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Biological Activities of Crystalline Pertussigen from *Bordetella pertussis*

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We studied various biological activities of crystalline pertussigen and found that in mice as little as 0.5 ng of pertussigen induced hypersensitivity to histamine, 8 to 40 ng induced leukocytosis, 2 ng increased production of insulin, 0.1 ng increased production of immunoglobulin E and immunoglobulin G1 antibodies to hen egg albumin, 9.5 ng increased susceptibility to anaphylactic shock, and 0.5 ng increased the vascular permeability of striated muscle. We also found that in Lewis rats 20 ng of pertussigen promoted the induction of hyperacute experimental allergic encephalomyelitis. Pertussigen given intraperitoneally was toxic to mice at a dose of 546 ng. Treatment of pertussigen with glutaraldehyde eliminated this toxicity. Mice immunized with 1,700 ng of detoxified pertussigen were protected against intracerebral challenge with 3×10^4 viable *Bordetella pertussis* cells. When as little as 0.5 ng of pertussigen was given intravenously to mice, the increased susceptibility of the animals to histamine could still be detected 84 days later. The biological properties of crystalline pertussigen indicate its similarity to leukocytosis-promoting factor, Islet-activating protein, late-appearing toxic factor, and mouse-protective antigen of *B. pertussis*.

Recently, pertussigen was obtained in a highly purified, crystalline form (1; J. J. Munoz and H. Arai, *in Proceedings of the International Symposium on Bacterial Vaccines*, in press). This substance, which originally was referred to by us and others as histamine-sensitizing factor, is a protein, but it is not yet clear whether it is composed entirely of amino acids (18). We have indicated previously (1, 18) that pertussigen is similar to leukocyte-promoting factor (LPF) (15), Islet-activating protein (IAP) (23), late-appearing toxic factor (10), and mouse-protective antigen (18), but most investigators do not agree that all of these substances are identical. In this work with highly purified pertussigen, we showed that LPF and IAP activities are found in pertussigen and that the doses of pertussigen required to produce these effects are as low or lower than those reported by other workers for their purified preparations. It should be stressed that the names given to this substance have been expressions of the activities studied in individual laboratories, and thus it is clear that on this basis alone, pertussigen should be identical to LPF, IAP, and late-appearing toxic factor. In our laboratory, we have shown that pertussigen is serologically identical to the LPF prepared by Arai and Sato (2) and Morse and Morse (15; unpublished data). Mizushima (*in Proceedings of the International Symposium on Bacterial Vaccines*, in press) has shown that LPF is identical to IAP. Thus, pertussigen is serologically similar if not identical to LPF and IAP. Dobrogosz et al. (6) have also shown that the pertussigen prepared by us and the LPF prepared by Morse produce similar sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein patterns.

The results of various tests performed with highly purified, crystalline pertussigen are described here.

MATERIALS AND METHODS

Preparation of pertussigen. Crystalline pertussigen was prepared as previously described (1; Munoz and Arai, in press). Solutions made in 50 mM tris(hydroxymethyl)aminomethane (pH 8)-1 M NaCl were standardized by their optical densities at 280 nm. An optical density at 280 nm of 1.00 corresponded to approximately 500 μg of protein per ml, as measured by the colorimetric method of Lowry et al. (13), using bovine serum albumin as a standard.

Detoxification of pertussigen. The toxicity of pertussigen could be reduced by treatment with glutaraldehyde as described elsewhere (Munoz and Arai,

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in press). The detoxification procedure consisted of treating pertussigen which had been dialyzed previously in 20 mM sodium phosphate containing 0.5 M NaCl (pH 7.6) with enough 0.2% glutaraldehyde solution made in the same buffer to bring the concentration of glutaraldehyde to 0.05%. This mixture was incubated at room temperature for 2 h, and then enough 0.2 M *l*-lysine solution (made in the same buffer) was added to bring the final concentration of *l*-lysine to 0.02 M. The mixture was incubated for 2 h at room temperature and then dialyzed for 2 days against 20 mM sodium phosphate buffer containing 0.5 M NaCl and 0.02 M *l*-lysine (pH 7.6).

