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Intracellular Localization of the Dermonecrotic Toxin of *Bordetella pertussis*

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Localization of the heat-labile dermonecrotic toxin of *Bordetella pertussis* strain 114 grown in chemically defined Stainer-Scholte medium was studied by using skin reactions in 4-day-old suckling mice as the assay for toxin. Through log phase and into stationary phase of growth the toxin was cell associated and not detected in the culture supernatant. Only about 4% of the activity present in a suspension of lysed cells was detected in a suspension of whole cells, and the dermonecrotic activity was not released by subjecting whole cells to osmotic shock, a procedure that releases proteins from the periplasmic space of many gram-negative bacteria. After cell lysis and preparation of soluble and membrane fractions, 73 to 80% of the activity in the cell lysate was recovered in the soluble fraction, with only 3 to 6% present in a membrane fraction. Further evidence for the intracellular cytoplasmic localization of the dermonecrotic toxin was the insensitivity of the toxin to trypsin treatment of whole cells. Treatment of whole cells with trypsin (80 μg/ml) for 20 min at 37°C did not decrease dermonecrotic or malate dehydrogenase activities, but did inhibit more than 95% of the extracytoplasmic adenylate cyclase activity. Identical trypsin treatment of a cell lysate decreased all the above activities by more than 90%.

The dermonecrotic toxin (DNT) from *Bordetella pertussis* is a heat-labile protein (3, 19) which is lethal to various animals (4, 17). Since the discovery of this toxin by Bordet and Gengou (4), it has been shown that toxin activity is increased after cell disruption (17), and Munoz et al. (18) found that after cell lysis most of the toxic activity was located in the soluble fraction of the lysate. These studies do not, however, rule out the possibility that DNT may occupy a periplasmic, rather than a cytoplasmic, location as has been recently suggested by Livey et al. (15) and Zakharova (22). The toxin has also been found in the supernatant of liquid cultures, but this has been assumed to be due to leakage of toxin from lysed cells (17). Lane (14) concluded, however, that the toxin was secreted by actively growing cells.

The DNT can cause damage to various tissues (1, 2, 12, 20) and may contribute to cellular damage observed after infection with *B. pertussis* (1, 2, 6, 21). This study was initiated to investigate the potential role of this toxin in the pathogenesis of clinical pertussis by clarifying its location in the organism. We now present evidence that the DNT is intracellular, not periplasmic, in location and is not secreted by actively growing cells.

MATERIALS AND METHODS

Chemicals. The following chemicals were obtained from Sigma Chemical Co.: deoxyribonuclease I; trypsin type III, 2× crystallized from bovine pancreas; β-nicotinamide adenine dinucleotide, reduced form; 3-(4,5 dimethylthiazolyl-2)-2,5-diphenyltetrazolium (MTT); cyclic adenosine 2′,3′-monophosphoric acid, sodium salt; *N*-tris(hydroxymethyl)methylglycine (Tricine); and *cis*-oxalacetic acid. Lysozyme (11,000 U/mg) and lima bean trypsin inhibitor were from Worthington Biochemicals Corp. Phenazine methosulfate and succinic acid were from Calbiochem, and crystalline bovine plasma albumin was obtained from Armour Pharmaceutical Co. All other chemicals were reagent grade and were obtained from usual commercial sources.

Growth and organism. *B. pertussis* 114 was grown in 2.8-liter Fernbach flasks containing 1.3 liters of modified Stainer-Scholte medium, as previously described (10). Growth was monitored by optical density at 650 nm, using a cuvette with a 1-cm light path in a Gilford model 250 spectrophotometer. Cells were harvested after 24 h by centrifugation at 16,000 × *g* for 15 min at 5°C. Cells were washed once with cold 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.3) containing 0.03 M NaCl and suspended 1:10 (wt/vol) in the same buffer.

