

Protective action of endotoxin on allergic phenomena

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E. coli endotoxin protects guinea pigs against the asthmalike syndrome resulting from exposure to histamine aerosols and delays the onset of bronchospasm induced by antigen inhalation in passively sensitized guinea pigs, preventing the anaphylactic death of the animals. It does not, however, suppress the Arthus reaction in rabbits. The antiallergic action exerted by endotoxin is a property of the entire molecule and is lost after its hydrolysis. Possible mechanisms to account for this endotoxin activity are discussed.

In 1957 Meier, Bein, and Jaques¹ reported that *Proteus vulgaris* polysaccharide protected guinea pigs sensitized with egg albumin from anaphylactic death if the animals were injected with the polysaccharide one hour before challenge with the egg albumin. Doses of 10 to 50 μ g per kilogram were able to suppress the Arthus reaction in rabbits and guinea pigs. The polysaccharide, however, failed to counteract the effect of histamine aerosol in guinea pigs and did not have any antihistaminic effect when tested in vitro by the Schultz-Dale procedure.

The present report describes experiments which show that lipopolysaccharide from enteropathogenic *Escherichia coli* possesses a definite ability to counteract the effect of histamine aerosols as well as the asthmalike syndrome resulting from exposure of egg albumin-sensitive animals to albumin aerosols. By contrast, the lipopolysaccharide was unable to suppress the Arthus reaction in rabbits.

MATERIALS AND METHODS

Local hybrid guinea pigs of both sexes with an average weight of 380 grams were used for some experiments and male albino guinea pigs with an average weight of 336 grams were used for other studies. All animals were kept on a pellet diet.*

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A 14 by 10 by 10 inch box with transparent sides was used in all experiments. Histamine and antigen solution were nebulized into the box by means of a de Vilbiss No. 40 nebulizer connected to a source of compressed air bubbled through water. The chamber was not airtight and allowed the aerosol fog to ooze from the seams. Three to 4 minutes of nebulization was allowed for the saturation of the chamber before the guinea pigs were exposed to the aerosols. As a rule, on exposure to a 1:2,000 dilution of histamine diphosphate,* the animals developed a sharply increased respiratory rate accompanied by coughing and went rapidly into shock. The first unequivocal sign of asthma served as end point and the guinea pigs were removed promptly from the aerosol and allowed to recover. The technique for the precise estimation of this end point may be found in the work of Noelpp-Eschenhagen.² This paper also gives recordings of the physiological changes in guinea pigs during exposure to histamine. Egg albumin powder† in 10 per cent solution in physiological saline was used for aerosolization. Difco lipopolysaccharide from *E. coli* 0111:B4 was used in solution in pyrogen-free water.

Guinea pigs were sensitized with a total of 40 mg. of egg albumin divided equally in two injections prepared and administered as follows: a 5 per cent albumin solution was made in physiological saline and emulsified with an equal volume of Difco complete Freund's adjuvant. The first injection was given intracutaneously. One week later a second injection was given intraperitoneally. Two weeks after the last injection the animals were used for the tests.

Rabbits were immunized with weekly intracutaneous injections of egg albumin in Difco complete Freund's adjuvant. Five to 6 injections were given, and 10 days after the last injection trial bleedings were made.

Experiment 1. Relation between the dose of endotoxin and the time of onset of histamine-induced asthma

Seven groups of 5 hybrid guinea pigs each were exposed to a 1:2,000 histamine aerosol and the average time of onset of signs of asthma was determined for each group. After a rest period of 24 hours, each group was injected intraperitoneally with increasing doses of endotoxin as follows: 0, 1, 2, 5, 50, 250, and 500 μ g. The first group, which did not receive endotoxin, was injected with 1.0 ml. of pyrogen-free water. All the animals were re-exposed to the histamine aerosol 1, 3, 5, 7, 24, and 48 hours after the endotoxin injection, and the average time of onset of signs of asthma in each group was calculated.

The results are graphically recorded in Fig. 1. The time in minutes of onset of asthma is plotted on the ordinates. The time interval in hours between the injection of endotoxin and the exposure to histamine is plotted on the abscissa. The rise of each curve, as compared with the control curve, represents the average protection against histamine bronchospasm induced by the endotoxin concentrations indicated in the diagram.

