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# Biological Activities and Chemical Composition of Purified Tracheal Cytotoxin of *Bordetella pertussis*

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**Specific destruction of ciliated epithelial cells lining the large airways is the primary respiratory tract cytopathology associated with human *Bordetella pertussis* infections. We have purified a single low-molecular-weight glycopeptide, tracheal cytotoxin (TCT), that appears to cause this pathology. By using a combination of solid-phase extraction and reversed-phase high-pressure liquid chromatography, about 700 nmol of biologically active peptide can be isolated from 1 liter of *B. pertussis* culture supernatant (approximately 60% yield). TCT at concentrations of 1 μM destroyed the ciliated cell population when incubated with respiratory epithelium in vitro. This concentration of TCT is similar to the concentrations found in the culture supernatant of growing *B. pertussis*. Purified TCT also inhibited DNA synthesis of hamster trachea epithelial cells in a quantitative, dose-dependent fashion. Endotoxin was not detected in the purified material, and neither *B. pertussis* nor *Escherichia coli* endotoxin could duplicate the biological activities of TCT. Amino acid and amino sugar analyses of purified TCT revealed the presence of glucosamine, muramic acid, alanine, glutamic acid, and diaminopimelic acid in molar ratios of 1:1:2:1:1. This suggests that TCT, the released ciliostatic principle of *B. pertussis*, is a disaccharide tetrapeptide subunit of peptidoglycan.**

*Bordetella pertussis* is a gram-negative bacterium that causes the human respiratory tract illness known as whooping cough (or pertussis). The characteristic pathology associated with the disease, which features specific bacterial colonization of and destruction of ciliated cells, was first described three-quarters of a century ago by Mallory and Horner (19). The consequences of this cytopathology can be severe, because the loss of ciliary activity significantly impairs mucus transport out of the respiratory tract. Accumulation of mucus, multiplying bacteria, and inflammatory debris in the absence of a functional mucociliary "escalator" give rise to a situation where coughing is the only remaining means to clear the blocked airways. Therefore, damage to the ciliated cells may generate the paroxysmal coughing episodes characteristic of pertussis as well as predispose the infected host to secondary pulmonary infections (24).

The cytopathological events during *B. pertussis* infection of the respiratory tract have been studied by using tracheal organ culture as an in vitro model system (7). With tracheal rings dissected from hamsters, infection with *B. pertussis* is similar to that in humans: a noninvasive, specific colonization of ciliated cells, followed by ciliostasis and the eventual extrusion of the ciliated cell population (23). Although there are a variety of biologically active virulence-associated macromolecules produced by *B. pertussis* (for a review, see reference 32), only a low-molecular-weight fraction of bacterial culture supernatant can mimic this ciliated-cell-specific pathology when incubated with tracheal organ cultures (15).

Furthermore, hamster trachea epithelial (HTE) cells (13), to which virulent *B. pertussis* can adhere, are unable to synthesize DNA in the presence of viable *B. pertussis*. This effect on HTE cells can be reproduced by the addition of the same low-molecular-weight fraction of culture supernatant that destroys tracheal organ culture (15). We speculate that this inhibitory activity of *B. pertussis* may delay the normal replacement of destroyed ciliated cells in vivo by blocking division and differentiation of the underlying regenerative basal cell population (18). This notion is consistent with the observation that the clinical course of pertussis is unchanged by antibiotic therapy initiated after the onset of coughing paroxysms (24). Viable *B. pertussis* organisms are no longer present, yet manifestations of active infection, including the coughing episodes, can continue for weeks.

To understand more fully the ciliated-cell-specific pathology that results from *B. pertussis* infections, we sought to purify the tracheal cytotoxin (TCT) activity of culture supernatants to homogeneity. Here we report the isolation and composition of TCT—a single molecule produced by *B. pertussis* that specifically destroys ciliated respiratory epithelial cells.