DNT and enzyme assays. DNT was assayed by using 4-day-old suckling mice [N:NIH (SW)]. Twofold

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serial dilutions of each test material were prepared in sterile saline, and 50 μl of each dilution was injected subcutaneously in the nuchal area of the mice. After 18 to 20 h dermonecrotic lesions were recorded. Controls which were always negative included saline and heated (56°C for 10 min) test samples. The purple-black lesions were scored as 4+, 3+, 2+, or 1+. The 4+ reaction often was accompanied by death, and the 1+ reaction was near the minimal observable necrosis. Each test sample was diluted to give a reaction spectrum from 4+ to negative. The number of dermonecrotic units per milliliter of undiluted sample was expressed as the reciprocal of that dilution which gave a 2+ necrotic lesion.

Adenylate cyclase activity was measured as previously described (10), except that the regenerating system for adenosine 5′-triphosphate was omitted because it was found to be unnecessary. Before assay of adenylate cyclase, whole cells and fractions containing ethylenediaminetetraacetic acid (EDTA) were dialyzed against 500 volumes of 60 mM Tricine (pH 7.5) for 1 h at 5°C with one change of buffer after 30 min.

Malate dehydrogenase was assayed at 23°C in a 1.0-ml reaction mixture as previously described (9).

Succinic dehydrogenase was assayed at 23°C in a reaction volume of 1.0 ml by the phenazine methosulfate-coupled reduction of MTT (13). The enzyme was preincubated for 10 min at 23°C in the presence of a solution containing 50 mM Tris-hydrochloride (pH 8.5), 16 mM KCN, and 15 mM sodium succinate. The reaction was initiated by the addition of phenazine methosulfate to 1.14 mM and MTT to 72.5 μM. The increase in absorbance at 570 nm was followed for 2 min, and 1 unit of activity was defined as 1 μmol of MTT reduced per min, using an extinction coefficient for MTT of 17 mM^{-1} cm^{-1} (13).

Cyclic phosphodiesterase activity was measured at 23°C in a reaction volume of 0.2 ml by the method of Brockman and Heppel (5), using cyclic adenosine 2′,3′-phosphate as substrate. Before assay of cyclic phosphodiesterase culture supernatants, whole cells and fractions containing EDTA were dialyzed as described above for the assay of adenylate cyclase.

The specific activity for DNT and each of the above enzymes was expressed as units per milligram of protein. Protein was determined with the Folin reagent (16), using bovine plasma albumin as the standard.

Osmotic shock. Cells were osmotically shocked by the method of Heppel (8), starting with washed cells suspended 1:40 (wt/vol) in the appropriate buffer.

Preparation of cell lysates and membranes. Cells were lysed by a lysozyme–freeze-thaw procedure as follows. Cells (1:40, wt/vol) in 10 mM Tris-hydrochloride (pH 7.8) were incubated for 30 min at 23°C after the addition of EDTA to 10 mM and lysozyme to 100 μg/ml. The mixture was frozen and thawed once and incubated for 3 min at 35°C and for an additional 15 min at 23°C. A homogeneous suspension was prepared after the addition of $MgCl_2$ to 20 mM and deoxyribonuclease to 10 μg/ml. Unless stated otherwise, cell lysates were prepared by this procedure.

For the preparation of spheroplast lysates, cells were suspended at a concentration of 1:40 (wt/vol) in 10 mM Tris-hydrochloride (pH 7.8) containing 20% sucrose and incubated for 25 min at 23°C after the addition of EDTA to 10 mM and lysozyme to 150 μg/ml. This mixture was centrifuged for 20 min at 23,000 × *g* and 5°C. The supernatant (spheroplast supernatant) was removed, and the pellet was suspended in 3 ml of 10 mM Tris-hydrochloride (pH 7.8) containing 20% sucrose plus 20 mM $MgCl_2$. Deoxyribonuclease was added to give a final concentration in the spheroplast lysate of 10 μg/ml, and this mixture was rapidly added to 20 volumes of cold 3.3 mM Tris-hydrochloride (pH 7.8).

