Endotoxin-Induced Hypersensitivity to Histamine in Mice

I. Contrasting Effects of Bacterial Lipopolysaccharides and the Classical Histamine-Sensitizing Factor of *Bordetella pertussis*

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ABSTRACT

PIERONI, ROBERT E. (Massachusetts Department of Public Health, Boston), EDWARD J. BRODERICK, AND LEO LEVINE. Endotoxin-induced hypersensitivity to histamine in mice. I. Contrasting effects of bacterial lipopolysaccharides and the classical histamine-sensitizing factor of *Bordetella pertussis*. *J. Bacteriol.* **91**:2169–2174. 1966.—The capacity of typhoid and possibly of pertussis endotoxins to induce histamine-shock susceptibility in some of the mice that survive graded doses of these endotoxins was confirmed. It was demonstrated, however, that pertussis endotoxin cannot be the main source of the typical histamine sensitization of pertussis vaccine. The following points are made. (i) With typhoid and pertussis endotoxins as inducers of histamine shock, no systematic relation between deaths and induction dose could be found, and 100% mortality could not be achieved. In contrast, with pertussis protective fraction as inducer, there was clear dose-response regression, with 100% mortality possible. (ii) The major part of the histamine-sensitizing activity of pertussis vaccine or its extracts was destroyed by trypsinization or by heating for 30 min at 100 C. These processes do not affect the histamine-sensitizing activity of the endotoxins. The implication for purified pertussis vaccine with high histamine-sensitization capacity is that endotoxin need not necessarily be present. The significance and possible mechanisms of action of endotoxin-induced histamine sensitivity are briefly discussed.

Since the discovery by Parfentjev and Goodline in 1948 of the histamine-sensitizing capacity of pertussis vaccine for certain mouse strains (13), there has been a growing body of evidence indicating the close association, if not the identity, of this histamine-sensitizing factor (HSF) and the antigen capable of conferring immunity against whooping cough (4, 10, 15, 19, 20). Recently we described a method for the extraction and partial purification of the protective antigen of pertussis, with use of the HSF activity as a time-saving "tracer" for the protective antigen (14). This work again confirmed the parallelism of these two activities.

Recently, Malkiel and Hargis reported that the lipopolysaccharide endotoxin of *Bordetella pertussis*, as well as that from various other gram-negative organisms, including *Salmonella typhosa*, was capable of sensitizing mice to histamine (9). If the protective and histamine-sensi-

tizing activities of *B. pertussis* are dual manifestations of a single bacterial component, and the latter activity can be ascribed in any considerable degree to the endotoxin of pertussis, then purification of the protective antigen might well result in a concentration of pyrogenic endotoxin. The possibility of preparing an atoxic purified pertussis vaccine would be remote indeed.

Earlier workers considered *B. pertussis* unique in its capacity to induce sensitivity to histamine in mice, after having failed to induce it with a wide variety of gram-negative organisms (6, 8) and their extracted endotoxins, including pertussis endotoxin (11, 19). In our earlier work (14), we found pertussis HSF to be susceptible to trypsinization, which is suggestive of protein rather than lipopolysaccharide composition. For these reasons we considered it important to confirm Malkiel and Hargis' finding of hista-

mine-sensitizing activity in endotoxins. This paper reports the finding of such activity in typhoid and possibly in pertussis endotoxins, with indications, however, that it differs in extent and kind from that of the main, nitrogenous HSF of pertussis vaccine.

MATERIALS AND METHODS

Bordetella pertussis cultures. Cohen and Wheeler medium was used in submerged, aerated culture and was harvested after 72 hr at 37 C. Cultures were inactivated by addition of 1:10,000 Merthiolate.

Mice. Female CFW mice, 12 to 18 g, were obtained from Carworth, Inc., New City, N.Y.

Test for histamine sensitization. Mice were inoculated intraperitoneally (ip) and 4 days later were ip challenged with 1.0 mg of histamine dihydrochloride in 0.5 ml of saline. Deaths were tabulated at 2 hr. Uninoculated controls run with each test invariably survived the histamine challenge and are therefore not included in the tables.

Preparation of pertussis protective fraction. Acetone-dried *B. pertussis* cells were repeatedly blended with glass micro-beads and renewed diluent for at least 5 cycles in a Waring Blendor. The pooled lysates, clarified at $37,000 \times g$ for 3 hr, were brought to 35% saturation with ammonium sulfate, and the precipitate was redissolved and dialyzed. A more detailed description of the process has been reported earlier (14).

