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The Protective Activity of Components of *Bordetella pertussis* Cell Walls

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Summary. A method for the preparation and purification of cell walls from *Bordetella pertussis* is described. After treatment with a detergent, sodium lauryl sulphate, followed by trypsin, or with four enzymes in succession, the material could induce protection against experimental intracerebral infection in mice. It is non-toxic and contains no histamine-sensitizing factor. Antisera prepared against the cell walls also gave protection. Agar-gel diffusion tests revealed the presence in the cell-wall material of lipopolysaccharide and at least one other component. These substances were apparently species specific. Evidence is also given to show the lack of protective activity in the lipopolysaccharide *per se*.

INTRODUCTION

Although the whole bacterial cells of *B. pertussis* are widely used as antigen for active prophylaxis, the incidence of moderate to severe reactions in the recipients of such vaccines stimulated investigations into the protective activities of various fractions of the bacteria. Among the first workers to show that the whole bacterial cell was not necessary for protection were Cruickshank and Freeman (1937). They treated *B. pertussis* cells with trypsin, then precipitated protective material with ethanol. That only a small portion of the bacteria was necessary for protection was subsequently indicated by the selective absorption of this entity on to red-cell stroma after ultrasonic disintegration of the bacteria (Pillemer, 1950; Pillemer, Blum and Lepow, 1954). This fraction was estimated to be less than 1 per cent of the nitrogenous material present in the ultrasonic extracts (Pillemer *et al.*, 1954).

More recently, two groups of workers have prepared cell-wall material from *B. pertussis* and have shown that it is a good protective agent, although relatively free from toxin and other antigens (Munoz, Ribi and Larson, 1959; Billaudelle, Edebo, Hammarsten, Hedén, Malmgren and Palmstierna, 1960). The present work reports a method for the purification of *B. pertussis* cell wall to produce protective material free from toxin and from histamine-sensitizing factor (HSF), an antigen with unusual pharmacological properties.

MATERIALS AND METHODS

Strains and Methods of Culture

Two strains of *B. pertussis* were used for the present work. Strain 4507 was isolated at the City Hospital for Infectious Diseases, Edinburgh, and was grown in the tris-resin liquid medium described in an earlier paper (Sutherland and Wilkinson, 1961). Cultures in Roux bottles or in 500 ml. Erlenmeyer flasks were incubated at 35° for 72 hours. Cells

of strain 18398, grown in a liquid medium similar to that of Cohen and Wheeler (1946), were kindly provided by Dr. A. F. B. Standfast (Lister Institute of Preventive Medicine, Elstree). The bacteria were washed once with saline containing merthiolate (0·01 per cent), then stored at −40° until required.

Bordet-Gengou medium (Cruickshank *et al.*, 1960, p. 231) was used for culture of material from mouse brains and for challenge cultures.

Active Protection Tests

Suitable groups of 6-week-old white mice (strain A2G, Laboratory Animals Centre, Carshalton), were injected intraperitoneally with 0·2 ml. of the protective agent. They were challenged 14 days later with an estimated 50,000 organisms harvested from a 24 hour Bordet-Gengou culture of the mouse-virulent *B. pertussis* strain 18/323 (Kendrick, Eldering, Dixon and Misner, 1947). The challenge dose, suspended in 1 per cent (w/v) casamino acid solution, was injected intracerebrally into the anaesthetized mice. Deaths occurring in the 48 hours following challenge were attributed to cerebral damage. Thereafter, the mice were observed for a further 12 days. All deaths were noted and cultures from the brains of dead mice were made on Bordet-Gengou medium. When *B. pertussis* was not isolated from the brain of a dead mouse, that fatality was not attributed to *B. pertussis* infection.

Passive Protection Tests

Groups of 8-week-old mice were injected intraperitoneally with 0·2 ml. of saline dilutions of the antiserum. The mice were challenged 4 hours later by the intracerebral route, using the same dose and conditions as in active protection tests.

Toxicity Tests

These were performed using 6-week-old mice, the animals being injected intraperitoneally with a saline suspension (0·2 ml.) of the material under examination. All deaths occurring until 48 hours after injection were regarded as being due to a toxin.

Histamine-Sensitization Tests

The method of Dolby (1958) was used.