It may be seen that with a dose of endotoxin as low as 1 μ g a slight protec-

*Nutritional Biochemicals Corporation.

†Baker Chemical Co.

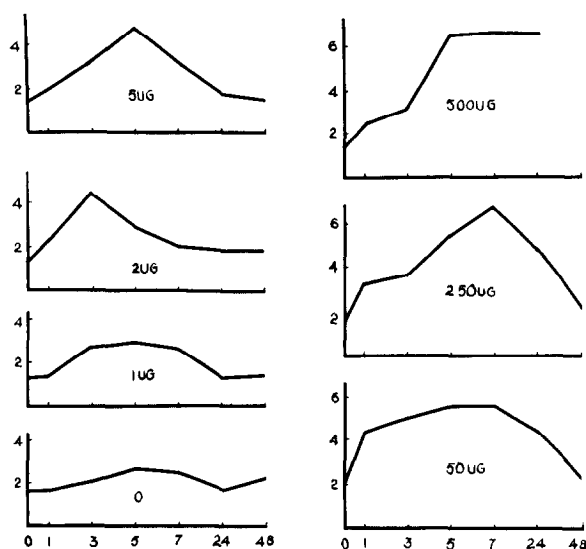


Fig. 1

Relation between the dose of endotoxin and the time of onset of signs of asthma in guinea pigs exposed to histamine aerosol. Ordinates: time in minutes required to elicit signs of unquestionable asthma. Abscissae: interval of time in hours between endotoxin injection and exposure to histamine aerosol.

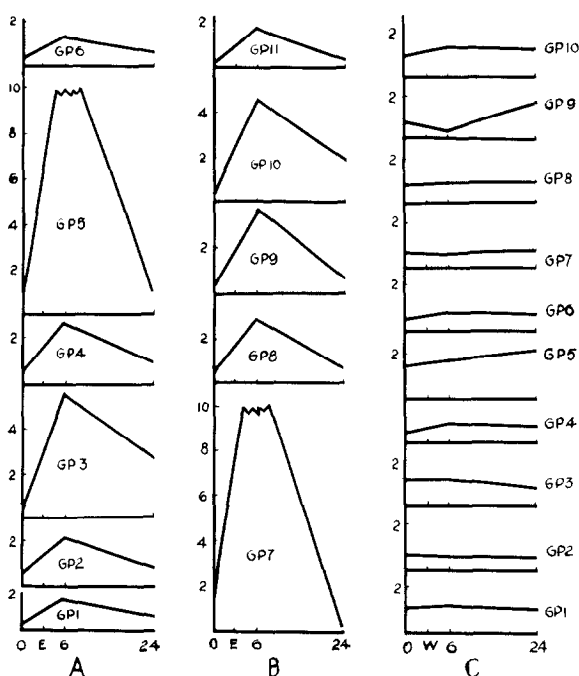
tive effect is already evident but is best defined at the 2 μg concentration. The optimal conditions for the demonstration of this effect occur approximately 6 hours after the 50 μg dose. The 500 μg dose was too toxic and caused the death of 4 of the 5 guinea pigs about 24 hours later.

Experiment 2. Individual variations in the time of onset of signs of histamine asthma in guinea pigs after the administration of 50 μg of endotoxin

A group of 21 male albino guinea pigs were exposed to an aerosol of histamine (1:2,000) and the time of onset of bronchospasm was recorded. After a 24 hour rest, 11 guinea pigs were injected intraperitoneally with 50 μg of endotoxin and 6 and 24 hours later were re-exposed to histamine. Ten control guinea pigs were injected with pyrogen-free water and were similarly re-exposed to histamine 6 and 24 hours later.

There was a marked individual variation in the degree of protection induced by endotoxin as is evident in Fig. 2. In all animals, however, there was a protective effect after the 6 hour period which had disappeared completely, in most cases, after a 24 hour rest period. The control guinea pigs gave a remarkably constant time of onset of bronchospasm in the 3 exposures to histamine.

