Primary Structure of the Peptidoglycan-Derived Tracheal Cytotoxin of *Bordetella pertussis*†

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ABSTRACT: The etiological agent of whooping cough, *Bordetella pertussis*, destroys the ciliated epithelial cells lining the large airways of infected individuals. This cytopathology can be reproduced in respiratory epithelium by tracheal cytotoxin (TCT), a small peptidoglycan-related molecule purified from the culture supernatant of growing *B. pertussis* organisms. Using fast atom bombardment mass spectrometry, we analyzed the positive- and negative-ion spectra of the purified, biologically active material and assigned a mass of 921 daltons to TCT. Analysis of fragment ions in these spectra as well as the spectra of the methyl ester and acetylated derivatives of TCT unambiguously defined the primary structure of TCT as *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramylalanyl-γ-glutamyldiaminopimelylalanine. TCT is therefore identical with the ciliostatic anhydropeptidoglycan monomer released by *Neisseria gonorrhoeae* and with the neurologically active slow-wave sleep-promoting factor FS_u. These and other structurally related glycopeptides containing muramic acid thus form a family of molecules with remarkably diverse biological activities.

Bordetella pertussis is a Gram-negative bacterium responsible for causing whooping cough (pertussis), a respiratory tract illness in humans. The organisms are noninvasive, and they specifically colonize and subsequently destroy ciliated cells lining the large airways (Mallory & Horner, 1912). The ciliated epithelium is an important part of a primary defense barrier that transports debris and particulate matter entrapped in mucus away from the lungs and toward the pharynx. Loss of the mucociliary clearance mechanism results in accumulation of mucus, multiplying bacteria, and inflammatory debris in the affected airway. These events associated with the specific cytopathology of pertussis can give rise to a life-threatening syndrome that features frequent airway blockage (prompting severe coughing episodes) as well as predisposition to secondary bacterial pulmonary infections [see Olson (1975)].

To study the primary respiratory tract pathology of *B. pertussis* infection, tracheal organ culture was developed as a model of infection (Collier et al., 1977). In vitro, viable *B. pertussis* organisms specifically colonize the ciliated cells, causing ciliostasis and the eventual extrusion of these cells from the respiratory epithelium. Reflecting the validity of this model system, these observations are indistinguishable from those made during studies of human infection with *B. pertussis* (Mallory & Horner, 1912). Although much investigation has centered around a number of biologically interesting molecules produced by *B. pertussis* [for a review, see Weiss and Hewlett (1987)], factors associated with the primary pathogenesis remained undetermined until 1982: a molecule called tracheal cytotoxin (TCT) from the supernatant of *B. pertussis* cultures was found to mimic the ciliated cell-specific destruction in tracheal organ culture (Goldman et al., 1982). TCT was thought to contain 15 amino acid residues and at least 2 amino sugars. Most notable, diaminopimelic acid and muramic acid were found in the active preparation, and these chemical moieties are known to be incorporated only into bacterial peptidoglycans (Schleifer & Kandler, 1972). Recently it has been possible to purify TCT to homogeneity and determine that this single molecule is capable of causing ciliated cell-specific respiratory tract cytopathology (Cookson et al., 1989). The purified and biologically active TCT contains amino acid and amino sugar residues consistent with its postulated peptidoglycan-like character (Cookson et al., 1989) and was confirmed to be a released fragment of *B. pertussis* peptidoglycan (Rosenthal et al., 1987).

In order to understand more fully the molecular events underlying the primary pathology of *B. pertussis* infections, we sought to determine the structure of TCT. Fast atom bombardment mass spectrometry (FABMS) can reveal unambiguous structural information about a sample of high purity (Barber et al., 1982). It has been previously used to determine the sequences of various fragments prepared from the purified intact peptidoglycan of whole bacteria (Martin et al., 1987). In this report, we use FABMS to determine the primary structure of TCT and discuss the biological implications of this structural assignment.

Materials and Methods

Preparation of TCT. TCT was purified from the supernatant of mid-to-late log phase *B. pertussis* cultures by using a combination of solid-phase extraction and reversed-phase HPLC as described previously (Cookson et al., 1989). We collected HPLC-purified TCT in polypropylene tubes that had been rinsed once with concentrated nitric acid, twice with water, and once with 100% acetonitrile prior to use. The volatile HPLC solvents were removed from the TCT sample by rotary evaporation in a Speed Vac concentrator (Savant, Farmingdale, NY). TCT was stored frozen at −70 °C until used for further study.