Preparation of Antisera in Rabbits

Antisera for passive-protection tests and for agar-gel diffusion tests were prepared as described by Dolby and Standfast (1958).

Agar-Gel Precipitation Tests

These were performed by the method of Crumpton and Davies (1956), a modification of the original method of Ouchterlony (1953).

Enzymes Used in Cell-Wall Treatment

Lipase and trypsin were obtained from L. Light & Co. (Colnbrook).

Lysozyme was prepared from egg white (Carter, 1949) and used without further purification.

Ribonuclease was isolated from bovine pancreas (Kunitz, 1941) and purified by chromatography on CM-cellulose (Taborsky, 1959).

Preparation and Treatment of Cell Walls

The bacteria were suspended in cold distilled water containing 0·01 per cent merthiolate, then disintegrated with ballotini glass beads in a Mickle tissue disintegrator (Salton, 1953). The unbroken cells were removed by centrifuging for 10 minutes at 2500 *g* in an angle centrifuge at 0°, the deposit being discarded. The supernatant was removed and centrifuged at 10,000 *g* for 15 minutes at 0°. The deposited cell walls were washed twice with cold 2·0 M sodium chloride solution and twice with cold distilled water. The absence of unbroken cells at this stage was confirmed by examination in the electron microscope. However, the cell walls still possessed adherent, electron-dense cytoplasmic material. Although the extraneous matter might be removed by further washings as shown by Billaudelle *et al.* (1960), other methods were tested. These involved digestion of the cytoplasmic material with enzymes or its removal by treatment with detergent.

In all the enzymic treatments, the cell walls were suspended at a concentration of approximately 1 per cent (w/v) in the appropriate buffer. For tryptic digestion, the enzyme at a final concentration of 0·01 per cent (w/v) was added to the cell-wall suspension in phosphate buffer (0·05 M, pH 8·0). The mixture was incubated at 37° for 3 hours with constant stirring. The treated cell-wall material was then harvested by centrifugation and washed once with cold distilled water. The product comprised 45 per cent of the dry weight of the original cell walls and was free from cytoplasmic material when examined in the electron microscope.

Lysozyme treatment was carried out in tris buffer (0·05 M,) pH 7·6, at 37° for 8 hours using 0·01 per cent (w/v) enzyme. The appearance of the cell walls was almost unchanged and 87 per cent of the original material remained. Much more material was removed when the cell walls were treated with lipase, the product comprising 40 per cent by weight of the original. The enzyme (0·02 per cent w/v) in this case was added to the cell-wall suspension in phosphate buffer (pH 7·0, 0·05 M) and the mixture was incubated at 37° for 6 hours. Examination of the product in the electron microscope revealed that part of the cytoplasmic membrane remained attached to the cell walls, but the other electron-dense cytoplasmic material had been removed.

Surface-active agents such as sodium dodecyl sulphate were used to disaggregate the lipid-containing cell walls of Gram-negative bacteria by Shafa and Salton (1960) and it seemed that detergents might also be suitable for removing cytoplasmic material, which was presumed to include lipid and lipoprotein, from the cell walls of *B. pertussis*. The cell walls were suspended in a solution of sodium lauryl sulphate (0·2 per cent w/v) and stirred at 37° for 30 minutes. After this treatment, the cell walls were washed once with distilled water. They comprised 42 per cent of the original dry weight and no trace of cytoplasmic material was observed in the product. In one case, the detergent treatment was performed at 50°, as it was found that at that temperature more material was removed from the cell wall. A combination of detergent treatment followed by trypsin digestion was also employed. The final product in this case was about 30 per cent of the original dry weight.

RESULTS

BIOLOGICAL PROPERTIES OF CELL-WALL PREPARATIONS

The protective activity of several of the cell-wall preparations was tested in mice and the results are shown in Table 1. It can be seen that the different treatments left the protective component largely unaffected. All the products still gave good protection at the

higher dose level employed. Tests for toxicity and for histamine-sensitizing activity were also performed and the results are shown in Tables 2 and 3 respectively. It was apparent that the toxicity of the original cell-wall material had been completely removed or destroyed by all the treatments except that with lysozyme. However, only one preparation, that digested with trypsin, failed to sensitize mice to histamine. It therefore appeared that, of the methods tested, only that involving trypsin produced cell-wall material free from toxin and from HSF.