For the preparation of soluble and membrane fractions, the above lysates were centrifuged at 75,000 × *g* for 30 min at 5°C. The supernatant (soluble fraction) was removed, and the pellet was suspended in 1 M NaCl containing 20% sucrose, layered on top of 60% sucrose containing 1 M NaCl, and centrifuged in a swinging bucket rotor at 64,000 × *g* for 2 h at 5°C. The membrane material recovered at the interface and the pelleted material were then washed with 60 mM Tricine, pH 7.5, resuspended in the same buffer, and assayed.

Trypsin treatment. Whole cells or cell lysates were suspended in 10 mM Tris-hydrochloride (pH 7.3) containing 30 mM NaCl to a concentration of 1.74 mg of protein per ml. An equal volume of a solution containing 60 mM Tricine (pH 7.5) and trypsin (80 μg/ml, final concentration) was then added, and after incubation for 20 min at 37°C lima bean trypsin inhibitor was added to 170 μg/ml. Control samples were treated as described above, except that trypsin was added after the addition of trypsin inhibitor. Before the whole cell samples were assayed, the cells were centrifuged and lysed by the lysozyme–freeze-thaw procedure.

RESULTS

Growth and toxin production. DNT activity was assayed in cells and in cell-free culture supernatants throughout the growth cycle of *B. pertussis* (Fig. 1). Cell-associated toxin was assayed after lysis of the cells. Throughout log phase and into stationary phase of growth the toxin was found to be only cell associated and was not detected in the culture supernatant. These culture supernatants were not concentrated before assay, but other culture supernatants from 24-h growth, which were concentrated 100 times by ultrafiltration (Amicon PM 10 membrane), permitted the detection of less than 0.01% of the DNT activity present in cell lysates.

Activities in fractions after cell lysis. To help determine the location of the cell-associated DNT, various enzymes were assayed along with DNT in cell fractions after cell lysis. It was expected that malate dehydrogenase (MDH) would be a cytoplasmic marker (9), succinic dehydrogenase (SDH) would be an inner membrane marker, and adenylate cyclase would be a periplasmic marker (9). Table 1 shows the results obtained after cell lysis by the lysozyme–freeze-thaw procedure. As expected, MDH behaved as an intracellular cytoplasmic marker.

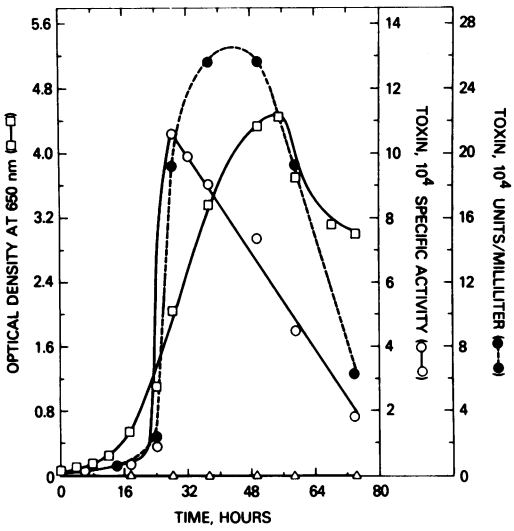

FIG. 1. *Growth of B. pertussis and dermonecrotic activity detected in lysed cells and culture supernatants. Growth conditions and the preparation of cell lysates by the lysozyme-freeze-thaw procedure were as described in the text. Culture supernatants were assayed within 4 h after centrifugation at 17,300 × g for 15 min at 5°C. Dermonecrotic activity in lysed cells is shown in units per milligram of protein* (○) *and in units per milliliter* (●). *Other symbols:* △, *DNT activity in culture supernatant;* □, *bacterial growth (optical density).*