Derivatization of TCT. Chemical modification of TCT allowed us to determine the number of carboxyl, amino, and

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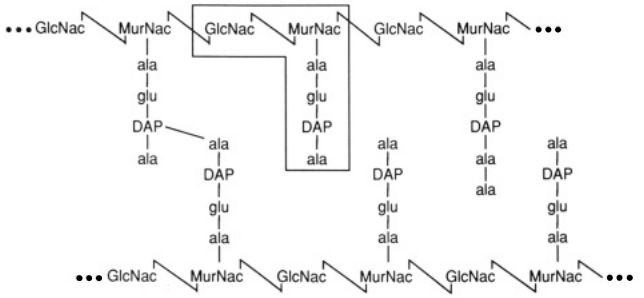

FIGURE 1: Proposed arrangement of the constituent residues of TCT. The composition of TCT predicts a simple structure for the molecule based upon the known structure of *B. pertussis* peptidoglycan, the precursor substrate from which TCT is derived. The $\beta(1\rightarrow4)$ glycosidic bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine residues making up the glycan backbone and the peptide bonds making up the side chains and peptide cross-links are shown. The abbreviations used are as follows: GlcNac, *N*-acetylglucosamine; MurNac, *N*-acetylmuramic acid; glu, glutamic acid; ala, alanine; DAP, diaminopimelic acid.

hydroxyl functional groups present on the TCT molecule. Free carboxyl groups were methylated with excess 1.5 N HCl/methanol at room temperature for 3 h. Hydroxyl and amino groups were acetylated with an excess of acetic anhydride/pyridine (1:1) solution at room temperature for 45 min. The products of these reactions were separated from the excess reagents by rotary evaporation in a Speed Vac concentrator. In addition, some samples of methylated toxin were subsequently acetylated to yield TCT derivatives with both modifications.

Fast Atom Bombardment Mass Spectrometry. Fast atom bombardment mass spectra were recorded by using a VG ZAB-SE double-focusing mass spectrometer equipped with a VG 11-250 data system (VG Instruments, Danvers, MA). We deposited compounds for study onto the sample probe in 1–2 μL of glycerol. The probe was then placed in the mass spectrometer through a vacuum lock such that the bombarding xenon atom beam (8-keV energy, 1-mA gun emission current) was directed upon the sample. The resulting ions were accelerated to + or −8-keV energy, mass separated, and detected at a scan speed of 15 s/decade with mass resolution of 2000 daltons.

Results

Determination of the Molecular Mass of TCT by FABMS. In a previous report (Cookson et al., 1989) we determined that purified, biologically active TCT is composed of glucosamine, muramic acid, alanine, glutamic acid, and diaminopimelic acid in molar ratios of 1:1:2:1:1. This composition, particularly the presence of diaminopimelic acid and muramic acid, and the observation that TCT is the major released peptidoglycan fragment of growing *B. pertussis* (Rosenthal et al., 1987) suggest that TCT is a monomeric disaccharide–tetrapeptide derived from peptidoglycan. The hypothesized TCT structure is shown superimposed upon the known cell wall structure of *B. pertussis* (Folkening et al., 1987) in Figure 1. When purified toxin was subjected to FABMS, a protonated molecule $(M + H)^+$ at m/z 922 was identified when positive ions were analyzed (Figure 2), and a deprotonated molecule $(M - H)^-$ at m/z 920 was identified when negative ions were analyzed (data not shown). These data indicate that the molecular mass of TCT is 921 daltons. This confirms previous reports (Goldman et al., 1982; Rosenthal et al., 1987) showing that the *B. pertussis* factor that is toxic for ciliated cells has a low molecular weight.