Assays for biological activities. Tests for increased susceptibility to histamine, induction of leukocytosis, protection of mice against intracerebral challenge with *Bordetella pertussis*, and enhancement of anaphylactic sensitivity of mice to hen egg albumin (HEA) were performed as previously described (18). The enhancement of the immunoglobulin E (IgE) class of immunoglobulins with specificity to HEA has also been described previously (5, 18), but the titration of mouse IgE with specificity to HEA was done by passive cutaneous anaphylaxis tests in Lewis rats. In the passive cutaneous anaphylaxis test, the rat skin detects only the IgE class of mouse immunoglobulin, not IgG1 (21). The backs of the rats were shaved the day before dilutions of mouse antisera were injected. Each of 15 sites on the back of each rat received 0.05 ml of the desired dilution. After 24 h each rat was challenged intravenously (i.v.) with 1 ml of a 0.5% solution of Evans blue in saline containing 0.5% crystalline HEA. Passive cutaneous anaphylaxis reactions were measured on the dermis side of the skin 30 min later. IgG1 antibody to HEA was measured by 2-h passive cutaneous anaphylaxis reactions in the skin of CFW mice that had received 0.05 ml of a dilution of serum that had been heated for 3 h at 56°C to inactivate the IgE. The mice were challenged i.v. with 0.2 ml of 0.5% Evans blue containing 0.5% HEA.

Experimental allergic encephalomyelitis (EAE) was induced in Lewis rats as described previously (4) by using the technique of Levine et al. (12).

Toxicity tests were performed in 3-week-old mice. Doses of pertussigen were given intraperitoneally (i.p.) in 0.2 ml of phosphate-buffered physiological saline (pH 7.2). The mice were weighed daily, and deaths were recorded.

Insulin determinations were performed in mice by a modification of the method used by Mori et al. (14). Briefly, CFW female mice received doses of pertussigen i.v., and 3 days later each received an i.p. dose of 25% glucose in physiological saline (0.02 ml/g of body weight). Exactly 15 min later the mice were anesthetized with ether and exsanguinated by cutting the axillary blood vessels. The blood was allowed to clot in test tubes, and the sera were removed after centrifugation at 1,000 × *g* for 0.5 h. Insulin levels in these sera were determined by a radioimmunoassay performed with reagents purchased from Amersham Corp., Arlington Heights, Ill. We used the exact procedures and reagents recommended by the manufacturer (Insulin RIA kit; catalog no. IM 78; Amersham Corp.). Briefly, 100 μl of binding reagent was added to 100 μl of serum, and the mixture was incubated for 45 min at 2 to 5°C, after which 100 μl of a solution containing ^{125}I-labeled insulin was added and the mixture was refrigerated at 2 to 5°C for 2.25 h. Then 700 μl of buffer was added, and the preparation was mixed and centrifuged for 25 min at 1,500 × *g*. The supernatant was decanted, and the tubes were drained carefully. The radioactivity in each tube containing precipitated material was determined with a gamma counter. A standard curve was constructed by using triplicate determinations of known concentrations of insulin. The unknown sera were diluted 1:2 with phosphate-buffered saline and were run in duplicate.

Increased permeability of tissue to radioactive human serum albumin was measured by the method of Leibowitz and Kennedy (11), as previously described (3). Hemagglutination tests were performed as described previously (18, 22).

Animals. Induction of EAE was done in Lewis rats reared at the Rocky Mountain Laboratories. All other assays were performed in CFW mice raised in our laboratory. For histamine sensitization tests we used 5- to 8-week-old female mice. The mouse protection tests were performed in 3-week-old male mice. The induction of leukocytosis and the increase in anaphylaxis were carried out in 5- to 8-week-old male mice.

Antisera. Antisera against pertussigen were raised in rabbits and sheep as described previously (16). Anti-pertussigen sera were also raised in 5- to 8-week-old CFW mice. One sublethal dose (about 0.2 μg with some preparations) was given i.p., and then four or five subcutaneous boosters (similar doses) were given at 2-week intervals. The mice were bled 1 week after the last booster dose.