TABLE 1

ACTIVE PROTECTION TESTS WITH CELL-WALL PREPARATIONS

Treatment of cell walls	*Dose/mouse*	*Deaths/Total No. of mice challenged*
Untreated	200 µg.	3/12
	100 µg.	4/12
	40 µg.	6/12
	10 µg.	9/12
Trypsin	100 µg.	4/12
	10 µg.	7/12
Sodium lauryl sulphate	200 µg.	2/12
	40 µg.	5/10
	10 µg.	9/10
Lipase	200 µg.	2/10
	40 µg.	7/10
	10 µg.	8/11
Unvaccinated controls		23/23

TABLE 2

TOXICITY TESTS WITH CELL-WALL PREPARATIONS

Treatment of cell walls	*Dose/mouse*	*Deaths/Total No. of mice*
Untreated	1·0 mg.	10/10
	100 µg.	1/10
Trypsin	1·0 mg.	0/10
	100 µg.	0/10
Lipase	1·0 mg.	1/10
	100 µg.	0/10
Sodium lauryl sulphate	1·0 mg.	0/10
	100 µg.	0/10
Lysozyme	1·0 mg.	10/10
	100 µg.	2/10

In an effort to reduce still further the amount of material which would have to be injected to induce protection, three combinations of treatments were attempted. In the first two of these methods, the detergent treatment, at either 37° or 50°, was followed by tryptic digestion. In the third method, the cell walls were successively treated with ribonuclease, lipase, trypsin and lysozyme. Viewed in the electron microscope, the products

TABLE 3

HISTAMINE-SENSITIZATION TEST WITH CELL-WALL PREPARATIONS

Mice sensitized with	*Dose/mouse*	*Deaths/Total No. of mice*
Controls		0/15
Untreated cell wall	1·0 mg.	2/10
	100 µg.	0/10
Trypsin cell wall	1·0 mg.	0/9
	100 µg.	0/10
Lipase cell wall	1·0 mg.	7/10
	100 µg.	0/10
Sodium lauryl sulphate cell wall	1·0 mg.	4/9
	100 µg.	0/10
Lysozyme cell wall	1·0 mg	3/10
	100 µg.	0/10

of these different treatments appeared to be fairly similar. The results of protection tests can be seen in Table 4. The material produced after serial digestion with the four enzymes, or that from sodium lauryl sulphate treatment at 37° followed by trypsin digestion, still gave good protection at doses of 100 µg. (equivalent to approximately 1·6 mg. of unbroken cells). However, the product of treatment with the detergent at the higher temperature

TABLE 4

ACTIVE PROTECTION BY CELL-WALL PREPARATIONS

Treatment of cell walls	*Dose/mouse*	*Deaths/Total No. of mice challenged*
Sodium lauryl sulphate at 37° then trypsin	100 µg.	2/16
	50 µg.	9/19
	10 µg.	13/14
Sodium lauryl sulphate at 50° then trypsin	100 µg.	16/18
	50 µg.	17/20
	10 µg.	20/20
Ribonuclease, lipase, trypsin and lysozyme	100 µg.	0/18
	20 µg.	6/18
	5 µg.	11/18
Unvaccinated controls		23/23

(50°) was almost non-protective. It is possible that at the higher temperature some essential bond was disrupted by the sodium lauryl sulphate and that the resultant fragments had lost the protective activity possessed by the whole entity.

AGAR-GEL DIFFUSION TESTS

The different cell-wall preparations were all examined by agar-gel diffusion against antisera to various *Bordetella* species and strains, and to *B. pertussis* cell walls. The treated

and untreated cell-wall samples were dispersed in saline by treating for 30 seconds in a MSE-Mullard ultrasonic disintegrator. The crude unwashed cell walls appeared to contain a very large number of antigens, probably as many as could be detected in the original cells. This was ascribed to the large amount of cytoplasmic material contaminating the walls. The number of components was reduced slightly by the routine washings in sodium chloride solution and in distilled water, but at least seven antigens were still present (Fig. 1). Treatment with trypsin or detergent alone reduced the number of lines to four or five. After serial digestion with the four different enzymes, only four lines of precipitation could be detected. The product of combined detergent and trypsin treatment gave two lines and possibly a third (Figs. 1 and 2). One of the precipitation lines was identified

