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Antibacterial Agents and Release of Periplasmic Pertussis Toxin from *Bordetella pertussis*

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Pertussis toxin accumulates in the periplasm of *Bordetella pertussis* prior to secretion, and we examined its fate following treatment with antimicrobial agents. Both antibiotics that inhibit protein synthesis (erythromycin and chloramphenicol), transcription (rifampin), or cell wall biosynthesis (cefoperazone and piperacillin) and magnesium sulfate (which inhibits transcription of pertussis toxin, but not bacterial growth) did not prevent release of preformed toxin. In contrast, agents that affect bacterial membranes, such as polymyxin B, lidocaine, procaine, and ethanol, inhibited release of preformed pertussis toxin. These results suggest new protein synthesis is not required for pertussis toxin secretion, but a functional membrane complex is required.

Pertussis toxin is a member of the AB_5 family of toxins, which includes cholera toxin, *Escherichia coli* heat-labile toxin, and Shiga toxin. It is a critical virulence factor for the gram-negative bacterium *Bordetella pertussis*, the causative agent of whooping cough (21, 22, 32). It is also the most complex bacterial toxin known, comprised of six subunits, called S1, S2, S3, S4, and S5 in a 1:1:1:2:1 ratio (14, 17, 27). S1 is the A component of pertussis toxin, the part of the toxin that mediates damage to host cells. S1 enzymatically attaches the ADP-ribose group from NAD onto mammalian GTP binding proteins, abolishing normal cellular regulation. S2 to S5 associate to form the B component, or B pentamer, which binds to the host cell and delivers S1 into the cytoplasm of target cells.

This complex structure of pertussis toxin seems to necessitate an equally complex pathway for assembly and secretion from the bacterium. Each of the toxin subunits is synthesized with an N-terminal signal sequence, which mediates secretion to the periplasm, where the signal peptide is removed and the subunits fold and assemble into holotoxin (17). Finally, secretion of the assembled toxin past the outer membrane is mediated by the proteins encoded by the *ptl* operon (PtlA through PtlI) (7, 33). Interestingly, AB_5 toxins have only been found in gram-negative bacteria. A possible explanation is that without the compartment provided by the periplasm of the gram-negative bacterium, the six toxin subunits would be unable to efficiently associate and assemble. Gram-positive bacteria lack this compartment and might release predominantly nonfunctional (and possibly immunizing) subunits.

While the periplasm may be needed for efficient assembly of the AB_5 toxins, it can still act as a barrier to toxin secretion, and strains producing AB_5 toxins tend to accumulate intracellular pools of toxin. A dose as small as 0.05 to 0.15 ng of pertussis toxin per ml can elicit a positive response in the Chinese hamster ovary (CHO) cell assay (9, 33), and we have observed that *B. pertussis* accumulates hundreds of nanograms of periplasmic pertussis toxin, a potentially significant dose. We were interested in examining what happened to this pool of toxin during antibiotic treatment.

In some situations, antibiotic treatment can cause the patient's condition to worsen instead of improve. Lysis of dead bacteria may release a larger dose of toxin or toxic components (such as LPS) than that released by living bacteria. It has been well established that during the most severe stage of pertussis, the paroxysmal phase, antibiotic treatment does not improve the patient's condition, suggesting that lasting damage mediated by the bacterial toxins and not the bacteria per se is responsible for the observed symptoms (3, 5, 21, 22, 26). Furthermore, anecdotal reports of a patient's condition worsening after antibiotic treatment suggest that release of preformed toxin could be a problem in whooping cough. In this study, we have investigated the effects of antibiotics and other agents on release of pertussis toxin.

B. pertussis is fastidious and can be challenging to grow. We have had success by incubating the bacteria in a thin layer of broth plated on an agar support. These cultures have a high surface/volume ratio, and the semisolid agar layer is a rich source of nutrient reserves. *B. pertussis* strain BP338 (31) was grown on Bordet-Gengou agar (BGA) containing 15% sheep's blood as previously described (31). After 24 h, the bacteria were harvested and adjusted to an optical density at 600 nm (OD_{600}) of 0.1 in Stainer Scholte broth, and 6.0 ml was plated per BGA plate. The bacteria were allowed to grow for 24 h at 37°C to accumulate intracellular pertussis toxin. In this study, growth was monitored by calculating the OD of the culture after it had been diluted to fall within a useful range (0.2 to 0.7).