Taken together, the composition and mass predict that TCT is a monomeric disaccharide–tetrapeptide. Since dimeric or larger subunits of peptidoglycan of similar composition would

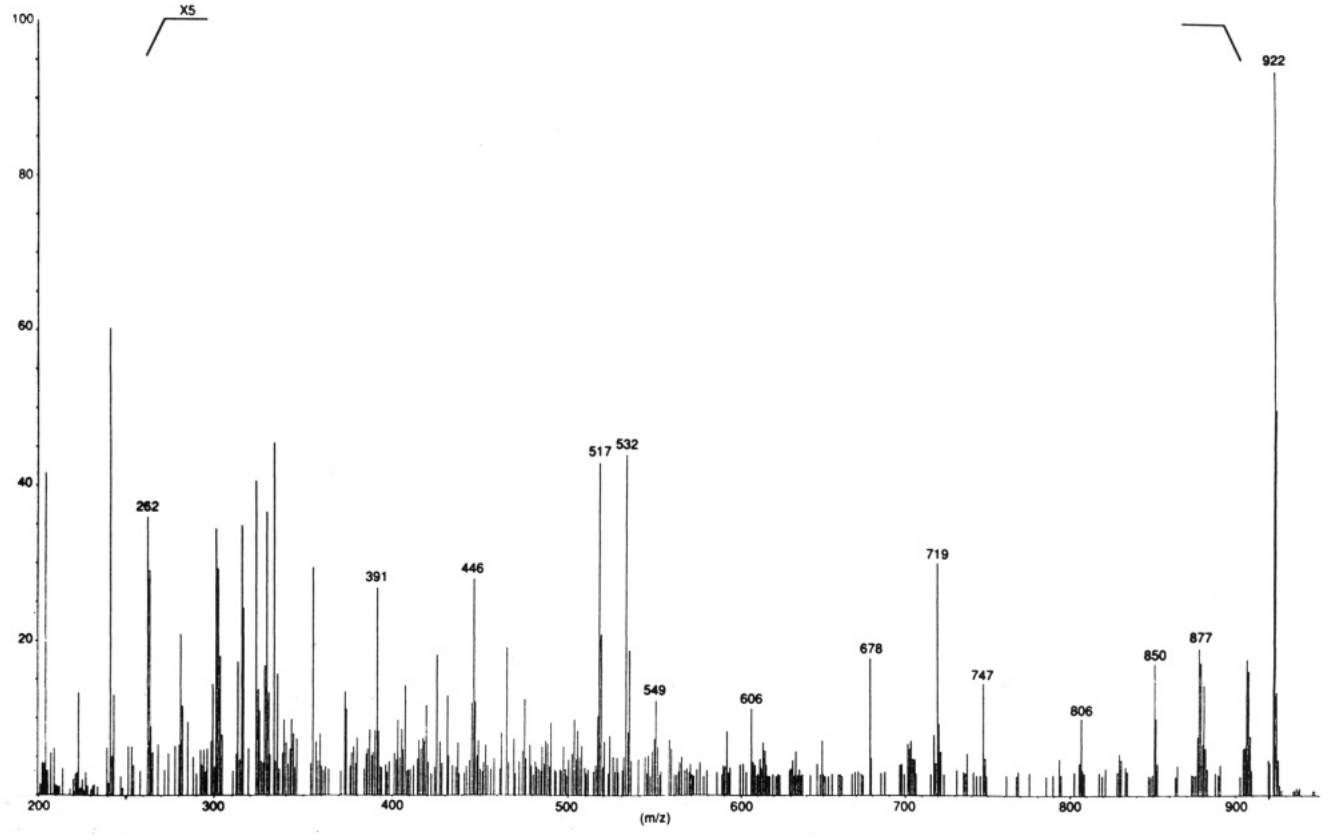

FIGURE 2: Positive-ion FAB mass spectrum of TCT, $(M + H)^+$ 922. Most of the fragment ions important for structural identification of TCT are labeled in the mass spectrum. By use of the data collection system, the background ions produced by the glycerol liquid matrix were subtracted from this spectrum.