These results confirm the observations made in the first experiment. The protective effect is demonstrable in both hybrid and albino guinea pigs. The latter, however, proved to be more sensitive to histamine aerosol and exhibited a greater degree of individual variation in their response to the protective action of endotoxin than the hybrid animals.

**Fig. 2**

Variations in the protective action of 50 μ g of endotoxin in albino guinea pigs exposed to histamine aerosol. Ordinates: time in minutes required to elicit signs of unquestionable asthma. Abscissae: interval of time in hours between endotoxin injection and exposure to histamine aerosol. *E* = endotoxin. *W* = water. Columns *A* and *B* = guinea pigs injected with endotoxin. Column *C* = control guinea pigs injected with water.

Table I. *Effect of endotoxin in anaphylactic guinea pig asthma*

Guinea pigs		Amount of anti-egg albumin serum injected for sensitization	Dose of endotoxin injected	Average time of onset of asthma in minutes	Animals killed by albumin exposure
No.	Type				
10	Hybrid	1 ml.	50 μ g	3.53	0
10	Hybrid	1 ml.	0*	1.56	5
10	Albino	1 ml.	100 μ g	7.60	0
10	Albino	1 ml.	0*	1.11	7

*Received 1 ml. of pyrogen-free water.

Experiment 3. Effect of endotoxin in anaphylactic guinea pig asthma

Forty guinea pigs were sensitized passively by the intraperitoneal injection of 1 ml. of rabbit anti-egg albumin serum. A preliminary test with varying amounts of serum has shown that 1 ml. produced optimal sensitization as indicated by the appearance of asthma in less than two minutes when these guinea pigs were exposed to an aerosol of 10 per cent egg albumin in saline.

Forty-eight hours later, 10 guinea pigs were injected intraperitoneally with 50 μ g of endotoxin and 10 with 100 μ g. The other 20 animals received an injection

tion of 1 ml. of pyrogen-free water for control purposes. Six hours after the injection all 40 guinea pigs were exposed to an aerosol of 10 per cent egg albumin and the time of onset of signs of asthma recorded. Table I gives the result of the experiment as well as the number of guinea pigs which died as a result of exposure to the antigen.

The endotoxin significantly delayed the onset of bronchospasm induced by antigen inhalation in the passively sensitized guinea pigs. The mean time required for the onset of respiratory difficulty with the 50 μ g dose was doubled, as compared to controls. A sevenfold prolongation of the onset of asthma was observed with 100 μ g of endotoxin. Further evidence of the marked protective effect of the endotoxin is the fact that 60 per cent of the animals exposed to the albumin aerosol without a preliminary endotoxin injection died in 1½ minutes or less of exposure while all test animals survived. These findings suggest that endotoxin administration results in interference at some stage in the process by which the antigen-antibody reaction gives rise to bronchospasm. The interference prevents the anaphylactic death of the animal.

Experiment 4. Effect of endotoxin on the Arthus reaction in the rabbit

Six mature white New Zealand rabbits immunized with egg albumin were given intracutaneous injections of 0.5 ml. of 5 per cent albumin in physiological saline. Similar injections were given to four normal rabbits. The immunized rabbits developed zones of skin necrosis at the site of the injection. Table II gives the diameter of the necrotic zone after 48 hours. No reaction was evident in the normal animals. Three days later, the intracutaneous albumin injections were repeated in the same group of animals, but 3 of the immunized animals were given 15 μ g per kilogram of endotoxin and the other 3 were given 30 μ g per kilogram one hour prior to the albumin injection. Two normal rabbits received only intradermal albumin injections for control purposes.

All the immunized rabbits injected with endotoxin developed zones of necrosis in 48 hours which were as large as those developed previously in the absence of endotoxin (Table II). Doses over 30 μ g per kilogram of endotoxin intravenously were not used as they produced signs of toxicity.

The results indicate that endotoxin in sublethal doses is unable to suppress the Arthus reaction in rabbits.