Preparation of pertussis endotoxin. Lipopolysaccharide (LPS) was extracted from the cell walls of two lots of pertussis bacilli, essentially according to the method of Westphal as described in Kabat and Mayer (5). Nucleic acids were removed from the first endotoxin preparation (ET no. 1) by centrifuging the pooled aqueous phases for 2 hr at 30,000 rev/min and discarding the supernatant fluid. Spectrophotometric analysis at 260 m μ indicated that over 95% of the nucleic acids was eliminated by this procedure. High-speed centrifugation was not carried out on the second endotoxin preparation (ET no. 2), and, consequently, it required a considerably higher dosage to effect an LD₅₀ in mice (Table 2). ET no. 2 was heated at 100 C for 30 min.

Commercial sources. LPS *S. typhosa* 0901, lots 371539 and 473454, were purchased from Difco. Both lots were prepared by the Westphal phenol extraction method, according to the manufacturers. Trypsin (1:300) and histamine dihydrochloride were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio.

Determination of the 50% histamine-sensitizing dose (HSD₅₀). The HSD₅₀ was calculated by the method of Reed and Muench.

Tryptic treatment of pertussis lysates. Preliminary experiments indicated that pertussis lysates were more refractory to trypsinization than known proteins such as tetanus toxin. Empirical increments in trypsin concentration and in incubation time at 37 C were successively tried and tested for reduction in HSF activity. The pH of experimental and control solutions was maintained constant at 7.8.

RESULTS

Effect of typhoid endotoxin on histamine sensitivity. Table 1 presents the results obtained after injecting five serial dilutions (50 to 800 μ g) of typhoid LPS, lot 471539, into groups of 10 CFW mice. Another group of 10 mice were inoculated with 1 ml of pertussis vaccine, lot 27, containing 13.5×10^9 organisms. Many of the mice receiving the higher doses of typhoid LPS died of endotoxic shock. These deaths usually occurred within 18 hr but were not tabulated until 96 hr, at which time the survivors were challenged with 1 mg of histamine dihydrochloride. In confirmation of Malkiel and Hargis, typhoid LPS sensitized some of these surviving mice to histamine death. However, at no dosage was the degree of sensitization as high as that conferred by pertussis vaccine which induced 100% mortality in the 10 mice challenged with histamine.

Effect of pertussis endotoxin on histamine sensitivity. Two pertussis LPS preparations (ET no. 1 and ET no. 2) were tested for their capacity to enhance sensitivity to histamine (Table 2). Although the purity of the two preparations differed markedly, the highest dose of each endotoxin injected effected 100% mortality from endotoxin shock. Each preparation sensitized a small percentage of the surviving mice to histamine shock. However, as was the case with typhoid endotoxin, the sensitizing capacity was in no way comparable to that induced by the parent pertussis vaccine which sensitized all 10 mice to histamine death.

Effect of pertussis protective fraction on histamine sensitivity. As we previously reported (14), most of the protective antigen and HSF can be recovered from a pertussis lysate in the precipitate after the lysate is brought to 35% saturation

TABLE 1. Effect of graded doses of typhoid endotoxin on histamine sensitivity of surviving mice

Endotoxin dosage*	Death rate	
	Toxic	Histamine
μ g		
50	0/10	0/10
100	2/10	2/8
200	5/10	2/5
400	6/10	0/4
800	10/10	—
Pertussis vaccine, 13.5×10^9 cells	0/10	10/10

* *Salmonella typhosa* 0901 LPS, lot 471539.

TABLE 2. *Effect of graded doses of pertussis endotoxin on histamine sensitivity of surviving mice*

Addition	Dosage	Death rate	
		Toxic	Histamine
ET no. 1	μg		
	37	0/5	1/5
	187	0/5	0/5
	1,870	5/5	—
ET no. 2	525	0/10	2/10
	1,050	0/10	1/10
	2,100	3/10	0/7
	4,200	9/10	0/1
	8,400	9/9	—
Pertussis vaccine	13.5×10^9 cells	0/10	10/10

with ammonium sulfate. The resulting supernatant fluid, although containing the bulk of the organic bacterial components, is low in both protective and HSF activities. The histamine-sensitizing effects of six such precipitates obtained from three different lysates are shown in Table 3. Toxic deaths were seldom encountered, occurring only when the amount of protective fraction injected far exceeded the HSD_{50} . There is clear evidence of histamine death-rate regression on dosage of pertussis protective fraction, with 100% mortality always obtainable. These were in sharp contrast with the characteristics of histamine shock when typhoid or pertussis endotoxins were the sensitizing agents (Tables 1, 2, and 5). Despite the fact that three different lysates were used in preparing the protective fractions, the HSD_{50} were notably constant, differing by less than twofold, with values lying between the extremes of 3.7 and 7.1 μg of nitrogen.