Very little activity was detected with whole cells, and the activity in the cell lysate was predominantly recovered in the soluble supernatant of the cell lysate with little activity in a membrane fraction. The specific activity of MDH in the soluble supernatant increased about twice relative to the cell lysate. Also as expected, SDH behaved as a membrane-bound enzyme. Considerable activity was measured in whole cells, and none of the activity in the cell lysate was recovered in the soluble fraction; however, it was found in the membrane fraction. The specific activity of SDH in the membrane fraction increased 1.5- to 2.0-fold relative to the lysate. The DNT behaved like an intracellular cytoplasmic constituent. Only 4 to 5% of the DNT activity could be detected when whole cells were used, and 73% of the activity in the cell lysate was recovered in the cell lysate supernatant. About 6% of the DNT was detected in a twice-washed membrane fraction. Like MDH, the specific activity of DNT in the lysate supernatant increased about twofold relative to the cell lysate. Table 1 also shows that only 12% of the adenylate cyclase activity was detected in the soluble fraction of the cell lysate and very little activity was detected in the membrane fraction. However, about 40% of the total adenylate cyclase activity was lost during fractionation, and this loss of activity makes the localization of this enzyme by its enzymatic activity uncertain.

TABLE 1. *Activities in cell fractions after lysozyme-freeze-thaw lysis of B. pertussis*[a]

Fraction[b]	Activity of:[c]			
	MDH	DNT	SDH	Adenylate cyclase
Cell lysate[d]	100	100	100	100
Whole cells	6	5	60	19
Cell lysate supernatant	80	73	0	12
Membranes washed	<0.01	9	32	6
Membranes washed twice	<0.01	6	28	0.4
Pellet	30	24	83	30

[a] The data in this table and in the following tables represent single sample determinations. However, each experiment was done at least twice and in some cases several times with similar results each time.

[b] The various fractions were prepared as described in the text. The pellet resulted from centrifugation to obtain purified membranes.

[c] Activities are expressed as the percentage of the total activity present in an equivalent amount of cell lysate.

[d] Total activities and specific activities in the cell lysate were as follows: MDH, 152 μmol/min and 0.7 U/mg of protein, respectively; DNT, 2 × 10^6 U and 9,000 U/mg; SDH, 24.2 μmol/min and 0.11 U/mg; and adenylate cyclase, 241 nmol/min and 1.1 U/mg.

Activities in soluble and membrane fractions after osmotic lysis of spheroplasts are shown in Table 2. Little inactivation of adenylate cyclase occurred during fractionation after osmotic lysis of spheroplasts. Of the activity present in the spheroplast lysate, 32.5% was in the soluble fraction and 13.7% was in the membrane fraction. Results with MDH, DNT and SDH are similar to those in Table 1. Of the activity present in the spheroplast lysate, 68% of MDH and 80% of DNT, but only 0.2% of SDH, were recovered in the soluble fraction; and only 0.2% of MDH and 3.2% of DNT, but 47.2% of SDH, were recovered in the twice-washed membrane fraction. Table 2 also shows the activities present in the supernatant of the spheroplasts. Only MDH (20%) and DNT (11.4%) had significant activity in this supernatant. This likely reflects lysis of some spheroplasts or leakage of material from the spheroplasts and not a periplasmic localization of these components. About 14% of the cell protein was also found in the supernatant of the spheroplasts, and the results in Tables 3 and 4 argue against a periplasmic or other extracytoplasmic localization of these activities.

TABLE 2. *Activities in cell fractions during preparation of membranes after osmotic lysis of B. pertussis spheroplasts*

Fraction[a]	Activity of:[b]			
	MDH	DNT	SDH	Adenylate cyclase
Cell lysate[c]	100.0	100.0	100.0	100.0
Whole cells	2.0	3.6	79.0	42.0
Supernatant of spheroplasts	20.0	11.4	0.1	1.6
Spheroplast lysate	47.0 (100)	68.5 (100)	89.0 (100)	40.0 (100)
Supernatant of spheroplast lysate	32.0 (68)	55.0 (80)	0.2 (0.2)	13.0 (32.5)
Membranes washed once	0.3 (0.6)	5.2 (7.6)	46.0 (51.6)	5.5 (13.7)
Membranes washed twice	0.1 (0.2)	2.2 (3.2)	42.0 (47.2)	0.4 (0.1)
Pellet	5.0 (11)	15.0 (22)	25.0 (28)	22.0 (55)

[a] The fractions were prepared as described in the text. The pellet resulted from centrifugation to obtain purified membranes.