Table II. *Effect of endotoxin on the Arthus reaction in the rabbit*

<i>Rabbit No.</i>	<i>Immunization status</i>	<i>Albumin injected intracutaneously</i>	<i>Zone* necrosis (mm.)</i>	<i>Endotoxin injected intravenously</i>	<i>Zone necrosis (mm.)</i>
1	Immunized	0.5 ml. 5%	28	30 μ g/Kg.	29
2	Immunized	0.5 ml. 5%	30	30 μ g/Kg.	28
3	Immunized	0.5 ml. 5%	25	30 μ g/Kg.	28
4	Immunized	0.5 ml. 5%	32	15 μ g/Kg.	28
5	Immunized	0.5 ml. 5%	25	15 μ g/Kg.	25
6	Immunized	0.5 ml. 5%	25	15 μ g/Kg.	25
7-12	Normal	0.5 ml. 5%	0	None	0

*Average diameter.

Experiment 5. Effect of hydrolysis of endotoxin on its ability to protect guinea pigs against histamine aerosols

This experiment was undertaken to determine whether the observed anti-histaminic effect of endotoxin is a property of the entire macromolecule or whether the haptene fragments liberated during the hydrolytic process are also active.

Procedure

A solution of 1,000 μ g per milliliter of Difco endotoxin O111:B4 was prepared in 0.1M acetic acid. Five milliliter aliquots were placed in glass tubes and sealed. Two tubes were kept in the refrigerator as controls, and the rest were placed in a boiling water bath. At intervals of 5, 15, 30, 60, 120, 240, and 360 minutes, two tubes were removed from the bath and cooled rapidly. Each solution was neutralized with N NaOH and its volume brought to 10 ml.

A group of 27 albino guinea pigs in which the onset of histamine asthma was less than 2 minutes was selected and divided in groups of three. Each group was injected intraperitoneally with 1 ml. of one of the hydrolyzed endotoxin solutions. As a control, one group was injected with 1 ml. of the unheated endotoxin and another group with 1 ml. of the neutralized acetic acid solution. Six hours after the injection and again 48 hours later the animals were exposed to histamine aerosol.

As an evaluation of the thoroughness of the hydrolytic process, the hydrolyzed aliquots were submitted to two additional procedures: (a) titration on the skin of normal rabbits according to Larson's procedure,³ and (b) gel diffusion by the Ouchterlony procedure. The skin of two normal white rabbits was well shaven and 0.2 ml. of each aliquot and the control solutions were injected intracutaneously. The amount of edema and erythema in 48 hours was measured after soaking the skin in alcohol. Gel diffusion plates made by the Ouchterlony⁴ procedure were run with all the aliquots by using rabbit O111:B4 antiserum. A summary of the results of this experiment is shown in Table III.

It is evident from these results that the protective action of endotoxin depends on the integrity of the macromolecule and that hydrolysis of the material results in a gradual loss of activity which is completely gone after 4 to 6 hours of heating. Only slight precipitating, skin-reactive, and histamine-protective activities were present after 2 hours of hydrolysis. The material liberated by prolonged hydrolysis (4 to 6 hours) was completely inactive.

DISCUSSION

The results of our experiments have shown that *E. coli* endotoxin exerts anti-allergic action in guinea pigs. It protects the animals against bronchospasm induced by histamine aerosols and against the asthmalike syndrome which results from exposure of passively sensitized animals to an aerosol of the specific antigen.

The Arthus reaction in rabbits was not suppressed by endotoxin injections in the experiments of this study. These results contrast sharply with those obtained by Meier, Bein, and Jaques¹ in their studies with *Proteus* polysaccharide

Table III. *Effect of hydrolysis of endotoxin on its ability to protect guinea pigs against histamine aerosols*

Time of hydrolysis (min.)	Av. histamine sensitivity before endotoxin (min.)	Av. histamine sensitivity 6 hr. after endotoxin (min.)	Av. histamine sensitivity 48 hr. after endotoxin (min.)	Reaction in rabbit		Gel diffusion (bands of precipitation)
				A	B	
Neutralized	1.13	1.61	1.22	0	0	0
0	1.09	6.34	1.56	20	26	2
5	1.03	4.86	1.41	16	18	2
15	1.08	4.77	1.60	10	12	2
30	1.37	4.90	1.36	5	12	2
60	1.43	3.28	1.75	2	10	2
120	1.16	3.38	2.00	0	7	1
240	1.18	1.55	1.07	0	5	0
360	0.97	1.74	1.23	0	0	0

which failed to counteract the effect of histamine but was able to suppress the Arthus reaction.