## MATERIALS AND METHODS

**Bacteria.** Virulent phase I *B. pertussis* Tohama I was stored at −70°C in a solution containing Casamino Acids (Difco Laboratories, Detroit, Mich.), $MgCl_2$, $CaCl_2$, and NaCl with 50% glycerol (30). Bacteria were grown on solid modified Stainer-Scholte medium (SSM) (16, 31) supplemented with 10% fresh defibrinated sheep blood in Alsevers (GIBCO Laboratories, Grand Island, N.Y.) by incubating at 37°C for 72 h in a humidified atmosphere containing 95% air–5% $CO_2$. We used the resulting growth to inoculate liquid medium consisting of SSM containing 10% Casamino Acids. After incubation on a rotary shaker (150 rpm) at 37°C for 24 to 48 h in an atmosphere of 95% air–5% $CO_2$, the late-log-phase cells were harvested by centrifugation (15 min at 2,200 × *g*), washed once with SSM, and suspended in fresh SSM without Casamino Acids. Using these organisms we inoculated fresh cultures to approximately $10^7$ bacteria per ml (based on standardized $A_{540}$) and incubated them on a rotary shaker as described above.

**Solid-phase extraction.** Bacteria growing in the mid- to late-log phase (≤4 × $10^9$ bacteria per ml) were centrifuged at 13,200 × *g* for 20 min at 4°C. The supernatant was filtered through 0.2-μm-pore-size cellulose-acetate membrane filter units (Corning Glass Works, Corning, N.Y.) and acidified to pH 3 with 100% trifluoroacetic acid (TFA) to a final concen-

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tration of 0.5%. Portions of this solution were poured into a glass column (1.5 by 22 cm), which served as a sample reservoir. At the tapered end of the column we attached a $C_{18}$ Sep-Pak (Waters Associates, Milford, Mass.) that had been prepared to receive the sample by wetting twice, first with 100% methanol and then with 0.1% TFA in water. All solutions were forced through Sep-Paks by pressurized air. After complete extraction of the sample of interest, the Sep-Pak was washed with two 10-ml volumes of 0.1% TFA in water. We then eluted some of the retained molecules by the addition of 3 ml of 20% *n*-propyl alcohol in 0.1% TFA and concentrated them to dryness in a rotary evaporator (Speed Vac Concentrator; Savant, Farmingdale, N.Y.). The resulting $C_{18}$ fraction was characterized by reversed-phase high-pressure liquid chromatography (HPLC) analysis (see below) and used for the subsequent solid-phase extraction.

We used quaternary methylamine (QMA) Sep-Pak cartridges (ACCELL media; Waters Associates) attached to the end of a 10-ml plastic syringe for anion-exchange solid-phase extraction. The exchange matrix was sequentially wetted with 10 mM ammonium acetate (pH 5.5) containing 20% methanol (running buffer), 10 mM ammonium acetate (pH 5.5) containing 1 M NaCl and 20% methanol (elution buffer), 100% methanol, and again with running buffer. After loading the $C_{18}$ fraction onto the QMA Sep-Pak cartridge in running buffer, we washed it with 5 ml of the same buffer and eluted the retained molecules with 5 ml of elution buffer. This eluate was concentrated to a volume of approximately 2.5 ml by rotary evaporation, acidified by the addition of 10% TFA to a final concentration of 0.5%, and desalted by subjecting it to $C_{18}$ solid-phase extraction as described above. After concentration to dryness by rotary evaporation, the anion-exchange-extracted sample was stored at −70°C before reversed-phase HPLC analysis.

**Reversed-phase HPLC.** We performed HPLC with Waters Associates equipment, except where noted. Aquapore Octyl ($C_8$) RP-300 cartridge columns (Brownlee Labs, Rainin Instrument Co., Woburn, Mass.) maintained at ambient temperature were used for all chromatographic separations: we employed a 130- by 4.6-mm (inner diameter) column containing 7-μm spherical particles of $C_8$-derivatized silica with a solvent delivery rate of 1 ml/min. UV-absorbing components of the column effluent were detected at 214 nm by using a Spectroflow 757 variable-wavelength UV detector (Kratos, Ramsey, N.J.), and peak areas and retention times were recorded by using a Hitachi Model D-2000 Chromato-Integrator (Hitachi, Ltd., Tokyo, Japan). We collected peaks of interest in borosilicate glass tubes and stored them at −70°C after removal of the volatile mobile phase by rotary evaporation. For making mobile phases, we filtered all solvents with a Millipore all-glass filtration apparatus with Durapore 0.45-μm-pore-size membranes (Millipore Corp., Bedford, Mass.) and degassed the acetonitrile, methanol, and purified water before mixing. All organic reagents were HPLC/Spectro grade (Pierce Chemical Co., Rockford, Ill.), and water was distilled and deionized (NANOpure; Barnstead Co., Newton, Mass.).