Effect of trypsin on pertussis-induced histamine sensitization. In an earlier study (14), it was found that the addition of trypsin to a pertussis lysate resulted in a reduction of its HSF activity. This effect would not be expected if the lipopolysaccharide of *B. pertussis* were primarily responsible for induction of histamine sensitivity. Because this initial observation was made with small numbers of test animals, we decided to expand the scale of this observation. The results of eight separate experiments with use of four different pertussis lysates at various dilutions and incubation periods are shown in Table 4. In each case, addition of trypsin diminished the capacity of the pertussis lysate to sensitize mice to histamine. Of a total of 102 mice sensitized with control lysates containing no trypsin, 69 (68%) died after his-

TABLE 3. *Effect of pertussis protective fraction on histamine sensitivity*

Test	Lot no.	Dosage (μg of nitrogen)	Death rate		
			Toxic	Histamine	HSD_{50} (μg of nitrogen)
11	III	0.6	0/8	1/8	6.8
		1.8	0/8	1/8	
		5.4	0/8	3/8	
		16.2	0/8	6/8	
		48.6	0/8	8/8	
13	III	4.2	0/8	2/8	7.1
		12.6	0/8	7/8	
		37.8	0/8	7/8	
16	IV	0.2	0/8	0/8	6.2
		2	0/8	0/8	
		20	0/8	8/8	
		200	3/8	5/5	
19	V	1.5	0/16	1/16	4.0
		7.5	0/16	13/16	
		37.5	0/16	15/16	
20	V	1.5	0/12	3/12	3.7
		7.5	0/12	8/12	
		37.5	0/12	12/12	
23	V	1.5	0/14	0/14	3.9
		7.5	1/14	11/13	
		37.5	1/14	13/13	

TABLE 4. *Effect of trypsin on pertussis-induced histamine sensitivity*

Lysate no.	Death rate	
	Trypsin-treated	Control
II	1/10	6/9
III	11/16	15/16
III	0/5	1/5
V	1/8	8/8
V	6/10	9/10
VII	2/18	11/18
VII	9/12	12/12
VII	1/24	7/24
Total	31/103	69/102

mine challenge. Injection of the trypsin-treated lysates resulted in sensitization of 31 out of 103 mice (30%) to histamine shock. The difference is highly significant ($P < 0.005$) and points to a proteinaceous substance as probably responsible for the major part of the pertussis-mediated hypersensitivity to histamine.

Effect of heat on histamine sensitivity induced by typhoid endotoxin. Most of the characteristic biological activities of endotoxins have been found to be heat-stable. The component of the pertussis bacillus primarily responsible for induction of histamine sensitivity, however, has been shown by several investigators to be heat-labile (7, 8, 12). It seemed of interest, therefore, to ascertain the effect of heat on the histamine-sensitizing capacity of typhoid endotoxin. Four groups of 20 mice each were sensitized with a second typhoid LPS, lot 473454, with use of threefold dilutions ranging from 27 to 729 μ g. Four other groups received similar injections, except that the endotoxin was heated at 100 C for 30 min in a water bath. Table 5 indicates that heat had no effect on the intrinsic lethality of the endotoxin preparations. Of 80 mice inoculated with dilutions of the unheated endotoxin, 16 succumbed to endotoxic shock, whereas after heating the endotoxin was lethal to 18 of the 80 mice tested. Similarly, upon challenge of the survivors with histamine, no significant difference was noted between the groups receiving the heated and the groups receiving the unheated preparations: 42% of the mice receiving un-

treated typhoid LPS succumbed to histamine challenge, compared with 45% of the animals receiving the heated preparations. The heat lability of pertussis HSF is apparent from the fact that all of the mice injected with unheated pertussis vaccine died after histamine challenge, whereas 90% of the mice receiving an equal number of cells heated at 100 C for 30 min survived the histamine challenge (Table 5).

DISCUSSION

If pertussis endotoxin were the main bearer of the histamine-sensitizing activity of pertussis vaccine or its protective fraction, then the problem of preparing a nontoxic, purified vaccine would be very great indeed. This is because of the considerable and growing evidence that protection and histamine sensitization may be functions of the same molecule. This study indicates, however, that pertussis endotoxin makes, at most, a minor contribution to the histamine-sensitizing activity of vaccine, and that such activity, arising from endotoxins in general, is different in kind from that of the typical HSF of *B. pertussis*.