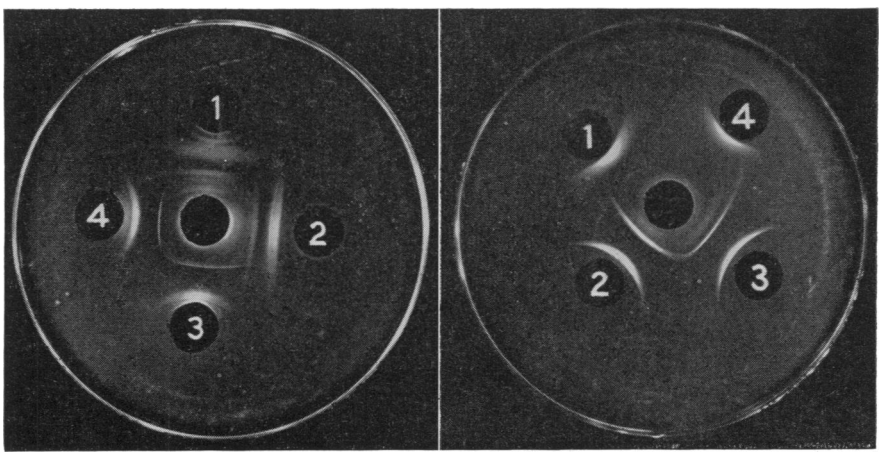

FIG. 1 FIG. 2

FIG. 1. Gel diffusion test. Central reservoir contains antiserum to whole cells of *Bordetella pertussis*. (1) Washed cell walls. (2) Cell walls treated with detergent at 37°. (3) Cell walls treated with four enzymes (see text). (4) Cell walls treated with detergent at 37° then trypsin.

FIG. 2. Gel diffusion test. Central reservoir contains antiserum to washed cell walls of *Bordetella pertussis*. (1) Lipopolysaccharide from whole cells. (2) Cell walls treated with detergent at 37° then trypsin. (3) Cell walls treated with four enzymes (see text). (4) Lipopolysaccharide from cell walls.

as being due to lipopolysaccharide. It was similar to the line formed by the product of phenol extraction of cells or cell walls (Fig. 2). Both it and the other line or lines were absent when tested against antisera to *B. parapertussis* and to *B. bronchiseptica*. The material treated with detergent at 50°, then with trypsin, gave no lines of precipitation with any antisera. There could be two explanations of this. The material might not have been finely enough divided to permit diffusion. This is unlikely, as it was not true of other cell-wall preparations and the lack of precipitation was consistent, despite repeated experiments with material dispersed by varying periods of ultrasonic irradiation. Alternatively, the treatment might have produced smaller molecules incapable of causing precipitation, e.g. degraded polysaccharide (Maclennan, 1960).

THE ROLE OF THE LIPOPOLYSACCHARIDE IN THE *B. pertussis* CELL WALL

The lipopolysaccharide is an essential component of the cell walls of Gram-negative bacteria, and *B. pertussis* is no exception. The isolation and properties of the lipopolysaccharide obtained from whole cells of this organism were reported by Maclennan

(1960). By using the phenol extraction method of Westphal, Luderitz and Bister (1952), the lipopolysaccharide has now been extracted from washed cell walls of *B. pertussis*. It resembled Maclennan's product in many respects and was shown by agar-gel diffusion to be immunologically homogeneous and identical with the product from whole bacteria. It comprised 20·4 per cent of the dry weight of the cell wall and approximately 48·5 per cent of the trypsin and sodium lauryl sulphate-treated preparation.

The lipopolysaccharide was not antigenic in mice or rabbits. It appeared to be a hapten, as it did give a line of precipitation with antisera prepared against whole cells or cell walls of *B. pertussis*. It did not induce protection in mice when administered in doses of 10–200 μg. per animal. This might have been because of its poor antigenicity. However, it was shown that the lipopolysaccharide produced by virulent strains of *B. pertussis* was immunologically identical with that produced by strains 51 and 2216, which are avirulent, poorly protecting strains of the organism (Sutherland, 1961).