Two distinct chromatographic systems were employed to purify TCT from solid-phase extracts: reversed-phase HPLC by gradient elution with methanol in a triethylamine acetate (TEA-acetate)-buffered mobile phase and gradient elution with acetonitrile in a TFA-buffered mobile phase. The TEA-acetate buffer was prepared as described by Matrisian et al. (21), except that a 0.01 M acetic acid solution was brought to pH 5.55 by the addition of triethylamine. The aqueous portion of the mobile phase (solution A) consisted of 0.01 M TEA-acetate (pH 5.55), and solution B consisted of solution A containing methanol at a final concentration of 30% (vol/vol). In the TEA-acetate-buffered system, a convex gradient from 0 to 7.5% methanol developed over a period of 30 min. This gradient was preceded by a 5-min isocratic loading and washing interval with A and followed by a 3-min isocratic elution with 3A:1B. Subsequent purging with B for 5 min removed any adsorbed molecules remaining on the column, and equilibration with A for at least 20 min before another sample injection insured reproducible chromatography.

For the TFA-buffered system, solution A consisted of 0.1% TFA in water (pH approximately 3), and solution B consisted of solution A plus acetonitrile at a final concentration of 30% (vol/vol). A linear gradient from 0 to 9.0% acetonitrile proceeded over an interval of 30 min. Other aspects of the TFA system were similar to those of the TEA-acetate system. In both buffer systems we found it to be extremely important that the final volume of the B solution contained the correct concentration of buffering agents, i.e., TFA or TEA-acetic acid.

**Analytical methods.** The Protein Chemistry Laboratory at Washington University School of Medicine conducted the amino acid and amino sugar analyses. Samples were hydrolyzed under vacuum in 6 N HCl for 24 h at 110°C for amino acid analyses or in 4 N HCl for 6 h at 100°C for amino sugar analyses. They identified amino group-containing components in the hydrolyzed samples by using standard techniques employing ion-exchange HPLC.

We examined test samples for contaminating endotoxin by the chromogenic *Limulus* amebocyte lysate assay (QCL-1000; Whittaker Bioproducts, Inc., Walkersville, Md.). Purified *Escherichia coli* lipopolysaccharide serotype O26:B6 (Sigma Chemical Co., St. Louis, Mo.) was used as a control for lysate reactivity, and purified *B. pertussis* lipopolysaccharide (List Biological Laboratories, Inc., Campbell, Calif.) was used to construct a standard curve. Each assay was carried out according to the recommendations of the supplier.

We determined protein concentrations with the Bio-Rad protein Assay (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as a standard.

**Assessment of biological activity.** HTE cells were isolated and cultured as described previously (13), except that we used 9 μg of gentamicin per ml instead of penicillin and streptomycin. HTE cells were harvested from nearly confluent monolayers, seeded at $1.5 \times 10^4$ cells per microtiter well in bicarbonate-buffered minimal essential medium containing 2.5% fetal bovine serum (HyClone, Logan, Utah), and incubated for 24 h at 37°C in a humidified environment containing 95% air–5% $CO_2$. We removed the medium from each well with gentle suction and replaced it with the appropriate control or test samples dissolved in 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered minimal essential medium (pH 7.3). Purified TCT was added in the presence of bovine serum albumin as a protein protectant and carrier; control samples also included bovine serum albumin. After incubation for 4 h at 37°C in a humidified environment (without $CO_2$), the DNA-synthetic ability of sample-treated cells and untreated control cells was quantitated by measuring [$^3$H]thymidine incorporation after serum stimulation as described previously (15).