[b] Activities are expressed as the percentage of the total activity present in an equivalent amount of cell lysate. Percentages in parentheses are relative to the spheroplast lysate.

[c] Total activities and specific activities in the cell lysate were as follows: MDH, 170.4 μmol/min and 1.84 U/mg of protein, respectively; DNT, 2.2×10^6 U and 24,000 U/mg; SDH, 31.3 μmol/min and 0.34 U/mg; and adenylate cyclase, 65.9 nmol/min and 0.7 U/mg.

Osmotic shock of *B. pertussis*. Many periplasmic proteins are released from gram-negative bacteria by subjecting the cells to osmotic shock (8). Since it was thought that some of the DNT might have a periplasmic location, the effect of osmotic shock on the release of DNT from *B. pertussis* was determined. Table 3 shows that no significant amounts of DNT were released either by osmotic shock or by treatment of the cells with 20% sucrose containing 2 mM EDTA. Sucrose-EDTA released 2 to 3% of the DNT and MDH, with about 3% release of the total cell protein. The shock fluid contained 4 to 6% of the DNT and MDH and about 6% of the total cell protein.

We believed that adenylate cyclase, previously shown to be extracytoplasmic in *B. pertussis* (9), and cyclic phosphodiesterase, which is a periplasmic enzyme in many gram-negative bacteria (8), would be positive controls for release of periplasmic constituents; but sucrose-EDTA treatment and osmotic shock together released only 1.0% of the adenylate cyclase and 17.2% of the cyclic phosphodiesterase. It was found, however, that significant amounts of both of these enzymes were extracellular. About 10% of the adenylate cyclase and 92% of the cyclic phosphodiesterase were found in culture supernatants from 24-h growth of *B. pertussis*, whereas less than 0.01% of the MDH and DNT were present in this same culture supernatant.

Effect of trypsin on cellular activities of *B. pertussis*. Further evidence that DNT is not extracytoplasmic in *B. pertussis* was obtained from the inability of trypsin to inactivate the toxin when whole cells were treated. Treatment of a cell lysate with 80 μg of trypsin per ml for 20 min at 37°C followed by the addition of trypsin inhibitor destroyed 93% of MDH, 60% of SDH, 97% of DNT, and almost 99% of adenylate cyclase (Table 4). However, identical treatment of an equivalent amount of whole cells followed by cell lysis resulted in no inhibition of DNT, MDH, or SDH, but 99% inhibition of adenylate cyclase. A reasonable interpretation of these results is that MDH, SDH, and DNT are intracellular components and that adenylate cyclase is external to the cytoplasmic membrane.

TABLE 3. *Activities in whole cells, shock fluid, and culture supernatants from B. pertussis*

Preparation[a]	Activity of:[b]			
	MDH	DNT	Adenylate cyclase	2',3' Cyclic phosphodiesterase
Cell lysate[c]	100.0	100.0	100.0	100.0
Whole cells	4.2	4.5	15.0	8.0
Sucrose-EDTA supernatant	2.6	1.8	0.3	4.2
Shock fluid	5.8	3.6	1.6	13.0
Shocked cells	5.5	4.5	14.0	2.2
Culture supernatant	<0.01	<0.01	10.0	92.0

[a] The various fractions were prepared as described in the text.

[b] Activities are expressed as the percentage of the total activity present in an equivalent amount of cell lysate.

[c] Total activities and specific activities in the cell lysates were as follows: MDH, 75.6 μmol/min and 0.97 U/mg of protein, respectively; DNT, 8.7×10^5 U and 11,400 U/mg; adenylate cyclase, 190.4 nmol/min and 2.4 U/mg; and cyclic phosphodiesterase, 32.9 μmol/h and 0.42 U/mg.