Bacteria from the 24-h cultures were harvested, pooled, and washed once in prewarmed broth. Control cells were suspended in fresh broth, and others were suspended in broth containing antibiotics and other test compounds added at the following concentrations: erythromycin, 10 μg/ml; chloramphenicol, 34 μg/ml; rifampin, 25 μg/ml; magnesium sulfate, 50 mM; polymyxin B, 50, 25, or 5 μg/ml; ethanol, 10%; lidocaine hydrochloride, 40 mg/ml; procaine hydrochloride, 40 mg/ml; cefoperazone, 100 μg/ml; or piperacillin, 100 μg/ml. Six milliliters was plated onto BGA and incubated at 37°C. Samples were taken at 2 h to examine the fate of the pool of preformed toxin and at 24 h to examine the contribution of newly synthesized toxin. The treated cultures were monitored for growth by OD and viability.

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TABLE 1. Bacterial growth, accumulation of pertussis toxin, and viability after treatment

Treatment[a]	Amt of intracellular pertussis toxin (ng/ml)[b]	OD_{600}[c]	OD_{600}[d]		Viability (10^{10} CFU/ml)[e]	
			Control	Treated (% of control)	Control	Treated (% of control)
Erythromycin (10 μg/ml)	78 ± 16	2.77 ± 0.49	11.6 ± 1.4	4.0 ± 1.2 (35)	3.0 ± 2.9	0.18 ± 0.09 (6)
Chloramphenicol (34 μg/ml)	124 ± 28	3.8 ± 0.05	12.3 ± 1.11	5.2 ± 0.33 (42)	1.4 ± 1.2	0.45 ± 0.3 (32)
Rifampin (25 μg/ml)	239 ± 61	3.4 ± 0.58	11.7 ± 0.17	3.3 ± 0.11 (28)	5.9 ± 0.1	0.48 ± 0.3 (8)
Piperacillin (100 μg/ml)	333 ± 53	3.98 ± 0.19	13.8 ± 0.46	7.5 ± 0.26 (54)	6.2 ± 0.4	0.5 ± 0.2 (8)
Cefoperazone (100 μg/ml)	333 ± 53	3.98 ± 0.19	13.8 ± 0.46	8.7 ± 0.26 (63)	6.2 ± 0.4	0.9 ± 0.1 (14)
Polymyxin B (50 μg/ml)	94 ± 12	2.98 ± 0.25	12.4 ± 0.59	3.3 ± 0.48 (27)	5.4 ± 0.7	<0.01 (<0.2)
Polymyxin B (25 μg/ml)	117 ± 30	3.6 ± 0.44	13.4 ± 0.70	3.8 ± 0.47 (28)	6.3 ± 0.6	0.14 ± 0.04 (2)
Ethanol (10%)	158 ± 24	3.56 ± 0.31	12.2 ± 0.87	11.1 ± 1.0 (91)	5.1 ± 0.9	4.1 ± 0.9 (80)
Lidocaine (40 mg/ml)	159 ± 28	3.65 ± 0.36	11.9 ± 0.23	3.2 ± 0.37 (27)	3.7 ± 0.3	0.045 ± 0.02 (1)
Procaine (40 mg/ml)	174 ± 29	3.77 ± 0.39	12.6 ± 0.69	4.7 ± 0.35 (37)	4.7 ± 1.0	0.34 ± 0.05 (7)

[a] Broth cultures were harvested at 24 h. The amount of intracellular pertussis toxin and the OD were determined. The cultures were split into treated (as indicated) and untreated control groups. OD and viability were determined after a 24-h incubation.
[b] Amount of intracellular pertussis toxin prior to treatment.
[c] OD_{600} prior to treatment.
[d] OD_{600} after 24 h of the treatment indicated.
[e] Viability after 24 h of the treatment indicated.