Tracheas dissected from young (6 to 12 weeks) male golden Syrian hamsters (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were used to prepare tracheal

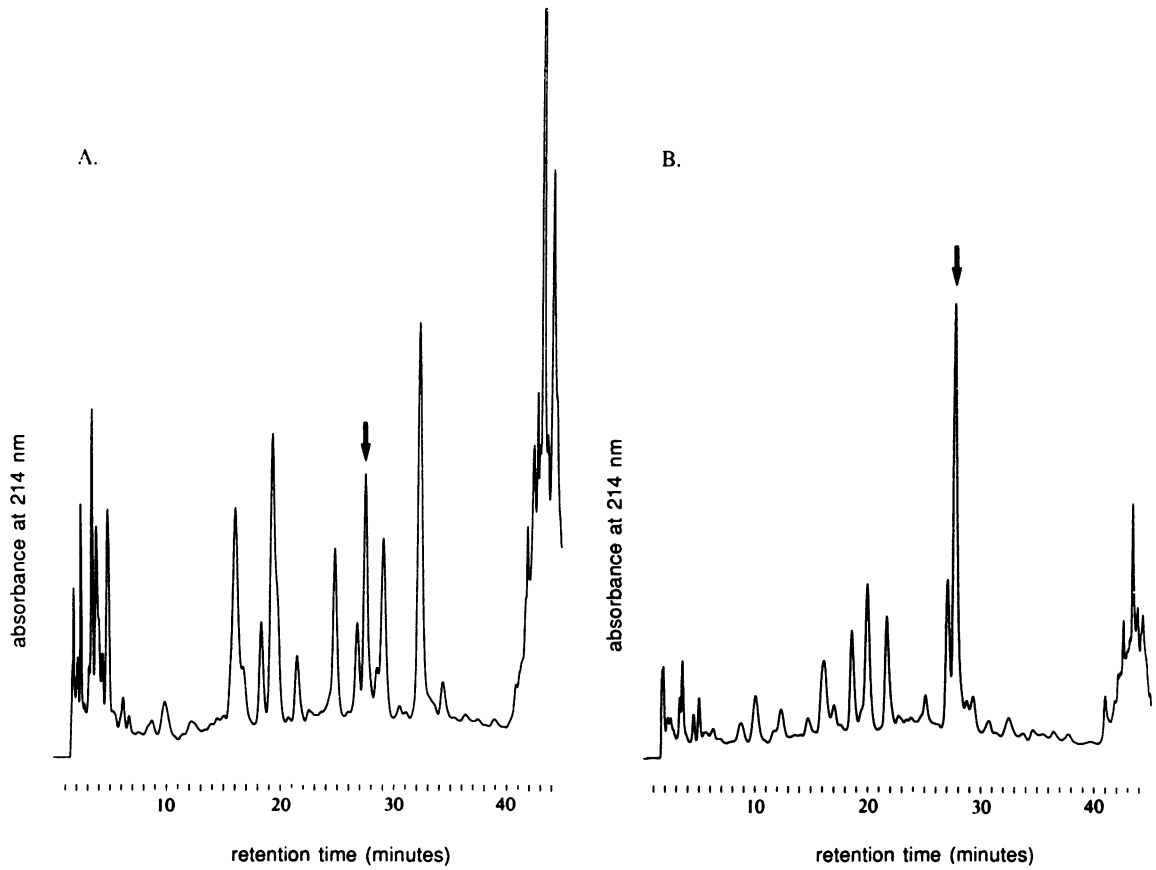


FIG. 1. TFA-buffered reversed-phase HPLC analysis of solid-phase extracts. The peak with toxic activity is indicated by the arrows. (A) Retained components from a $C_{18}$ extract of 25 ml of *B. pertussis* culture supernatant (0.128 $A_{214}$ units full scale). (B) Anionic components obtained by ion-exchange extraction of the $C_{18}$ extract in A (0.064 $A_{214}$ units full scale).

rings manually (6) or with a tissue chopper (custom design by Richard McDonald; details available upon request). Cultures were grown in nutrient mixture F-12 (GIBCO) buffered with 5 mM HEPES (pH 7.3) in a humidified environment at 37°C. To evaluate the biological activity of sterile test samples, we observed rings for pathology at 24-h intervals with an inverted light microscope under ×10 and ×20 power.

## RESULTS

**Extraction of TCT from culture supernatants.** To devise an effective purification scheme, we chose solid-phase extraction to prepare samples for more rigorous isolation procedures. In the first step of a rapid method, we used a reversed-phase matrix ($C_{18}$) to concentrate tracheal cytotoxic activity from the culture supernatant of *B. pertussis* growing in the mid- to late-log phase. Once the supernatant had been acidified and passed through the $C_{18}$ Sep-Pak, nonspecifically bound substances were washed away and adsorbed molecules were selectively eluted by the addition of 0.1% TFA in water containing 20% *n*-propyl alcohol. Under these conditions, a peptide-rich fraction can be obtained that is free of large polypeptides, salts, and free amino acids (2–4, 29). HPLC analysis of this relatively complex extract revealed the presence of one peak with cytotoxic activity (Fig. 1A).