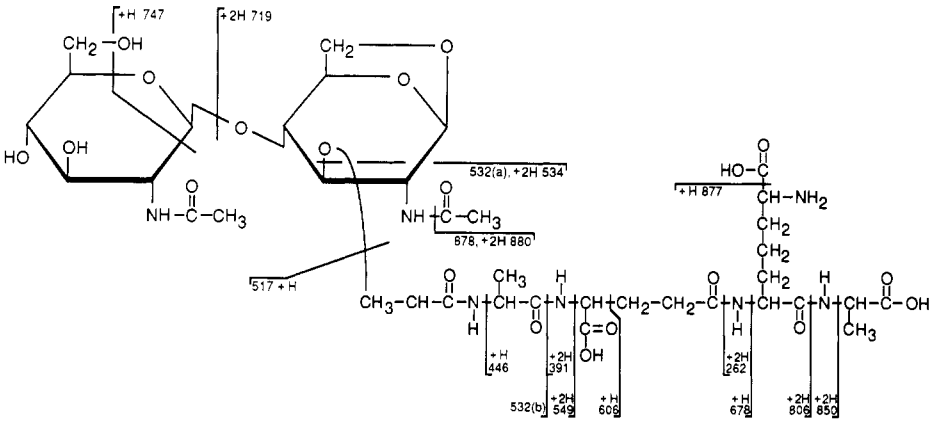

FIGURE 3: Sequence ions associated with $(M + H)^+$ 922 and the structure of TCT: *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-alanyl-γ-glutamyldiaminopimelylalanine. The numerical values represent the mass of various fragment ions resulting from cleavage along the indicated bonds. "+H" or "+2H" notation indicates proton transfer to the observed ion.

exhibit molecular weights of at least 1860 (M_r of a dimeric disaccharide–tetrapeptide), we can exclude the possibility that TCT exists as a polymeric disaccharide–tetrapeptide. The data also indicate that the molecule contains 1,6-anhydromuramic acid, as the corresponding disaccharide–tetrapeptide containing a hydrated muramic acid residue would exhibit a protonated molecule at m/z 940 [or TCT $(M + H)^+ + 18(H_2O)$] and a deprotonated molecule at m/z 938 [or TCT $(M - H)^- + 18(H_2O)$]. Thus, composition and mass data lead us to believe that the structure of TCT is *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramylalanylglutamyldiaminopimelyl-alanine, a monomeric disaccharide–tetrapeptide with M_r = 921. However, composition and mass are not definitive indicators of structure. Conclusive data confirming the proposed structure of TCT follows from the experiments presented below.

Primary Structure of TCT. Molecular ions are usually the most abundant ions found in the FAB mass spectrum of a sample, and these ions indicate the molecular weight of the sample compound(s). Additional information can be found as a result of sample compound fragmentation, a process that is often inherent to ionization during FABMS and produces structurally relatable fragment ions below the mass of the molecular ion (Barber et al., 1982). In order to detect the associated fragment ions from TCT and use them to determine the primary structure, highly purified and concentrated material (approximately 10 μg/μL) was required. Preparation of biologically active toxin in sufficient quantity and purity for this task has recently been described (Cookson et al., 1989), and the FAB mass spectrum of this material is shown in Figure 2. "N-" and C-terminal fragment ions, defined by whether or not an observed fragment ion contains the carboxyl terminus of the parent TCT ion, provide sequence information from both ends of TCT [for clarity, "N-terminal" fragment ions are defined as the observable ions containing the saccharide ("N-terminal") portion of TCT created by cleavage in the C-terminal peptide portion of the molecule]. Since a number of these data present overlapping sequences, they assign the primary structure of TCT with a high degree of certainty.

Positive ion spectra proved to be the most useful for fragment ion analysis; therefore, the fragment ions (Figure 2) and their associated structural assignments (Figure 3) are related to the $(M + H)^+$ 922 of the parent TCT molecule. The mass differences between the N-terminal ions at m/z 850, 678, and 549 determine the peptide sequence from the carboxyl terminus of the toxin to be alanine–diaminopimelic acid–glutamic acid. The remainder of the peptide portion of TCT was surmised from the C-terminal ions: m/z 532 corresponds to the mass of a lactyl tetrapeptide; and m/z 391 corresponds to the same fragment missing the N-terminal lactylalanine residue, i.e., a Glu-DAP-Ala-COOH tripeptide fragment ion. Other C-terminal ions, m/z 719, 534, and 517, relate saccharide information specifically indicating the loss of *N*-acetylglucosamine, the anhydrosaccharide, and the entire disaccharide from $(M + H)^+$ 922, respectively. Together, the fragment ions determine the primary sequence of tracheal cytotoxin: *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramylalanylglutamyldiaminopimelylalanine.