In an attempt to elucidate the role of the lipopolysaccharide in protection against experimental infection, antisera prepared against whole cells and against cell walls were absorbed with the lipopolysaccharide until no further precipitation ensued (Cruickshank *et al.*, 1960, p. 327). The absorbed sera were then tested along with the corresponding unabsorbed sera to determine their ability to promote passive protection in mice. The results of this experiment are shown in Table 5. The antisera prepared against whole

TABLE 5

PASSIVE PROTECTION TESTS WITH RABBIT ANTI-PERTUSSIS SERA, BEFORE AND AFTER ABSORPTION WITH *Bordetella pertussis* LIPOPOLYSACCHARIDE

Mice previously injected with serum prepared against	*Serum*	*Dose/mouse*	*Deaths/Total No. of mice challenged*
Whole cells of *B. pertussis*	Unabsorbed	0·1 ml.	4/12
		0·025 ml.	5/10
		0·006 ml.	9/10
	Absorbed with lipopolysaccharide*	0·1 ml.	2/10
		0·025 ml.	6/10
		0·006 ml.	10/10
Trypsin-treated cell walls	Unabsorbed	0·1 ml.	4/12
		0·025 ml.	7/12
		0·006 ml.	9/12
	Absorbed with lipopolysaccharide*	0·1 ml.	4/12
		0·025 ml.	5/13
		0·006 ml.	8/12
Unprotected mouse controls			17/17

* The lipopolysaccharide was extracted from cell walls of *B. pertussis* strain 4507 (Westphal *et al.*, 1952).

cells and against cell walls all gave good protection and this was undiminished following absorption with lipopolysaccharide. It thus seemed that the precipitating antibodies against the lipopolysaccharide complex were not involved in protection against intracerebral challenge in mice.

DISCUSSION

Several workers have shown that *B. pertussis* cell walls can protect against experimental intracerebral infection in mice. Munoz *et al.* (1959) prepared cell walls after disintegra-

tion of the bacteria with glass beads, using differential centrifugation and thorough washing. Electron microscopic examination of these cell walls showed that small amounts of cytoplasmic material and of cell membranes still adhered to them. This preparation was a good protective agent and was practically non-toxic, but it did possess considerable histamine-sensitizing activity. Cell walls with similar properties, free from adherent material, were also obtained following disruption of the bacteria in a press (Billaudelle *et al.*, 1960). However, these workers did not report any test for histamine sensitization by their preparations.

Since the identity of the HSF was shown to differ from that of the antigen protecting against experimental intracerebral infection (Dolby, 1958), it seemed desirable to eliminate this component with its unusual pharmacological activity. This has apparently been achieved by the method of detergent and trypsin treatment described above. Even so, the preparation is still chemically complex and contains one component, the lipopolysaccharide, which may be unnecessary for protection. However, the removal of the lipopolysaccharide would be somewhat difficult and would require fairly drastic methods (e.g. phenol extraction) which would destroy the protective activity of the residue. It is also possible that the presence of the lipopolysaccharide is of value because of the adjuvant effect obtained with such compounds. Thus it may be particularly important in multiple vaccines.

It is interesting that in a micro-organism such as *B. pertussis*, which produces toxin, HSF, haemagglutinin and other antigens, the protective activity should rest mainly in the cell wall. This situation is not unique among genera of the *Parvobacteriaceae*. Similar situations exist in *Pasteurella tularensis* (Shepard, Ribi and Larson, 1955), in *P. pestis* (Keppie, Cocking and Smith, 1958) and in *Brucella abortus* (Markenson, Sulitzeanu and Olitzki, 1962). In these three organisms, cell-wall material gave good protection against experimental infection. The material from *Br. abortus* was prepared by ultrasonic treatment and it was reported that there was an actual gain in protective activity following bacterial disintegration, this being attributed to the increased dispersion achieved (Markenson *et al.*, 1962).

Our results confirm those of earlier workers who have shown that only a small part of the cell of *Bordetella pertussis* is necessary for protection. In the sodium lauryl sulphate–trypsin preparation, the material used to induce protection comprises approximately 6 per cent of the dry weight of the whole bacterial cells.

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