The amount of toxin activity was quantified by using the CHO cell assay as previously described (4, 9, 33). Intracellular toxin was released by treatment with lysozyme and EDTA as previously described (4, 33). The amount of secreted toxin was determined from filter-sterilized culture supernatants that were dialyzed against phosphate-buffered saline at 4°C for 5 h using a Slide-A-Lyzer 10K (Pierce, Rockford, Ill.) to remove agents that might be toxic to the CHO cells. Control experiments were performed to determine if the compounds directly affected pertussis toxin—for example, had a denaturing effect on the protein. Supernatants from untreated cultures were incubated with the compounds for 2 h at 37°C and dialyzed as described above. Only lidocaine had a direct effect on the pertussis toxin protein, reducing activity by fourfold.

In these studies, after 24 h the bacteria had grown to an OD_{600} of between 2.77 and 3.98 and had accumulated between 78 and 333 ng of periplasmic pertussis toxin per ml (Table 1). Since the amount of intracellular toxin present at 24 h could vary, secretion of pertussis toxin by bacteria treated with various compounds was always compared to that of control cells from the same initial culture. As a point of reference, little toxin release was seen from the pertussis toxin secretion mutant. After 2 h in culture, the Ptl secretion mutant BPM3171 released only 8.3 ng/ml, or 17% as much pertussis toxin as wild-type BP338 grown under the same conditions (Table 2, BPM3171), and only 24 ng/ml, or 2% as much as the wild type, after 24 h in culture (Table 3, BPM3171).

Effect of protein synthesis inhibitors on pertussis toxin secretion. Erythromycin, a bacteriostatic agent that blocks protein synthesis by binding to the 50S ribosomal subunit, is commonly used to treat patients with pertussis and their contacts (2, 7). Erythromycin, added at 10 μg/ml, appeared to be effective, as evidenced by a failure of the treated bacteria to grow and some loss of viability (Table 1). When pertussis toxin secretion was examined after 2 h (Table 2, erythromycin), the treated cells had released more toxin (56.7 ng/ml) than the untreated cells (48.4 ng/ml). This was slightly more than half of the intracellular pool of 78 ng of pertussis toxin per ml (Table 1). After 24 h, the untreated cells secreted 936 ng of toxin per ml (Table 3, erythromycin), which exceeded the 78 ng of toxin per ml initially present in the periplasm, suggesting that new synthesis was contributing to the secreted pool. In contrast, after 24 h, the erythromycin-treated cells had released only 150 ng/ml, about half of which could be accounted for in the preformed pertussis toxin pool.

Similar results were obtained with two other antibiotics. Chloramphenicol also inhibits protein synthesis by binding to the 50S ribosomal subunit, but at a different site than erythromycin. Rifampin inactivates the DNA-dependent RNA polymerase. Both antibiotics are bacteriostatic, and as observed with erythromycin, the treated cells stopped growing and viability was slightly affected (Table 1). As was observed with erythromycin, after 2 h, the antibiotic-treated cells released slightly more pertussis toxin than the control cells (Table 2), and most of the extracellular toxin could be attributed to the pool of preformed toxin. After 24 h, the antibiotic-treated cells released reduced amounts of pertussis toxin in comparison to those of the untreated controls (Table 3).

$MgSO_4$ was examined because, unlike the antibiotics, it inhibits the expression of the *bvg*-mediated virulence genes in *B. pertussis*, but does not affect bacterial growth (11, 15). Treatment with $MgSO_4$ did not affect secretion of preformed pertussis toxin after 2 h (Table 2).

TABLE 2. Secretion of pertussis toxin after 2 h

Treatment group	Amt of toxin secreted (ng/ml)[a]	
	Control	Treated
BPM3171 (secretion mutant)	8.32 ± 1.3*	ND[b]
Wild-type BP338 (control versus treated)		
$MgSO_4$	48.4 ± 8.3	56.7 ± 4.3
Erythromycin	48.4 ± 8.3	56.7 ± 4.3
Chloramphenicol	91.5 ± 34.5	124.8 ± 25.0
Rifampin	45.0 ± 3.1	66.6 ± 10.5
Piperacillin	83.2 ± 10.5	74.9 ± 15.8
Cefoperazone	83.2 ± 10.5	99.8 ± 0
Polymyxin B (50 μg/ml)	111.5 ± 31.9	29.6 ± 6.2*
Polymyxin B (25 μg/ml)	77.4 ± 21.3	31.2 ± 7.1*
Ethanol	99.8 ± 15.1	26.0 ± 7.6*
Lidocaine	149.7 ± 44.3	65.5 ± 13.9*
Procaine	124.8 ± 43.2	20.3 ± 2.3*

[a] Amount of pertussis toxin secreted in 2 h from secretion mutant BPM3171 or wild-type BP338 treated as indicated. *, values statistically significantly different from control BP338 values ($P < 0.05$).
[b] ND, not determined.