RESULTS

Crystalline pertussigen was highly active in a number of biological assays. The following activities, which are commonly attributed to LPF and IAP, were observed: (i) 0.5 ng of pertussigen (expressed as weight of protein) sensitized 50% of the mice tested to histamine; (ii) the dose that induced leukocytosis (a doubling of the leukocyte count in the peripheral blood) in CFW male mice was 8 to 40 ng/mouse; (iii) the dose that induced an increased secretion of insulin due to a glucose load in mice was 2 ng; and (iv) pertussigen had 5×10^3 hemagglutinating units per mg of protein (1 hemagglutinating unit was the amount of pertussigen that produced granular agglutination of 50 μl of a 0.5% suspension of chicken erythrocytes). The agglutination was typically granular and different from the even agglutination caused by the filamentous hemagglutinin (8).

In addition, crystalline pertussigen was toxic to mice at an i.p. dose of 546 ng/mouse (dose that killed 50% of the mice tested). Typically, the deaths were delayed, as has been reported for preparations of late-appearing toxic factor (10). When 5 μg of pertussigen was administered, most mice did not gain weight, and deaths occurred by day 5. The last mouse died on day 8

(Fig. 1). A 1-μg dose of one preparation killed four of five mice. The mice that received the 1-μg dose gained weight from days 2 to 5 and then remained at nearly constant weights until they died (Fig. 2). One mouse that survived the 1-μg dose gained weight at a normal rate.

When pertussigen was treated with glutaraldehyde, its toxicity and some of its other activities were reduced. The histamine-sensitizing and hemagglutinating activities were reduced markedly. The histamine-sensitizing activity was less than 25% of the original value (dose that sensitized 50% of the mice tested, >16 ng/mouse), and the hemagglutinating activity was less than 4% of the original value (the original material agglutinated chicken erythrocytes at a dilution of 1:1,200, whereas the glutaraldehyde-treated material produced only questionable agglutination of a similar suspension of erythrocytes at a dilution of 1:40 [lowest dilution tested]). The reduction of toxicity allowed the demonstration of mouse-protective activity at a dose of 1.7 μg (dose that protected 50% of the mice tested). Previous preparations of pertussigen made either from cells grown in shake cultures, treated with acetone, and then extracted (18, 20) or from cells collected from shake cultures and then extracted with 1 M NaCl–0.05 M sodium pyrophosphate–0.5% Triton X-100 (pH 8) and further purified by column chromatography (unpublished data) were not as toxic (mice tolerated doses of 10 μg without any obvious toxic effects, and 1.8 μg protected mice from intracerebral infection with *B. pertussis* [20]) as the crystalline preparations used in this study. In addition, glutaraldehyde-treated pertussigen showed active specific precipitation with antibody.

The ability of pertussigen to stimulate an IgE response to HEA is shown in Fig. 3. The mice received 100 μg of HEA i.p. and different amounts of pertussigen i.v. at zero time. On day 21 the mice received an i.p. booster dose of 5 μg of HEA. The animals were bled 7 days after the booster dose. Each serum was titrated for its content of anti-HEA IgE and IgG antibody by

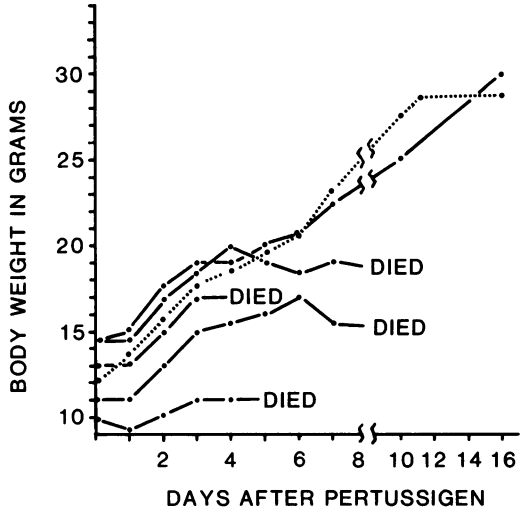

FIG. 2. *Body weights of five individual mice after i.p. inoculation of 1 μg of pertussigen (solid lines). For comparison, the dotted line shows the average weight gain of five mice that received diluent only. The weight gains of the individual mice in this control group were uniform and were very close to the average values shown in this figure.*

