rabbit, 18 hrs. after endotoxin injection	>1000	220
Rabbit, 48 hrs. after endotoxin injection	>1000	29

Size of inoculum: 230/0. 1 ml.

Endotoxin derived from *Sal. enteritidis* was given iv 5 μg per animal.

DISCUSSION

The induction of non-specific resistance to infections by endotoxins is regarded as a phenomenon embracing many species and strains of bacteria. Indeed it was shown that the administration of these substances is followed by increased resistance to *Sal. typhosa*, *E. coli*, *Pseudomonas*, *Klebsiella*, and *Staphylococcus* (1, 3, 4). Administration of endotoxin produces changes in both humoral and cellular systems of the animals; some of these changes seem to be associated with the increased resistance to infection. The increase in the phagocytic activity of RES would appear to be nonspecific since injection of endotoxin is followed by improved ingestion of many types of particles such as bacteria, carbon, and starch (2). The humoral changes effected by the administration of endotoxin include an increase in titers of bactericidal antibodies to many strains of enterobacteriaceae (6). These antibodies are specific and have opsonizing capabilities. Thus, they are intimately involved in the defense of the body against infection (7).

The streptococcus was selected for our studies because the factors participating in the elimination of this bacterium are relatively well defined (8). The virulent streptococcus is engulfed and killed by the phagocytes only in the presence of type-specific antibodies directed against the M substance located on the cell's surface. This was shown *in vitro* with bactericidal tests utilizing

normal fresh human blood supplemented with anti-M immune serum, and *in vivo* by using immune rabbit serum to protect mice against lethal doses of virulent streptococci (9).

In our present work we failed to detect any beneficial effect of endotoxin administration alone on infections caused by highly virulent strains of streptococci. If, however, type-specific serum was injected into the animals, a completely different response to endotoxin was obtained. Animals pretreated with endotoxin were protected by very small amounts of anti-M antibodies. In the absence of the endotoxin stimulation, the same amount of antibodies had no effect on the infection.

An indication of the mechanism which may be responsible for the combined effect of endotoxin and antibody is suggested from experiments with fresh rabbit blood. It was reported that rabbit blood has poor bactericidal activity against streptococci even in the presence of a potent immune serum (5). We demonstrated that the blood from rabbits which were first injected with endotoxin became strongly bactericidal in the presence of antiserum, apparently due to an increased activity of the phagocytic cells present in their blood. It seems, therefore, that in endotoxin-treated mice RES is activated to such an extent that it is capable of destroying virulent streptococci with the aid of smaller amounts of opsonins than are ordinarily required.

The question may be posed as to why endotoxin by itself suffices to induce nonspecific resistance to certain species of bacteria and not to others, like streptococci. In our previous work we demonstrated that antibodies to Gram-negative bacteria are found in many species of mammals under natural conditions and that the level of these antibodies increase following the injection of endotoxin (10). These "natural" antibodies serve as opsonins and participate in the defense of the body to infection. With our presently available techniques we were unable to detect in non-immunized mice or rabbits any type of substances having such opsonizing properties for virulent types of streptococci. Although it remains to be proved, it is very tempting to postulate that wherever non-specific resistance can be induced by endotoxin alone, both the humoral and the cellular factors are available to the animals at the time of infection. On the other hand, in cases where administration of endotoxin alone is unable to provide protection, one of the factors may be missing or may be present in an unsatisfactory amount. In any case, it is clear that the administration of endotoxin proves to be advantageous to the animals as indicated by the improved performance of their reticuloendothelial systems.

SUMMARY

Endotoxins derived from several species of Gram-negative bacteria, while inducing non-specific resistance to typhoid bacilli in mice, failed to increase the resistance of these animals to infection with virulent strains of Group A strepto-

cocci. However, if administration of endotoxin was followed by injection of minute amounts of type-specific antiserum, a substantial degree of protection against the streptococcal pathogen was obtained. The same amount of type-specific antiserum given to the animals by itself did not have any effect on the outcome of the streptococcal infection. Fresh rabbit blood, obtained from animals pretreated with endotoxin, together with anti-M protein immune serum, was found strongly bactericidal for streptococci. These observations suggest that, at least with regard to streptococcal infection, both humoral and cellular factors are required for induction of non-specific resistance.

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BIBLIOGRAPHY

1. Rowley, D., Rapidly induced changes in the level of non-specific immunity in laboratory animals. *Brit. J. Exp. Path.*, 1956, **37,** 223.
2. Benacerraf, B., Sebestyen, M. M., and Schlossman, S., A quantitative study of the kinetics of blood clearance of P^{32}-labelled *Escherichia coli* and staphylococci by the reticuloendothelial system, *J. Exp. Med.*, 1959, **110,** 27.
3. Dubos, R. J., and Schaedler, R. W., Reversible changes in the susceptibility of mice for bacterial infections. I. Changes brought about by injection of pertussis vaccine or of bacterial endotoxins, *J. Exp. Med.*, 1956, **104,** 53.
4. Springer, G., Protection of mice against lethal staphylococcus infection by *Escherichia coli* O_{86} fractions, *Science*, 1961, **134,** 335.
5. Rothbard, S., Bacteriostatic effect of human sera on group A streptococci, *J. Exp. Med.*, 1945, **82,** 93.
6. Michael, J. G., Whitby, J. L., and Landy, M., Increase in specific bactericidal antibodies after administration of endotoxin, *Nature*, 1961, **191,** 296.
7. Whitby, J. L., Michael, J. G., Woods, M. W., and Landy, M., Symposium on bacterial endotoxins. II. Possible mechanisms whereby endotoxins evoke increased non-specific resistance to infection, *Bact. Rev.*, 1961, **25,** 437.
8. Lancefield, R. C., Specific relationship of cell composition to biological activity of hemolytic streptococci, *Harvey Lectures*, 1940–41, **36,** 251.
9. Fleck, D. G., Mouse protection and enhancement of phagocytosis by antisera to *Streptococcus pyogenes*, *Brit. J. Exp. Path.*, 1956, **37,** 406.
10. Michael, J. G., Whitby, J. L., and Landy, M., Studies on natural antibodies to Gram-negative bacteria, *J. Exp. Med.*, 1961, **115,** 131.