TABLE 3. Secretion of pertussis toxin after 24 h

Treatment group	Amt of toxin secreted (ng/ml)[a]	
	Control	Treated
BPM3171 (secretion mutant)	24.2 ± 4.2*	ND[b]
Wild-type BP338 (control versus treated)		
Erythromycin	936 ± 180	150 ± 29*
Chloramphenicol	936 ± 180	175 ± 25*
Rifampin	1,560 ± 540	175 ± 83*
Piperacillin	1,830 ± 278	250 ± 0*
Cefoperazone	1,830 ± 278	333 ± 53*
Polymyxin B (50 μg/ml)	1,410 ± 345	80.2 ± 29*
Polymyxin B (25 μg/ml)	1,248 ± 279	116 ± 17*
Ethanol	2,251 ± 155	187 ± 43*
Lidocaine	1,502 ± 159	70 ± 14*
Procaine	1,445 ± 261	34 ± 6.0*

[a] Amount of pertussis toxin secreted in 24 h from secretion mutant BPM3171 or wild-type BP338 treated as indicated. *, values statistically significantly different from control BP338 values ($P < 0.05$).
[b] ND, not determined.

Effect of cell wall inhibitors on pertussis toxin secretion. In general, *B. pertussis* strains are resistant to penicillin and cephalosporins; however, piperacillin and cefoperazone have been shown to have activity against *B. pertussis* (10). After 24 h, the OD of the piperacillin-treated cells was half that of the untreated cells, but the viability was reduced to 8% (Table 1). Similarly cefoperazone caused a reduction in viability (14%) without greatly affecting OD (63%). Neither piperacillin nor cefoperazone affected toxin secretion after 2 h (Table 2), but both antibiotics had an effect after 24 h (Table 3).

Effect of membrane-damaging agents on pertussis toxin secretion. Polymyxin B is a cationic antibiotic that exerts its bactericidal activity by binding to the negatively charged lipopolysaccharide on gram-negative bacteria and disrupting the bacterial outer membrane (29). We found that at 25 or 50 μg/ml, polymyxin B was bactericidal, as evidenced by a failure to grow and a loss of viability (Table 1), but it was not bactericidal at 5 μg/ml (data not shown).

In contrast to the other antibiotics, treatment with polymyxin B significantly reduced the amount of extracellular pertussis toxin released by the bacteria. After 2 h, cultures treated with 50 μg of polymyxin B per ml released 27% as much as control cells, while cultures treated with 25 μg of polymyxin B per ml released 40% as much as control cells (Table 2). The control cells secreted more than a microgram of toxin after 24 h, while the cells treated with 50 μg of polymyxin B per ml released 76 ng of toxin per ml (Table 3), or 81% of the preformed toxin pool of 94 ng/ml (Table 1).

Other compounds known to affect the membranes of bacteria were also examined. Ethanol changes the fluidity of membranes and is accompanied by decreases in functional capacities (24), including prevention of secretion signal cleavage in bacteria (19), which should prevent maturation of pertussis toxin. Treatment with 1% ethanol did not affect bacterial growth or toxin secretion (data not shown). Treatment with 10% ethanol had only a slight effect on bacterial growth and viability (Table 1), but had a large effect on pertussis toxin secretion. Ethanol caused a statistically significant decrease in the amount of pertussis toxin secretion at both 2 h (Table 2) and 24 h (Table 3).

Lidocaine and procaine are anesthetics that are antimicrobial (25). They have been shown to bind to the membranes of bacteria, alter their structure and fluidity (20), and disrupt membrane potential (18). Procaine, like ethanol, has been shown to inhibit signal peptide cleavage (12).