Amino acid analysis of partially purified material suggested that TCT may have significant anionic or acidic character. Therefore, anion exchange was chosen to fractionate acidic molecules from basic and/or neutral molecules present in the TCT-enriched eluate from the $C_{18}$ extraction. The $C_{18}$ extract was loaded onto the strong anion-exchange QMA Sep-Pak in low-salt running buffer (pH 5.5) and washed with the same to remove any nonspecifically bound components. Adsorbed molecules with presumed anionic character were then eluted from the QMA Sep-Pak with high-salt elution buffer, also at pH 5.5. Optimal extraction of toxic activity into the eluted fraction occurred at pH 5.5; a lower pH resulted in decreased recovery, whereas a higher pH increased the proportion of contaminating molecules harvested without a significant increase in yield. Figure 1B shows a typical chromatogram of the UV-absorbing components in the effluent from this ion-exchange solid-phase extraction. Comparison of the HPLC profiles of the $C_{18}$ (Fig. 1A) and QMA (Fig. 1B) extracts indicates that the complexity of the fraction containing activity has been significantly reduced and that several components with HPLC retention times similar to the peak with cytotoxic activity have been removed.

**Purification of TCT from culture supernatant extracts.** TCT was completely purified from the ion-exchange extract described above by using two successive reversed-phase HPLC separations. First, the QMA eluate was loaded onto a $C_8$ column in TEA-acetate buffer (pH 5.55) and separated by gradient elution with methanol. A typical chromatogram resulting from this purification step is shown in Fig. 2A. Amino acid analysis of the biologically active fraction (data