The observed differences between *Proteus* and *E. coli* polysaccharides suggest the possibility that the various polysaccharides obtainable from different gram-negative bacteria may vary widely in their biologic properties depending perhaps on variations in the chemical and physical configuration of their molecules.

The observed effect could be dependent on the complex series of physiologic reactions initiated by endotoxin and, according to some investigators, mediated through an adrenergic mechanism.⁵⁻⁹ These investigators demonstrated that endotoxins do significantly stimulate the adrenal output of catecholamines and corticosteroids. Rosenberg and associates⁶ found that following endotoxin injection, epinephrine levels rise immediately and soon return to control levels but norepinephrine remains slightly elevated. They postulated that the release of these substances produces hemodynamic changes which lead to shock. Other investigators^{10, 11} have made extensive studies on the nature of endotoxin shock and its relation to adrenal products. The results of the experiments by Meleyeo, Pruzansky, Feinberg, and Kantor¹² do not favor this mechanism. They found that a streptococcal infection was able to inhibit anaphylaxis induced by albumin aerosols in sensitized guinea pigs, in spite of bilateral adrenalectomy. However, adrenalectomy does not necessarily obliterate an adrenergic mechanism because the sympathetic nervous system remains intact and extra-adrenal tissue might be present.

Greisman¹³ has made an attempt to elucidate the basic problem of endotoxin activity, whether it acts directly at the cellular level or through an intermediate step whereby the composition of the blood is altered. He studied the effect of *E. coli* endotoxin on the isolated guinea pig ileum and found that it was capable of activating a histamine-like releasing factor upon in vitro incubation with fresh normal rat plasma. The endotoxin per se was unable to liberate histamine directly. He suggested the hypothesis that endotoxin activates plasma histamine releasing factors which may account for certain of the pathophysio-

logic alterations which characterize the endotoxin response. Studies *in vivo* have also demonstrated the release of histamine following endotoxin injections. Hinshaw, Vick, Carlson, and Fan¹⁴ injected lethal doses of endotoxin in dogs and observed a marked fall in whole blood histamine with a concomitant rise in plasma histamine. In addition, it was shown¹⁵ that a conversion of histidine to histamine occurs at an accelerated rate following endotoxin injection. The possibility that endogenous histamine could be the mediator stimulating the adrenal cortex has been postulated by other workers.¹⁶

These findings, as well as our own, lead us to speculate that the same mechanisms by which lethal doses of endotoxin lead to shock, may give, with minute sublethal doses, a net antiallergic effect resulting from gentle adrenal stimulation.

There are, however, other possible explanations for the beneficial effect of sublethal endotoxin injections. Meier, Bein, and Jaques¹ favor the hypothesis of a direct or indirect interference with a cellular constituent involved in the antigen-antibody reaction or causing the release of factors which become fixed to the cells rendering them less sensitive to the anaphylactic action.

In our experiments, endotoxin failed to suppress the Arthus reaction. We consider this as evidence that endotoxin does not prevent an antigen-antibody reaction from taking place but rather modifies the results of the immunologic reaction.

More intensive study is required to determine if endotoxin induces the release of a factor or factors capable of inactivating histamine or blocking the cell receptors for the drug. From this point of view, the findings of Braun and Kessel¹⁷ are promising. They have shown that endotoxin seems to be able to react with cells of the macrophage-monocyte system either directly or by combining with specific cell-bound antibodies which results in an alteration of cell membrane permeability and leakage of intracellular DNA breakdown products. The possible action of these or other cell breakdown products upon the histamine cell receptors or the metabolic processes of the effector cells deserves more investigation.