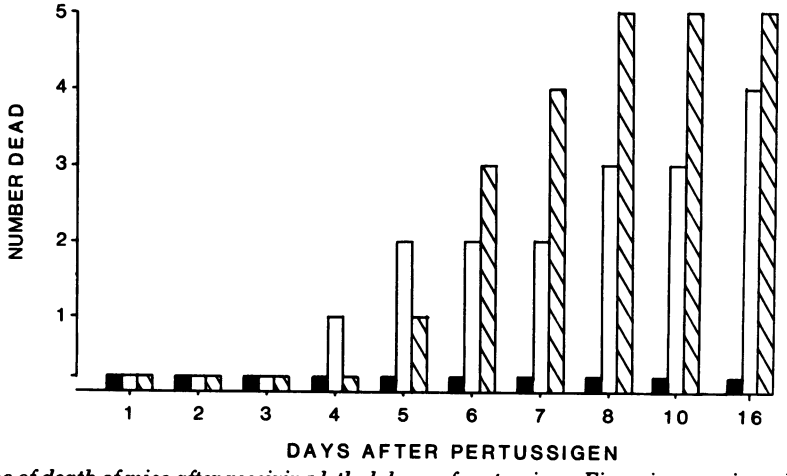

FIG. 1. *Time of death of mice after receiving lethal doses of pertussigen. Five mice were inoculated for each test group. Mice that received 0.1 to 0.2 μg of pertussigen survived (data not shown), as did mice that received diluent only. Solid bars, control mice; open bars, mice that received 1 μg of pertussigen at zero time; cross-hatched bars, mice that received 5 μg of pertussigen at zero time.*

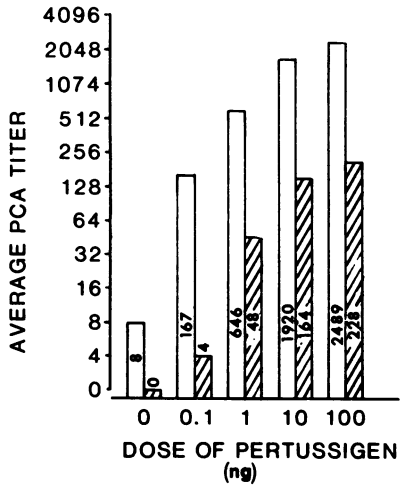

FIG. 3. *Increases in IgE (open bars) and IgG1 (cross-hatched bars) antibodies to HEA in the sera of mice immunized with 100 μg of HEA i.p. and different doses of pertussigen i.v. After 21 days each mouse received a booster dose of 5 μg of HEA, and 7 days later the animals were bled. Each bar represents the geometric mean titer of five different sera.*

the passive cutaneous anaphylaxis test. The results showed that as little as 0.1 ng of crystalline pertussigen increased the production of IgE with specificity to HEA. As the dose of pertussigen was increased, the titer of IgE also increased; a 1,000-ng dose of pertussigen was too toxic and killed 11 of 14 mice. The highest average titer obtained was 2,489; this was in the group that received 100-ng doses of pertussigen. Control mice receiving HEA alone had undetectable titers of IgE with specificity to HEA.

The titer of antibody against HEA of the IgG1 class was only slightly increased with a dose of 0.1 ng of pertussigen, and at the 100-ng dose the average titer obtained was 228. Although this was about 10 times lower than the IgE titers, it did not mean that on a weight basis there was 10 times more IgE produced, because IgE is more efficient in inducing passive cutaneous anaphylaxis than IgG1 antibody is. Nevertheless, the increase in IgE was as great as the increase induced by other methods of stimulating this type of antibody (9).

The ability of pertussigen to accelerate the induction of EAE was tested by giving 200 mg of guinea pig spinal cord emulsion mixed with different amounts of crystalline pertussigen to Lewis rats i.p. The day of onset and degree of paralysis were recorded each day for 14 days, and the total EAE score was calculated as previously described (4). The results of a representative test are given in Table 1 and show that 20 ng of pertussigen increased the EAE score significantly.

Crude preparations of pertussigen change the permeability of striated muscle to human serum albumin (3). Table 2 shows that 0.5 ng of crystalline pertussigen induced a significant increase in the permeability of thigh muscles to ^{125}I-labeled human serum albumin.

