Release of Pertussis Toxin and Its Interaction With Outer-membrane Antigens

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The absence of subunit S3 in cell-associated pertussis toxin (PT) from a mutant of *Bordetella pertussis* which failed to produce cell-free toxin suggested that this subunit was involved in the release of PT into the culture medium. The addition of methylated β -cyclodextrin (MCD) to the culture medium caused a small but consistent increase in the release of lipopolysaccharide (LPS) by four wild-type strains of *B. pertussis*. Since previous studies have shown that MCD also enhances the levels of PT in culture supernates, it seemed probable that the increased shedding of outer-membrane vesicles (OMV) may explain the increased levels of both cell-free PT and LPS. Release of PT was inhibited in media buffered with HEPES but was unaffected in Tris/HCl buffer. This suggested that in addition to shedding of the outer membrane, increased permeability and greater destabilization of the outer membrane, as caused by Tris/HCl buffer, may be important in the release of PT. Our data do not support the idea that PT is packaged into OMV because only an insignificant proportion (0.01%) of the total cell-free PT was associated with LPS. The association of PT with small micelles derived from outer-membrane amphiphiles may be more important since the LPS content of PT purified from culture supernates (containing no large OMV) was nearly 18% by weight.

INTRODUCTION

Pertussis toxin (PT) is one of the protein toxins produced by virulent-phase cells of *Bordetella pertussis*, the causative agent of whooping cough (Wardlaw & Parton, 1983). This toxin (117 kDa) is composed of five dissimilar subunits, S1 (28 kDa), S2 (23 kDa), S3 (22 kDa), S4 (11.7 kDa) and S5 (9.3 kDa) (Tamura *et al.*, 1982) and occurs in both cell-free and cell-associated form in exponentially-growing cultures of *B. pertussis* (Perera *et al.*, 1987). Although a substantial amount of PT occurs as cell-free protein, the mechanism by which this toxin is released is not known.

In general, the ways in which proteins are transported across the outer membrane of Gram-negative organisms are poorly understood. It has been proposed that the heat-labile enterotoxin of *Escherichia coli* is released in outer-membrane vesicles (OMV) which are blebbed off from the cell surface (Gankema *et al.*, 1980). However, this mechanism seems to be incompatible with the failure to demonstrate an outer-membrane precursor for this toxin (Hirst *et al.*, 1984). The release of cholera toxin *in vivo* may also depend on an increase in outer-membrane permeability resulting from the 'detergent-like' action of intestinal bile salts on the cell surface (Fernandes & Smith, 1977). Release of extracellular proteins such as phospholipase C and alkaline phosphatase from *Pseudomonas aeruginosa*, however, does not depend on an increase in outer-membrane permeability (Poole & Hancock, 1983). A possible role for Bayer's junctions (zones of

Abbreviations: MCD, methylated β -cyclodextrin; OMV, outer-membrane vesicles; PT, pertussis toxin.

Fig. 1. Isolation of supernates and OMV fractions for analysis for PT.

adhesion between the plasma and outer membranes) in mediating the secretion of exotoxin A of *P. aeruginosa* has been suggested (Lory *et al.*, 1983) and secretion of α -haemolysin from *E. coli* is mediated by two outer-membrane proteins of M_r 46000 and 62000 thought to be in the outer membrane (Hartlein *et al.*, 1983; Wagner *et al.*, 1983). More recent studies by Mackman *et al.* (1985), however, indicate that these proteins mediating release of α -haemolysin are not located in the outer membrane.

Our objectives were two-fold: first, to ascertain whether increased outer-membrane permeability is necessary for the release of PT, and secondly, to determine whether or not cell-free toxin remains associated with outer-membrane antigens to constitute mixed micellar or vesicular packages of toxins similar to those postulated by Parker *et al.* (1985).