This qualitative difference between vaccine sensitization and endotoxin sensitization consists in the fact that with endotoxins we could find no systematic relation between dose and histamine mortality, and could not achieve 100% mortality with any dose (Tables 1, 2, and 5). These results are in agreement with the findings of Malkiel and Hargis (9). None of their endotoxin preparations was as effective as pertussis vaccine in inducing hypersensitivity to histamine. They also reported a lack of linearity between the dose of certain endotoxins used as sensitizing agents and the degree of sensitivity to histamine engendered. These authors cited several other activities of endotoxins in which the absence of dose-response regressions were noted. In sharp contrast, however, Table 3 indicates that increasing doses of pertussis-protective fraction caused increasing mouse mortality upon challenge with a fixed dose of histamine. Thus, dose-response regressions permitting computation of median sensitizing dosage were always available from pertussis HSF, and 100% lethality of mouse groups could always be obtained. The latter characteristics of pertussis sensitization are obtainable whether the purified protective antigen or the crude cellular vaccine (16) is used, indicating that the endotoxin component of crude vaccine makes, at most, a minor contribution to such sensitization.

In further differentiation of the endotoxic and the vaccine mediators of histamine susceptibility in mice, we showed that tryptic digestion

TABLE 5. *Effect of heating typhoid endotoxin on the histamine sensitivity of mice surviving graded doses**

Addition	Dosage	Death rate†	
		Toxic	Histamine
	μ g		
Unheated typhoid endotoxin.....	27	0/20	9/20
	81	0/20	14/20
	243	1/20	4/19
	729	15/20	0/5
Heated typhoid endotoxin.....	27	0/20	9/20
	81	0/20	8/20
	243	2/20	9/18
	729	16/20	2/4
Unheated pertussis vaccine.....	13.5×10^9 cells	0/10	10/10
Heated pertussis vaccine.....	13.5×10^9 cells	0/10	1/10

* Strain used was *Salmonella typhosa* 0901 LPS, lot 473454. Heat treatment was at 100 C for 30 min.

† Total death rate for unheated typhoid endotoxin was 16/80 toxic and 27/64 histamine; for heated typhoid endotoxin, 18/80 toxic and 28/62 histamine.

and heat at 100 C abolished the major part of the histamine-sensitizing capacity of pertussis vaccine or its extracts. Heating, however, did not affect either the direct toxicity of typhoid endotoxin or the lethal rate of histamine shock induced in the survivors. That LPS endotoxin is unaffected by trypsin, which indeed, is a standard reagent in its purification, requires no experimental demonstration.

Confirmation of the fact that endotoxins are capable of altering the reactivity of mice to histamine could possibly prove of more than academic interest. Bacterial endotoxins have long been the object of extensive investigation because of their diverse effects on the physiological responses of experimental animals and man. Recently, Schayer (17) proposed the concept of "induced synthesis of histamine" resulting from endotoxin-mediated activation of the enzyme histidine decarboxylase in an attempt to elucidate and causally integrate several of the biological activities of endotoxins. He found (18) that the injection of mice with bacterial endotoxins, including that of *B. pertussis*, results in a rapid increase in histidine decarboxylase activity which reaches a peak value in about 6 hr and which returns to essentially normal levels within 24 hr. In contrast, he reported that mice inoculated with whole pertussis vaccine display maximal histidine decarboxylase activity in 3 or 4 days, at which time such mice are most sensitive to exogenously administered histamine. Schayer proposed that, in pertussis-vaccinated mice, the release into certain tissues of newly synthesized histamine through the action of histidine decarboxylase results in an increased tissue permeability, and, ultimately, an enhanced susceptibility to histamine challenge. It should be noted, however, that in the present experiments we were able to demonstrate augmented sensitivity to histamine 4 days after injection of mice with endotoxins—at a time when Schayer reported normal histidine decarboxylase activity (18).

Recently, Fishel and co-workers (2) postulated that pertussis-induced hypersensitivity to histamine was possibly a result of a functional imbalance between the α - and β -receptors of the adrenergic division of the autonomic nervous system. He found that the injection of a β -adrenergic blocking agent, dichloroisoproterenol, resulted in an enhanced susceptibility to histamine in normal mice, whereas an antagonistic α -adrenergic blocking agent, dibenzylamine, protected *B. pertussis*-sensitized mice from the lethal effects of histamine. Pieroni and Levine (Bacteriol. Proc., p. 63, 1966) and Bergman and Munoz (1) confirmed these results. In reviewing some of the recognized actions of endotoxins, as well as the autonomic nervous system, on vascu-

lar reactivity, Fishel et al. (3) discussed the interesting possibility that certain of the biological activities of bacterial endotoxins, including the Schwartzman phenomenon, might also be mediated through adrenergic mechanisms. The role of the autonomic nervous system in endotoxin-induced hypersensitivity to histamine, as well as certain characteristics of this phenomenon, are currently under investigation and will be the subject of a further report.

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