The duration of histamine hypersensitivity was tested by giving groups of 70 mice either 0.5, 2.5, or 12.5 ng of crystalline pertussigen i.v. and then determining the i.p. 50% lethal dose of histamine 7, 21, and 84 days later (Table 3). The 50% lethal doses in normal mice were >18.75, 16.79, and 16.28 mg for challenges on days 7, 21, and 84, respectively. The 50% lethal doses of histamine at 21 and 84 days for the groups of

TABLE 1. *Enhancement of experimental allergic encephalomyelitis in Lewis rats by pertussigen*

Dose of pertussigen (ng/rat)[a]	No. paralyzed/no. tested	EAE score[b]		
		Severity	Acuteness	Total
0	1/5	1	0	1
4	1/5	3	0	3
20	3/5	9	6	15
100	5/5	12	11	23

[a] Each rat received i.p. 200 mg of guinea pig spinal cord and pertussigen at one of the doses indicated.

[b] The total score was calculated by adding the severity of paralysis score to the acuteness score for each rat. The severity of paralysis was scored as follows: 0, no symptoms; 1, slight ataxia and slight paralysis of tail and hind legs; 2, definite paralysis of tail and hind legs; 3, complete paralysis of tail and both hind legs but animal still capable of moving about by means of front legs; and 4, totally paralyzed, lying on side. The acuteness was scored as follows: 5, paralysis appeared on day 5; 4, paralysis appeared on day 6; 3, paralysis appeared on day 7; 2, paralysis appeared on day 8; 1, paralysis appeared on day 9; and 0, paralysis appeared on any day beyond day 9. Thus, the total score is a reflection of the acuteness and severity of EAE.

TABLE 2. *Increased permeability of muscle induced by pertussigen*[a]

i.v. dose of pertussigen (ng/mouse)	Extravascular blood equivalent[b]	P[c]
0(buffer only)	8.713 ± 0.167[d]	
0.5	10.232 ± 0.478	<0.025
2.5	10.167 ± 0.279	<0.001
12.5	12.574 ± 0.545	<0.001

[a] Vascular permeability was measured by the method of Leibowitz and Kennedy (11).

[b] The extravascular blood equivalent was a relative measurement of the amount of radioisotope-labeled albumin that came out of the vascular bed.

[c] Probabilities were calculated by Student's t test.

[d] Mean ± standard error of the mean.

mice that received from 0.5 to 12.5 ng of pertussigen ranged from 0.59 to 0.82 mg and were not significantly different. It should be noted that if only one 0.5-mg dose of histamine had been used for challenging mice, a marked decrease in sensitivity would have been apparent by day 21, as previously reported by others (18).

Pertussigen also enhanced the anaphylactic sensitivity of mice that received 500 μg of HEA i.p. mixed with different doses of pertussigen (Fig. 4). A dose of 9.5 ng was the 50% effective dose of pertussigen (i.e., the dose that increased fatal anaphylactic sensitivity in 50% of the mice).

DISCUSSION

Our findings clearly show that crystalline pertussigen has many of the biological activities that have been observed in animals which receive whole-cell pertussis vaccine (18). Furthermore, they show that crystalline pertussigen has all of the activities described for LPF, IAP, and late-appearing toxic factor and that the doses required are equal to or lower than those that have been reported previously.

Serologically, LPF and IAP are identical (Mizushima, in press), and pertussigen is identical to LPF (unpublished data). The so-called late-appearing toxic factor was recognized as being similar to LPF by the workers that described it (10). Thus, pertussigen is serologically identical to LPF and IAP.

Dobrogosz et al. (6) found that LPF and pertussigen are similar as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Although we have crystallized pertussigen, the exact chemical nature of this substance is still unknown. We have found that crystalline pertussigen is principally protein and contains most of the common amino acids. Other workers have reported that their purified preparations (LPF [15] and IAP [23]) were composed entirely of amino acids, whereas Arai and Sato (2) reported that their purified preparation of LPF contained carbohydrate and lipid. More work will be needed to settle the exact chemical nature of pertussigen.

Pertussigen was highly active in all of the biological tests used. Only 0.5 ng of pertussigen per mouse sensitized CFW female mice to histamine, and as little as 0.1 ng/mouse administered with HEA increased the production of IgE with specificity to HEA. In CFW male mice, 8 to 40 ng of pertussigen was required to induce leukocytosis (with other preparations, we found an effective dose of 100 to 500 ng [1]). The 8- to 40-ng dose is similar to the value which Morse and Morse (15) reported for their preparation.