METHODS

Bacterial strains and growth conditions. The *B. pertussis* strains used were 18334 (Connaught Laboratories, Toronto), Tohama (kindly provided by Dr C. R. Manclark), 18323, 77/18319 (a 1977 isolate from Glasgow) and PT mutant Bp 357 (kindly provided by Dr A. Weiss). Bacteria were grown in the liquid medium of Imaizumi *et al.* (1983a) with or without methylated β -cyclodextrin (MCD). In some experiments, organisms were grown in medium buffered with 0.05 M-HEPES, pH 7.4 (in lieu of Tris/HCl) and containing no MCD. Cultures were incubated for either 24 or 48 h at 37 °C as described previously (Perera *et al.*, 1985).

Culture supernates and cell pellets. Bacteria were harvested by centrifugation at 10000 g for 30 min at 4 °C. The resulting supernate (SI) was filtered initially through a 0.45 μ m pore-size membrane (Millipore) and was either concentrated 15-fold by ultrafiltration (Amicon Hollowfibre, 5000 Da cut-off) or used without further treatment (see Fig. 1).

Preparation of OMV. The 15-fold concentrate of culture supernate SI (from 3 litres of culture medium) of strain 18334 was ultracentrifuged at 105000 g for 2 h at 4 °C. The supernate (SII) was withdrawn carefully and stored frozen at -20 °C. The pellet (PI) containing crude OMV was resuspended in 1.0 ml 0.01 M-sodium phosphate, 0.15 M-NaCl buffer, pH 7.4 (PBS). Purified OMV were obtained by further fractionation by isopycnic sucrose-density-gradient centrifugation as described previously (Perera & Freer, 1987). Fractions containing purified OMV (PII) were dialysed against 100 vols distilled water, lyophilized and reconstituted in 1.0 ml PBS.

Fig. 2. SDS-PAGE of PT preparations. Lanes: 1, molecular mass markers solubilized under reducing conditions; 2, PT from mutant Bp 357 (15 µg); 3, PT from strain 18334 (10 µg). PTs were solubilized under non-reducing conditions. The gel was stained with Coomassie brilliant blue. The positions of subunits S1 to S5 of PT are indicated.

Fetuin affinity chromatography. Fractions PII and SII (1.0 and 3 ml respectively) were applied to a fetuin-Sepharose 4B column (2 ml) and PT-containing fractions were obtained as described elsewhere (Sekura *et al.*, 1983; Perera *et al.*, 1985). Eluates PII-E and SII-E, as well as the fractions which bound to fetuin (PII-B and SII-B), were dialysed and concentrated by lyophilization as described above.

Preparation of immunosorbent column. A globulin-rich fraction containing rabbit antibodies to PT was prepared as described previously (Perera *et al.*, 1985). Antibodies were coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions.

Partial purification of cell-associated PT from Bp 357 by immunosorbent chromatography. Bacteria (from 3 litres of culture) were resuspended in 30 ml PBS, and after disruption by three passages through a French press, a cytoplasmic fraction was prepared as described previously (Perera & Freer, 1987; Perera *et al.*, 1985). Pertussis toxin in the extract was partially purified by immunosorbent chromatography on an anti-PT Ig-Sepharose 4B column (bed volume 30 ml). Bound PT was eluted with 3 M-KSCN in PBS and the toxin was concentrated and dialysed as reported previously (Perera *et al.*, 1985).

Purified PT from strain 18334. This was prepared as described elsewhere (Sekura *et al.*, 1983; Perera *et al.*, 1985).

SDS-PAGE. Samples were solubilized in buffer containing 1% (w/v) SDS with either 2.5% (v/v) 2-mercaptoethanol (Sigma) (reducing conditions) or 4 M-urea (non-reducing conditions) and heated to 100 °C for 5 min. Solubilized samples were electrophoresed on 17% (w/v) acrylamide resolving gels as described previously (Perera *et al.*, 1985). Peptides (and LPS) in gels were visualized by silver-staining according to the method described by Oakley *et al.* (1980). Some gels were silver-stained specifically for LPS (Tsai & Frasch, 1982).