Lidocaine and procaine were somewhat bactericidal to *B. pertussis*. Both compounds affected the growth and viability of the bacteria (Table 1). Like the other compounds that affect bacterial membranes, lidocaine and procaine also caused a statistically significant reduction in pertussis toxin release. After 2 h, lidocaine-treated cells released 44% as much as the untreated controls, and procaine-treated cells released only 16% as much as the control (Table 2). Very little additional release occurred after 24 h (Table 3), and the total amount of extracellular toxin was much less than that contained in the periplasmic pool at the beginning of the treatments (Table 1).

Our results suggest that new protein synthesis is not necessary for the release of the preformed pool of toxin, since antibiotics that inhibited protein synthesis (erythromycin, chloramphenicol, and rifampin) did not affect release of preformed toxin. In addition, $MgSO_4$, which specifically inhibits transcription of the virulence factors, but does not affect growth, did not inhibit the release of the preformed toxin. As expected, these treatments did inhibit new toxin synthesis. For the antibiotics, we cannot determine if secretion or nonspecific leakage was responsible for toxin release, but for $MgSO_4$, which does not affect bacterial growth, true secretion was probably occurring. Similarly, cephalosporins did not inhibit the release of performed toxin, but did inhibit new toxin production.

Interestingly, membrane-active compounds do appear to inhibit the release of periplasmic pertussis toxin. These include polymyxin B, ethanol, and the local anesthetic procaine. Lidocaine appeared to have a direct effect on pertussis toxin activity, and separate effects on secretion are difficult to assess. In other systems, these agents have been shown to inhibit secretion to the periplasm (6, 12, 30), and our results suggest they block secretion of pertussis toxin past the outer membrane as well. Polymyxin B was bactericidal, and one might predict that this would promote release of the toxin from the dead bacteria, but that did not appear to be the case. The action of ethanol was interesting. It had little effect on bacterial growth or viability; however, ethanol seemed to have a lasting inhibitory effect on secretion.

The role of antibiotic for treatment of *E. coli* O157:H7 infections is controversial and is an interesting example of a situation in which antibiotic treatment can harm the patient (8, 28). Release of the AB_5 toxin Shiga toxin by *E. coli* O157:H7 can cause progression to the serious, sometimes fatal complication hemolytic-uremic syndrome (8, 28). It was once thought that antibiotic treatment was harmful because it promoted release of preformed toxin; however, recent studies suggest the explanation may be more complicated. Shiga toxin is encoded on a lysogenic phage, and phage induction and lysis are required for toxin production and release (16, 23). It seems counterintuitive for bacterial death to be necessary for toxin release; however, it has been proposed that the rare bacterium that undergoes a lytic cycle produces phage that infect other susceptible *E. coli* present in the gastrointestinal tract, while O157:H7 lysogens are immune to superinfection (23). This implies that most of the Shiga toxin will be produced by the resident *E. coli* in the gastrointestinal tract, not O157:H7. If this is the case, the potential harmful or beneficial effects of antibiotic treatment may be as dependent on the properties of resident bacterial flora as on the infecting O157:H7 strain. Nonantibiotic treatment strategies for hemolytic-uremic syndrome have been proposed. These include absorbing the toxin with resins (1) or neutralizing the toxin with antibodies (13).

In contrast to Shiga toxin, pertussis toxin has a dedicated

secretion apparatus and can be released by viable bacteria. Prior to secretion, however, the bacteria accumulate significant levels of pertussis toxin, and we have found that in short-term studies, bacteria treated with erythromycin (the current antibiotic of choice) release as much pertussis toxin as the untreated controls. It would be beneficial to devise strategies that minimize the dose to patients. Adsorbing pertussis toxin from the respiratory tract would not be as easy as removing Shiga toxin from the gastrointestinal tract. Generating neutralizing antibodies to pertussis toxin is a goal of the pertussis vaccine program, but is not achieved in all cases. We found that membrane-active agents, such as polymyxin B, ethanol, and local anesthetics, can reduce pertussis toxin release. While these agents are not likely to be clinically useful for treating pertussis, these observations suggest directions for further investigation to improve clinical treatment for pertussis and possibly other toxigenic pathogens.

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