Pertussigen induced an increased production of insulin by the β-cells of the pancreas of mice at a dose of 2 ng, which is as low, as has been reported previously (23). All of the other activities described here have not been studied with purified preparations by other workers. Hyperacute EAE was induced in rats by 20 ng of pertussigen, permeability of striated muscle was induced in mice by as little as 0.5 ng, and active anaphylaxis was induced by about 10 ng.

Our preparations of crystalline pertussigen were toxic to mice at a dose of 546 ng. Typically, the toxic action was delayed. Most of the deaths at a dose of 1,000 to 5,000 ng occurred 5 to 8 days after i.p. injection. These preparations were too toxic to test for protective activity against intracerebral challenges with *B. pertussis*. At the well-tolerated doses of 100 to 200 ng, the preparations failed to protect mice. Pertussigen could

TABLE 3. *Duration of increased susceptibility to histamine after administration of pertussigen*

i.v. dose of pertussigen (ng/mouse)	50% Lethal dose of histamine (mg) on the following days after pertussigen administration:[a]		
	7	21	84
0 (buffer only)	>18.75	16.79	16.28
0.5	<0.5	0.77	0.79
2.5	<0.5	0.72	0.82
12.5	<0.5	0.61	0.59

[a] The 50% lethal dose was the amount of histamine base (given as histamine diphosphate) that killed 50% of the mice within 2 h after i.p. administration. Each value was calculated from the results obtained by challenging three groups of 10 mice each with different doses of histamine.

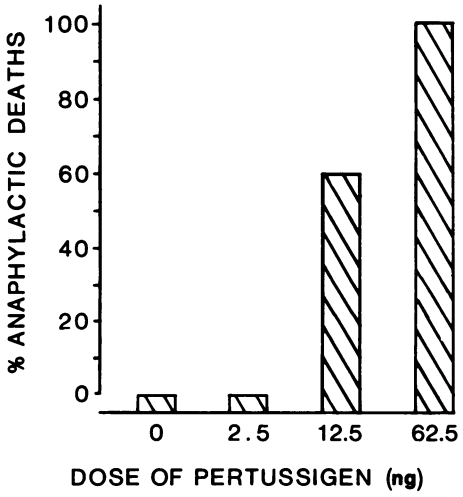

FIG. 4. *Increases in anaphylactic deaths induced by pertussigen in mice after single i.p. injections of 0.5 mg of HEA mixed with different doses of pertussigen at zero time. The mice were challenged i.v. on day 15 with 0.5-mg amounts of HEA.*

be detoxified easily by glutaraldehyde, and in this form protective activity was demonstrated at a dose of 1.7 μg. This amount is as low as has ever been reported for a *B. pertussis* protective antigen.

We should state here that previous work in our laboratory produced pertussigen preparations that were well tolerated by mice at doses of 10 μg (20; unpublished data). These preparations were made either from acetone-extracted cells that had been grown in Casamino Acids liquid medium with constant agitation or from cells grown under similar conditions and treated with 0.5% Triton X-100 in 1 M NaCl–0.05 M sodium pyrophosphate (pH 8). Under these conditions pertussigen was not as toxic and protected mice from infection at doses similar to doses of detoxified crystalline pertussigen. Compared with the activities of crystalline pertussigen, the other activities of these preparations were inferior. For example, the histamine-sensitizing activity of one of these preparations (19) was 260 ng/mouse, compared with 0.5 ng/mouse for the crystalline preparation. Thus, it seems that the toxicities of our previous preparations were reduced, as well as some of the other activities.

Previously, we reported that one of the properties of pertussigen was its ability to remain active for a long time in mice, as judged by the persistence of the increased susceptibility of mice to histamine after one injection of pertussigen (17). This was confirmed in the present work with crystalline pertussigen. At 84 days after receiving as little as 0.5 ng of pertussigen, mice still showed a marked increase in sensitivity to histamine. This is indeed a remarkable property and may explain why pertussigen is so active in mice.

The mode of action of pertussigen is not completely known. Fishel et al. (7) and we (18, 19) have postulated that it interferes with some functions of adrenaline, but the mechanisms of this interference are not yet clear. Now that pertussigen has been obtained in a highly purified form, it should be easier to determine not only its mode of action but also its exact chemical composition.

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