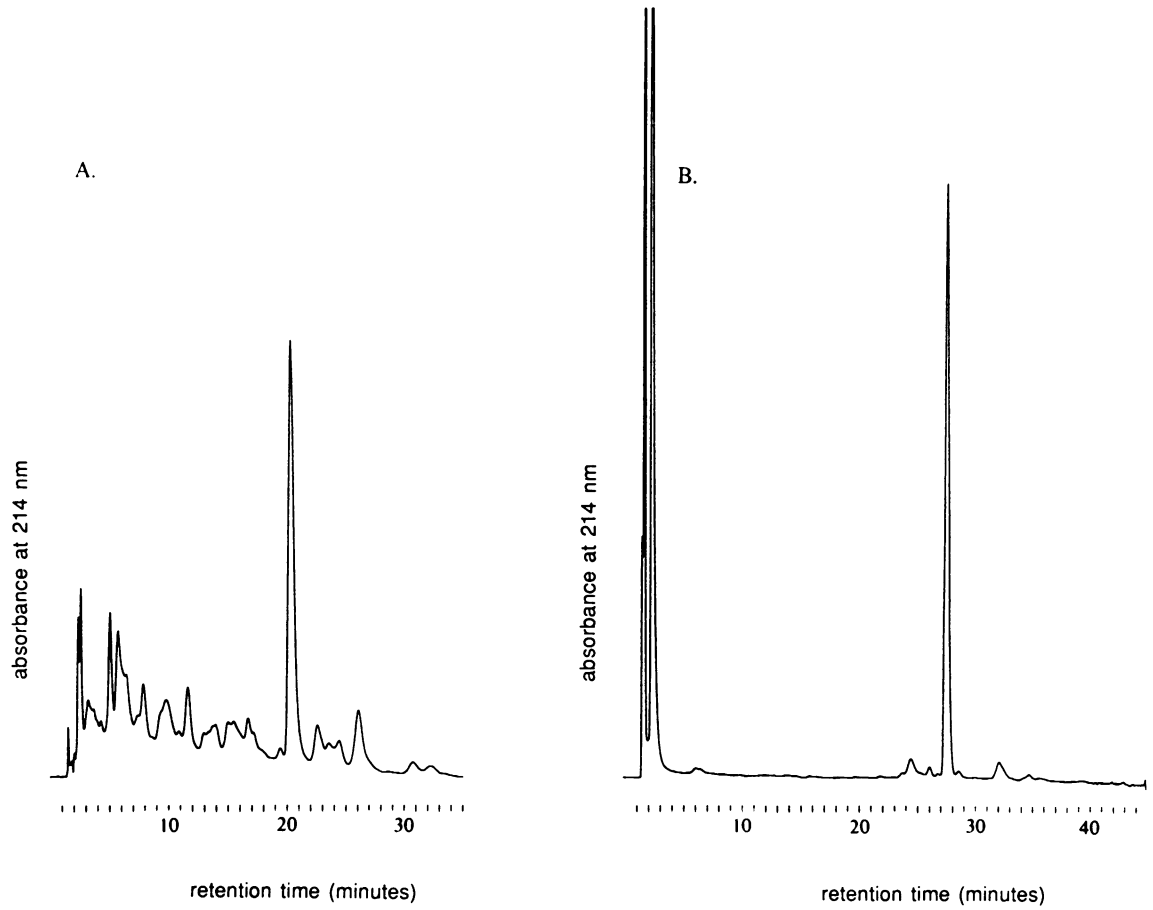


FIG. 2. Purification of TCT from solid-phase extracts of *B. pertussis* culture supernatants. (A) TEA-acetate-buffered reversed-phase HPLC of the anionic components from 16 ml of *B. pertussis* culture supernatant. Toxic activity is present only in the predominant peak of the chromatogram (0.064 $A_{214}$ units full scale). (B) TFA-buffered reversed-phase HPLC of the collected peak in A. The harvested peak from this procedure is biologically active TCT, free from all contaminants (0.032 $A_{214}$ units full scale).

not shown) indicated that the TEA-acetate system effectively separated TCT from other acidic peptides contained in the ion-exchange extract; however, nonproteinaceous, UV-absorbing contaminants were present in the collected material. To separate TCT from these contaminating molecules, the concentrated eluate containing activity from the TEA-buffered HPLC separation was subjected to reversed-phase HPLC in a TFA-buffered system (Fig. 2B). The single harvested peak from this procedure was homogeneous, free of all contaminants, and biologically active (see below). It should be noted that throughout purification the only fractions capable of destroying ciliated respiratory epithelium contained this peak.

Table 1 summarizes a typical purification of TCT from 25 ml of mid-log-phase *B. pertussis* Tohama I culture supernatant. The effectiveness of this protocol was evaluated by determining the amount of biological activity present in a sample after each step (see below). About 700 nmol of TCT could be purified from 1 liter of culture supernatant in high yield (63%), indicating that TCT was present in supernatants at a concentration in excess of 1 μM.

**Biological activity.** Dose-dependent inhibition of DNA synthesis in HTE cells was used as a method of quantitatively measuring TCT activity. This activity is also present in the toxic fractions that cause ciliated-cell-specific cytopathology in tracheal organ cultures. Using both organ culture and HTE cells as biological test systems for the material purified by the HPLC-based protocol, we proved that both activities of *B. pertussis* culture supernatants are due to intoxication by the same molecule. Ciliary activity in hamster tracheal rings was completely annihilated after 96 h of

TABLE 1. Summary of recoveries in the purification steps of TCT

| Isolation procedure | U of activity[a] | % Yield | Sp act[b] |
|---|---|---|---|
| Solid-phase extraction | | | |
| $C_{18}$ Sep-Pak | 112.2 | 97[c] | 1.95 |
| QMA Sep-Pak | 91.2 | 78.8 | 4.35 |
| Reversed-Phase HPLC | | | |
| 0.1 M TEA-acetate (pH 5.55) | 80.9 | 69.9 | 26.6 |
| 0.1% TFA (pH 3.0) | 73.3 | 63.4 | 26.6[d] |

[a] One unit of activity is equal to the amount of toxin required to generate a one-half-maximal inhibition of DNA synthesis. Purification was performed by starting with 25 ml of culture supernatant.

[b] Specific activity is defined as units of activity per microgram of protein.

[c] Based on elution of purified [$^3$H]diaminopimelic acid-labeled TCT present in mock culture supernatant filtrates. Radiolabeled TCT was prepared as described previously (26).

[d] Although an increase in specific activity was not obtained, significant resolution from nonproteinaceous, UV-absorbing contaminants was achieved by this final chromatographic step.