Quantitative methods. The concentration of LPS [throughout, LPS refers to LPSII of LeDur *et al.* (1980)] in gels was estimated by laser densitometry as follows. Gels with known amounts (ranging from 0.1 to 10.0 µg) of LPS (from *B. pertussis* strain 165, LIST Biological Laboratories Inc.) as well as samples to be analysed were subjected to SDS-PAGE as described above. The silver-stained bands of LPS were then scanned using a laser densitometer (LKB Ultrosan, model 2202) linked to a recording integrator (LKB, model 2220). The concentration of LPS in the samples was estimated by reference to a standard plot of peak area (obtained from densitometry) plotted against log concentration of LPS.

Fig. 3. (a) SDS-PAGE of culture supernates from four strains of *B. pertussis* stained for LPS. Lanes: 1, 3, 5 and 7, supernates of strains Tohama, 18323, 77/18319 and 18334, respectively, with MCD; 2, 4, 6 and 8, corresponding supernates without MCD; 9-13 show purified LPS, 0.1, 1, 2, 5 and 10 µg, respectively. The positions of LPSI and LPSII are shown. (b) Densitometric tracing of (a).

Table 1. Concentrations of LPS (estimated by densitometry) in culture supernates of four strains of *B. pertussis* grown in the presence and in the absence of MCD

Strain	MCD	LPS (µg ml ⁻¹)*
18334	-	10.3
	+	13.7
77/18319	-	3.0
	+	5.5
18323	-	10.1
	+	10.5
Tohama	-	11.6
	+	15.1

* Mean values from two experiments.

The concentration of PT was determined by an enzyme-linked immunosorbent assay (ELISA) as described previously (Perera *et al.*, 1986b). Protein was estimated according to the method of Bradford (1976).

RESULTS

Factors affecting release of PT

Analysis of culture supernates of mutant Bp 357 (grown in the presence or absence of MCD) by ELISA revealed no detectable PT (<30 ng ml⁻¹). However, some cell-associated toxin similar to that reported previously for strain 18334 (Perera *et al.*, 1986a) was detectable in sonicates of whole Bp 357 cells (unpublished data). Examination of partially purified cell-associated PT from Bp 357 by SDS-PAGE under non-reducing conditions showed a complete absence of subunit S3 (Fig. 2, lane 2), but as with 18334 (lane 3), mature S1, S2, S4 and S5 subunits were present in PT of mutant Bp 357. Both toxins were analysed under non-reducing conditions, which give better separation of the subunits.

An earlier study (Perera *et al.*, 1986a) showed that addition of MCD to the growth medium of four strains of *B. pertussis* (18334, 77/18319, 18323 and Tohama) increased the amount of cell-free PT by two- to sevenfold as revealed by the histamine-sensitizing assay. The amount of LPS (as a marker for OMV) present in the culture supernates of these strains was determined by densitometry after SDS-PAGE (Fig. 3a, b). Both LPSI and LPSII (LeDur *et al.*, 1980) were detectable in supernates of strains Tohama, 18323 and 18334 (lanes 1 + 2, 3 + 4 and 7 + 8, respectively). The concentrations of LPS as estimated by densitometry are shown in Table 1.

Fig. 4. SDS-PAGE of some of the fractions derived from PI. Lanes: 1, PI (25 µg); 2, PII-E (10 µg); 3, PII-B (2 µg); 4, molecular mass markers (as for Fig. 2); 5, LPS (2 µg). All samples were solubilized under reducing conditions, and the volumes of fractions PI, PII-E and PII-B were equivalent relative to the original culture volume. The gel was silver-stained. The positions of subunits S1-S4,5 of PT are indicated.

Table 2. PT and LPS contents of OMV and supernates obtained following ultracentrifugation of a culture supernate of strain 18334

Fraction	PT		LPS		PT:LPS ratio
	Concn* (µg ml ⁻¹)	Total† (µg)	Concn* (µg ml ⁻¹)	Total† (µg)	
PII	0.5	0.5	512.0	512.0	1:1280
PII-E	0.1	0.1	530.7	530.7	1:5300
PII-B	0.3	0.3	43.2	43.2	1:144
SI	1.5	4500.0	ND	—	—
SII	21.2	4240.0	151.5	30300.0	1:7.2
SII-E	<0.03‡	<6.00‡	125.0	25000.0	1:4167
SII-B	15.4	3080.0	2.7	420.0	1:0.2

ND, Not determined.