In examining our results in the light of the findings of other investigators, we conclude that the ability of sublethal doses of endotoxin to prevent anaphylactic bronchospasm, death from anaphylactic shock, and simple histamine bronchospasm while at the same time failing to exert a similar protective action on the Arthus reaction is evidence that endotoxin does not prevent the antigen-antibody reaction from taking place but rather interferes in the subsequent physiopathologic processes induced by the immune reaction. Though the adrenergic mechanism can account for the results obtained, the possibility that endotoxin macromolecules may have chemical determinants with a direct antihistaminic activity cannot be ruled out at present until a larger number of lipopolysaccharides is studied in this respect. Likewise, we must rule out the possibility that cellular breakdown products may interfere in the elicitation of the pharmacological activity of the mediators responsible for the allergic reaction.

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REFERENCES

1. Meier, R., Bein, H. J., and Jaques, R.: The Action of Bacterial Polysaccharides on Allergic Phenomena, *Internat. Arch. Allergy* **11**: 101, 1957.
2. Noelpp-Eschenhagen, I., and Noelpp, B.: New Contributions to Experimental Asthma, *Progress in Allergy*, Basle-New York, 1954, S. Karger, vol. 4, p. 361.
3. Larson, C. L., Ribí, E., Milner, K. C., and Lieberman, J. E.: A Method for Titrating Endotoxic Activity in the Skin of Rabbits, *J. Exper. Med.* **111**: 1, 1960.
4. Ouchterlony, O.: Antigen-Antibody Reactions in Gels, *Acta path. et microbiol. scandinav.* **26**: 507, 1949.
5. Cannon, W. B.: The Emergency Function of the Adrenal Medulla in Pain and the Major Emotions, *Am. J. Physiol.* **33**: 356, 1914.
6. Rosenberg, J. C., Lillehei, R. C., Longerbeam, J., and Zimmermann, B.: Studies on Hemorrhagic and Endotoxin Shock in Relation to Vasomotor Changes and Endogenous Circulating Epinephrine, Norepinephrine and Serotonin, *Ann. Surg.* **154**: 611, 1961.
7. Egdahl, R.: The Differential Response of the Adrenal Cortex and Medulla to Bacterial Endotoxin, *J. Clin. Invest.* **38**: 1120, 1959.
8. Heiffer, M. H., Mundy, R. L., and Mehlman, B.: Adrenal Catechol Amine Concentrations Following Endotoxin Administration, *Fed. Proc.* **18**: 66, 1959.
9. Melby, J. C., Egdahl, R. H., and Spink, W. W.: Secretion and Metabolism of Cortisol Following Injection of Endotoxin, *J. Lab. & Clin. Med.* **56**: 50, 1960.
10. Spink, W. W., and Vick, J.: Evaluation of Plasma Metaraminol and Hydrocortisone in Experimental Endotoxin Shock, *Circulation Res.* **9**: 184, 1961.
11. Lillehei, R. C., and Maclean, L. D.: Intestinal Factor in Irreversible Endotoxin Shock, *Ann. Surg.* **148**: 513, 1958.
12. Meleyco, L. N., Pruzansky, J. J., Feinberg, S. M., and Kantor, S. Z.: Inhibition of Anaphylaxis in the Guinea Pig by Local Streptococcal Infection, *J. ALLERGY* **30**: 257, 1959.
13. Greisman, S. E.: Activation of Histamine-releasing Factor in Normal Rat by *E. coli* Endotoxin, *Proc. Soc. Exper. Biol. & Med.* **1031**: 628, 1960.
14. Hinshaw, L. B., Vick, J. A., Carlson, C. H., and Fan, Y. L.: Role of Histamine in Endotoxin Shock, *Proc. Soc. Exper. Biol. & Med.* **104**: 379, 1960.
15. Hinshaw, L. B., Jordan, M. M., and Vick, J. A.: Mechanism of Histamine Release in Endotoxin Shock, *Am. J. Physiol.* **200**: 987, 1961.
16. Lecompte, J., Van Cauwenberge, H., and Palem-Uliers, M.: Adrenal Cortex Stimulation by the Liberation of Endogenous Histamine in Man, *Acta allergol.* **14**: 291, 1959.
17. Braun, W., and Kessel, R. W. I.: Cytotoxicity of Endotoxins in Relation to Their Effects on Host Resistance, in *International Symposium on Endotoxin*, New Brunswick, N. J., 1963, Institute of Microbiology, Rutgers, The State University. In press.