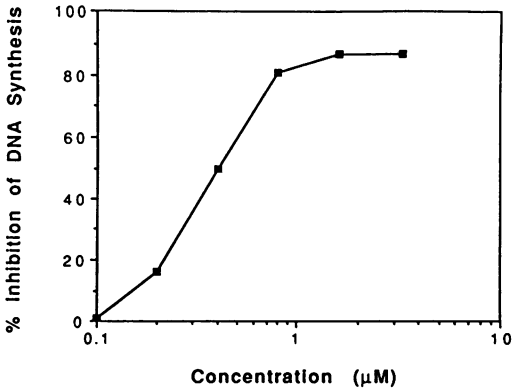


FIG. 3. Dose-response analysis of purified TCT: inhibition of HTE cell DNA synthesis. All points were calculated by comparing cells treated with TCT with cells treated with the control sample in triplicate. Each point represents the average of values <10% from the standard deviation of the mean.

incubation with purified TCT at 1 μM and after 48 to 72 h when incubated with 3 μM TCT. Figure 3 is a representative dose-response curve showing inhibition of DNA synthesis in HTE cells by purified TCT. A threshold for an inhibitory response was observed, and maximal inhibition occurred at concentrations of about 1 μM. This is in contrast to the lower specific activity and more shallow dose-response curve reported with less purified material (14, 15). We attribute this improved biological activity to the removal of contaminating molecules interfering with this assay for tracheal cytotoxic activity. Importantly, TCT is found in *B. pertussis* culture supernatants at concentrations sufficient to elicit the destruction of respiratory epithelium (see above).

To establish that the observed biological responses were in fact due to TCT and not to contaminating endotoxin, test samples were evaluated for the presence of endotoxin by using the *Limulus* amebocyte lysate assay. No detectable endotoxin (<10 fg per 6.5 μg of TCT) was present in samples that had been subjected to either TFA- or TEA-buffered reversed-phase HPLC. In $C_{18}$ solid-phase extracts of *B. pertussis* culture supernatants, we detected endotoxin concentrations as high as 10 ng/ml. However, purified *B. pertussis* or *E. coli* endotoxin at concentrations as high as 0.1 μg/ml neither inhibited DNA synthesis of HTE cells nor caused ciliated-cell-specific cytopathology in organ cultures (data not shown). This strongly suggests that contaminating endotoxin is not responsible for the biological activities described above for purified TCT.

**Composition.** To characterize further the biologically active material, we subjected samples of TCT to amino acid and amino sugar analyses (Table 2). The previously reported composition of partially purified toxic activity prepared by conventional chromatography (12, 15) indicated the presence of contaminating amino acids, amino sugars, or peptides. HPLC analysis of such preparations revealed multiple peaks, confirming the presence of impurities not removed by gel filtration chromatography and high-voltage paper electrophoresis (data not shown). A single UV-absorbing peak was harvested in the final step of the HPLC-based purification protocol (Fig. 2B), suggesting that the composition of TCT reflects its identity as a single glycopeptide. Its constituent residues are typical components of *B. pertussis* peptidoglycan (9) and of bacterial peptidoglycan in general (27). The molar ratios of amino acids and amino sugars in purified samples are consistent with the notion that TCT is a disaccharide-tetrapeptide subunit of peptidoglycan.

TABLE 2. Composition of purified *B. pertussis* tracheal cytotoxin

| Amino acid or amino sugar | Molar ratio[a] | No. of residues |
|---|---|---|
| Glucosamine | 0.83 | 1 |
| Muramic acid | 0.78 | 1 |
| Alanine | 1.7 | 2 |
| Glutamic acid | 1.0 | 1 |
| Diaminopimelic acid | 0.72 | 1 |

[a] Relative to glutamic acid.

## DISCUSSION

In this report, we have described the purification of a single peptidoglycan-related molecule called TCT from the supernatant of growing *B. pertussis*. TCT causes ciliated-cell-specific respiratory tract pathology in vitro that is indistinguishable from the primary pathology seen in human pertussis infections. In addition, TCT inhibits DNA synthesis of HTE cells in a quantitative, dose-dependent fashion, a finding that may also have pathogenic significance in vivo.