TABLE 4. *Effect of trypsin on cellular activities of B. pertussis*

Treatment[a]	Activity of:			
	MDH (μmol/min)	DNT (U)	SDH (μmol/min)	Adenylate cyclase (nmol/min)
Cell lysate control	2.93	33,920	0.52	1.25
Cell lysate plus trypsin	0.21	1,018	0.21	0.02
Whole cells control	3.15	36,000	0.42	0.46
Whole cells plus trypsin	3.29	36,000	0.43	0.006

[a] Equivalent amounts of cell lysate and whole cells were incubated at 37°C with or without trypsin and assayed as described in the text.

DISCUSSION

The results of this study show that the heat-labile DNT from *B. pertussis* strain 114 is an intracellular cytoplasmic component that is not secreted by actively growing cells. DNT activity could not be detected in culture supernatants, and in cell lysates the majority of the toxin was associated with the soluble fraction. This soluble toxin did not appear to be free in the periplasmic space since osmotic shock did not release DNT activity and trypsin treatment of whole cells did not inactivate the DNT but did completely inactivate the extracytoplasmic adenylate cyclase. These results do not support the suggestion that the heat-labile toxin may be periplasmic (15, 22) or the interpretation of Lane (14) that the toxin was released by actively growing cells. Lane measured the toxin in cell suspensions, not in culture supernatants, and the toxicity he observed was not sufficiently quantitated to determine the percentage of the total activity detected. Furthermore, Lane stored the cells at 2°C for 1 week before testing for toxicity and presented no data indicating that the toxicity was not due to release of the toxin from the cells during this storage period. Our results are in accord with other studies and do not appear to be peculiar to *B. pertussis* strain 114 or to the growth conditions we employed. Munoz et al. (18) used strain 2927 grown on Bordet-Gengou agar and found the toxin associated with the soluble fraction of a cell lysate. Livey et al. (15), who used strain 18334 grown in a liquid shake culture, found no toxin in the culture supernatant.

In addition to demonstrating and quantitating the intracellular localization of DNT, this study raises an important point about its possible role in clinical whooping cough. In studies with chicken embryos (7) and with tracheal ring explants (6), it was noted that generalized damage to the respiratory epithelium did not occur. Cytotoxicity was restricted to those ciliated epithelial cells to which organisms had attached. Assuming that DNT may be involved in this apparent direct contact-mediated cytotoxicity, these observations and the data presented herein are consistent with the concept that DNT is not a true exotoxin. The release or transfer of DNT may require direct contact between the target epithelial cell and the bacterial organism.

This study also presents data relevant to the localization of adenylate cyclase, which could have an important regulatory role in *B. pertussis* (8a, 11). Previous work presented evidence for the extracellular and extracytoplasmic location of this enzyme (9). The cell-associated extracytoplasmic enzyme was postulated to be in the periplasm, that space between the cytoplasmic membrane and the outer membrane. We confirmed that a small percentage of the enzyme is secreted by actively growing cells and that most of the activity is cell associated and extracytoplasmic. Trypsin treatment of cell lysates inactivated the intracellular cytoplasmic MDH and DNT, the membrane-bound intracellular SDH, and adenylate cyclase; but when whole cells were treated, only the adenylate cyclase was inactivated, indicating that the adenylate cyclase is extracytoplasmic. However, our data argue against the enzyme being free in the periplasmic space. The enzyme was not released after osmotic shock of *B. pertussis* or by the formation of spheroplasts. After osmotic lysis of the spheroplasts, significant amounts of adenylate cyclase were found both in the soluble fraction and in a membrane fraction with the activity in the membrane fraction easily removed by washing. A plausible interpretation of these data is that the cell-associated adenylate cyclase is loosely attached to a cell envelope component with an extracytoplasmic exposure. However, significant loss of adenylate cyclase activity did occur with some fractionation procedures. This raises the possibility that the lability of the enzyme may differ depending on the state of the enzyme in various isolated fractions. This lability makes the precise localization of adenylate cyclase by its enzymatic activity uncertain.

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