* Mean values from two experiments.

† Total amount after adjusting for original volume of 3 litres.

‡ Background level.

Strain 77/18319 released three- to fourfold less LPS than the other three strains examined. In the presence of MCD, the LPS content of supernates from strains 18334 and Tohama increased by nearly 13% compared with only 2% in strain 18323. Strain 77/18319 showed an increase of approximately 30%.

The substitution of HEPES buffer for Tris/HCl buffer in MCD-free media caused a three- to fourfold reduction in the release of PT by strain 18334 as revealed by ELISA. Organisms grown in media buffered with HEPES released approximately 15–20% less LPS into the culture than those grown in comparable media buffered with Tris/HCl.

Association of PT with OMV

Vesicles sedimented by ultracentrifugation of the culture supernate of 18334 (fraction PI) were further fractionated by sucrose-density-gradient centrifugation in order to remove contaminating free toxin particles. The buoyant density of the OMV fraction (PII) was similar to that reported previously for outer membranes (Perera & Freer, 1987). Toxin particles adsorbed to the surface of OMV in PII were removed by fetuin-affinity chromatography and the fractions recovered from the column were examined by SDS-PAGE (Fig. 4). Most outer-membrane proteins and LPS present in PI (lane 1) were also detectable in the eluate PII-E (lane 2) whereas PII-B contained mainly PT and LPS (lane 3). Subunits S4 and S5 of PT could not be separated under reducing conditions and were therefore referred to as S4,5 (Perera *et al.*, 1985).

The LPS and PT contents of fractions PII, PII-E and PII-B were estimated by densitometry and ELISA respectively (Table 2). The amount of PT in PII corresponded to only 0.01% of the total cell-free PT (PII + SI) in 3 litres of culture medium. Fraction PII-E contained approximately fivefold less PT than PII. Consequently, PII-E was proportionately enriched for LPS as judged by the PT:LPS ratio. Conversely, PII-B contained approximately threefold more PT and 12-fold less LPS than PII-E. This meant that PII-E and PII-B shared 25% and 75% respectively of the PT associated with the recoverable PII fraction.

Fractions SII, SII-E and SII-B were also examined for peptides and LPS by SDS-PAGE (data not shown). Fraction SII, even though ultracentrifuged, contained LPS together with subunits of PT and other peptides. Fraction SII-E contained no PT and SII-B contained mainly PT with a trace of LPS. All three fractions contained mainly LPSII. The amounts of PT and LPS are summarized in Table 2. Fraction SII contained 98% of the total (PI + SII) LPS in culture. (The remaining 2% of the LPS was associated with the sedimentable OMV of fraction PII.) The absence of PT subunits in fraction SII-E was confirmed when no PT was detectable by ELISA. The LPS content of this fraction also dropped by nearly 18% compared to SII. The PT:LPS ratio of SII-E changed by nearly 580-fold as a result of the removal of PT by the column. Fraction SII-B, on the other hand, was enriched for PT and contained nearly 73% of the PT present in SII. The LPS content of SII-B was nearly 18% by weight. According to PT:LPS ratios, fractions derived from PII appeared to contain less PT and more LPS whereas the reverse seemed to be true for those derived from SII.

DISCUSSION

The presence of cell-associated PT (unpublished data) with no detectable cell-free toxin in supernates of mutant Bp 357 confirmed the earlier findings of Weiss *et al.* (1983). According to these workers, this mutant, derived by Tn5 mutagenesis, was partially deficient in PT production or unable to export and secrete the toxin normally. Recently, Nicosia *et al.* (1986) reported the insertion of Tn5 in the S3 gene. Our observation that subunit S3 could not be detected in cell-associated PT of Bp 357 substantiated the above finding. It is not known, however, whether Bp 357 lacks the ability to synthesize S3 completely or is unable to process the precursor of S3 to mature S3. However, it appears that mature subunit S3 is indeed required for release of PT into the culture medium. The localization of the remaining four subunits in this mutant remains to be investigated.