The purification scheme utilizes both solid-phase extraction and reversed-phase HPLC technologies to harvest biologically active TCT; the technique is rapid and delivers a high yield of TCT that is free from all contaminants. Solid-phase extraction was a useful method to employ because it is simple, fast, inexpensive, and reproducible while offering an intermediate level of chemical selectivity with high load capacity. This allowed us to concentrate the tracheal cytotoxic activity present in 1 liter of bacterial culture supernatant by over 1,000-fold (reversed-phase matrix) and to partition it with molecules of acidic character from basic and neutral populations (ion-exchange matrix). TCT was separated from this mixture of acidic molecules by reversed-phase HPLC in buffer systems at a pH above (pH 5.55) and below (pH 3) the $pK_a$ of the constituent acidic residues of TCT. These buffers presumably induced changes in the polarity of the molecule, correspondingly altered its chromatographic character, and permitted us to isolate TCT to homogeneity. Thus, we determined that a single molecule from the culture supernatant of *B. pertussis* was capable of causing the primary respiratory tract pathology associated with pertussis.

The speed and efficiency with which the separation can be performed makes the method useful as an analytical tool. This has allowed us to examine other members of the *Bordetella* genus, which infect a variety of warm-blooded hosts, for the ability to produce TCT. We found that representative strains of each *Bordetella* species release measurable amounts of TCT (10; B. T. Cookson and W. E. Goldman, J. Cell. Biochem. **11B**:124, 1987). This is consistent with the observations that all virulent *Bordetella* strains cause remarkably similar illnesses and that they specifically colonize and destroy ciliated cells in the respiratory epithelium of their respective hosts. Furthermore, it supports the notion that TCT, a single molecule that can mimic the primary cytopathology of these infections, is a conserved virulence determinant of all *Bordetella* species.

This analytical system can resolve various classes of peptidoglycan fragments in addition to TCT, the major fragment released by growing *B. pertussis* (26). Although the

released material represents only a small percentage of the macromolecular peptidoglycan of *B. pertussis* organisms, our experiments show that TCT is produced at levels sufficient to evoke significant biological effects. Since TCT is undetectable by pulse-chase-type experiments designed to look at turnover of macromolecular peptidoglycan (25, 28), detecting soluble fragments released by bacterial microorganisms may require more direct methods (like those described here). The use of HPLC to separate fragments of purified peptidoglycan has been reported by workers in several laboratories (8, 11, 20), but its use has been limited to the analysis of enzymatic digestions of high-molecular-weight cell wall material precipitated by hot sodium dodecyl sulfate. TCT, for instance, is refactory to commonly used protein precipitation procedures (unpublished observations). This suggests that implementation of solid-phase extraction to harvest peptide extracts would greatly expedite the analysis of solutions thought to contain soluble peptidoglycan fragments. This technique could be widely applicable to avenues of research such as the investigation of other mucosal pathogens for production of TCT or TCT-like molecules, the detection of peptidoglycan in host tissue (see below), the analysis of antibiotic-induced bacterial autolytic products, and the study of recycling of peptidoglycan for new cell wall synthesis in bacteria.

The peptidoglycan-derived composition of TCT establishes that it is one of the muramyl peptides (muramic acid-containing glycopeptides), a family of molecules with diverse biological activities. Well-documented effects of these molecules include pyrogenicity, adjuvanticity, arthritogenicity, stimulation of leukocytes to produce interleukin-1, and induction of slow-wave sleep (for reviews, see references 1, 5, and 17). Of particular interest is the observation that *Neisseria gonorrhoeae* releases anhydromuramic acid-containing disaccharide peptides (27, 28) that are capable of causing ciliated-cell-specific damage to fallopian tube mucosa (22). Results reported here and our collaborative work with Rosenthal et al. (26) indicate that TCT has an analogous disaccharide-tetrapeptide arrangement. Presumably TCT is derived from macromolecular *Bordetella* peptidoglycan, the end product of a multigene biosynthetic pathway. We therefore conclude that TCT is not a clonable gene product and that biochemical approaches will be necessary to elucidate further the pathobiological roles of TCT in disease. We have accomplished the first step of such an approach by developing a rapid method of isolating completely purified and biologically active TCT in high yield. Homogeneous material will allow us to look at host cell receptors and/or target sites through which TCT exerts its toxic effects on respiratory epithelium. Armed with powerful separation and analysis tools and the appropriate biological model systems, we are now exploring the toxic effects of TCT on epithelia.

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