Many Gram-negative bacteria shed the outer membrane in the form of vesicles which contain LPS (Work *et al.*, 1966; Devoe & Gilchrist, 1973; Perera *et al.*, 1982). Morse & Morse (1970) reported similar OMV in culture supernates of *B. pertussis*. In order to elucidate whether the shedding of the outer membrane is important in the release of PT, the concentration of LPS (as a measure of OMV content) in culture supernates was monitored under conditions where the production of PT was enhanced by the addition of MCD. A small but consistent increase in the

shedding of LPS by four different strains of *B. pertussis* was observed. This effect was probably due to the interaction of MCD with the fatty acids of LPS by weak hydrophobic interactions, since according to Imaizumi *et al.* (1983*a, b*) MCD may bind fatty acids. Cyclodextrins have also been shown to form inclusion compounds with soluble and insoluble substances (Saenger, 1980).

The effect of destabilization of the outer membrane on PT production was investigated by comparison of toxin levels in the presence of Tris/HCl and HEPES buffer. It has been shown that whereas Tris interacts with the outer membrane and increases the permeability, HEPES buffer has no such effect (Irvin *et al.*, 1981; Poole & Hancock, 1983). Organisms grown in media buffered with Tris/HCl released three- to fourfold more PT than those grown in HEPES-buffered media. Although the LPS contents of the supernates failed to reflect the difference in the levels of PT observed, this suggested that Tris/HCl had some effect on the release of PT, possibly by changing the fluidity of the outer membrane. Thus the release of PT may depend on at least two permeability functions of the outer membrane, viz. vesicularization as promoted by MCD and destabilization.

Although very little is known about the role of OMV in the delivery of toxins *in vivo*, some of the earlier studies *in vitro* have shown that some enzymes and virulence factors of bacterial pathogens are associated with outer-membrane material (Pugsley *et al.*, 1986; Gankema *et al.*, 1980; Austin-Prather & Booth, 1984; Gulig *et al.*, 1984). Unlike some other Gram-negative pathogens, *B. pertussis* does not shed the outer membrane profusely (unpublished data). Furthermore, the large particles which sediment following ultracentrifugation contain only 2% of the total LPS, suggesting that most of the remaining LPS is non-particulate, possibly existing as small micelles. Contrary to a previous report by Parker *et al.* (1985), we found that an insignificant proportion (0.01%) of the total PT was associated with fraction PII, which contained the sedimentable OMV. It is not known what proportion of these OMV has PT associated with them.

The ability of PT to bind to the sialic-acid-containing protein fetuin (Sekura *et al.*, 1983) was exploited in order to separate OMV which contained PT trapped inside (fraction PII-E) from those which contained the toxin either externally or both internally and externally. According to the data obtained, only 25% of the OMV-associated PT appeared to be inside vesicles. The proportion of PT which was bound to the small micelles in fraction SII could not be determined. However, the LPS content of the PT-containing fraction SII-B was estimated as 18%. It is not known whether the PT in the above fractions is attached to LPS or outer-membrane proteins. The ability of subunits S2, S3 and S4 of PT to bind to lipid micelles (Montecucco *et al.*, 1986) may favour the hypothesis that PT binds to the lipid A moiety of LPS by weak hydrophobic interactions. By comparison of PT:LPS ratios, it can be concluded that the PT contents of LPS-containing fractions derived from SII were, in general, greater than those derived from PII. This may suggest a preferential association of PT with small micellar forms of outer-membrane components as opposed to large vesicles. It is not known whether this association occurs during or after release of PT.

In conclusion, the association of PT with OMV appears to be of doubtful significance. OMV may however be more important in the packaging of other virulence factors of *B. pertussis*.

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