Other fragment ions present in the TCT FAB mass spectrum corroborate the data discussed above; for instance, the signal at m/z 606 (Figure 2) indicated that glutamic acid was present as isoglutamic acid. If a normal glutamic acid residue were in its place and fragmentation occurred at the analogous position in the peptide backbone, we would have seen an ion at m/z 634 corresponding to 606 + 28 ($-CH_2CH_2$). This observation not only provided information supporting the correct structural assignment of TCT but indicated that the peptide bond of the Glu-DAP sequence was via the γ-COOH of glutamic acid. A γ-linked glutamic acid is typical of the peptide construction found in bacterial peptidoglycan (Schleifer & Kandler, 1972), the proposed precursor of TCT. Interestingly, the characteristic mass spectrum of TCT (shown in Figure 2) is identical with the spectrum of an anhydro monomer prepared by enzymatic digestion of purified macromolecular peptidoglycan from *Neisseria gonorrhoeae* (Martin et al., 1987).[1] Since this is essentially an in vitro preparation of TCT from *N. gonorrhoeae* murein, their work supports the proposal that the toxin originates from peptidoglycan and reveals a striking biological precedent for the structure we have presented.

FABMS of Acetylated and Methylated Derivatives of TCT. Individual aliquots of TCT were subjected to methylation and/or acetylation to determine the number of functional carboxyl, amino, and hydroxyl groups on the molecule. The FABMS data obtained from these derivatives are listed in Table I. Reaction of TCT with acetic anhydride/pyridine promotes the acetylation of hydroxyl and basic amino groups. The resulting acetylation increases the molecular mass by 42 daltons for each reacted group, and thus the total increase in mass reveals the number of these functional groups available for derivatization. As shown in Table I, ions corresponding

[1] The interested reader is referred to this work, because the selectivity associated with the use of tandem FABMS allowed characterization of some fragment ions for the peptidoglycan disaccharide tetrapeptide M_r = 921 in addition to the ones reported here.

Table I: Observed Ions for the Methylated and/or Acetylated Derivatives of TCT

obsd m/z	ratio[a]	corresponding TCT structure
	Acetylated Derivatives	
1048	1.0	triacetylated
1090	1.7	tetraacetylated
	Methyl Esters	
936	4.1	monomethyl ester
950	11.1	dimethyl ester
964	1.0	trimethyl ester
	Acetylated Methyl Esters	
1062	1.25	triacetylated monomethyl ester
1076	1.0	triacetylated dimethyl ester
1090	–[b]	triacetylated trimethyl ester
1104	3.6	tetraacetylated monomethyl ester
1118	1.7	tetraacetylated dimethyl ester
1132	1.1	tetraacetylated trimethyl ester

[a] Peak height ratio of ion abundance. [b] Ratio of the triacetylated trimethylated TCT m/z 1090 is not included, since this species is indistinguishable from the nonmethylated tetraacetylated TCT at m/z 1090.

to tri- and tetraacetylated derivatives were observed in the FAB mass spectrum of acetylated TCT. This indicates a total of four reactive hydroxyl and amino groups; we interpret this as confirmation of three hydroxyl groups on the *N*-acetylglucosamine residue and a free amino group on the side chain of the diaminopimelic acid residue (see Figure 3). Furthermore, four reactive acetylation sites support the assignment of 1,6-anhydromuramic acid to TCT, since a hydrated muramyl residue with hydroxyls at carbons 1 and 6 would have added two acetylation sites per molecule and therefore formed hexaacetylated derivatives (m/z 1192).

Reaction of free carboxylic acid functions with HCl/methanol converts them to methyl ester groups (–$COOCH_3$), thereby increasing the mass of the methylated molecule by 14 daltons for each carboxyl group present. Similar to the analysis of the acetylation products, it is possible to determine for a given molecule the number of free carboxyl groups by observing the mass increase after methylation. FABMS of methylated TCT revealed the presence of three ions corresponding to mono-, di-, and trimethyl esters (Table I). Formation of the trimethyl ester indicates that three carboxyl groups are available as methylation sites on TCT. Consistent with the toxin structure presented, the C-terminal alanine, the ϵ-COOH of diaminopimelic acid, and the α-COOH of glutamic acid provide three methylation-susceptible –COOH groups per molecule (see Figure 3). Finally, the number of carboxyl, amino, and hydroxyl moieties of tracheal cytotoxin was confirmed in the same experiment by subjecting methylated toxin to acetylation as described above. Since the methylation of TCT produced three detectable products and acetylation produced two, acetylation of the methyl esters could produce six different products. As expected, the FAB mass spectrum of acetylated TCT methyl esters revealed six ions corresponding to the tri- and tetraacetylated derivatives of the mono-, di-, and trimethyl esters (Table I). Detection of a tetraacetylated trimethyl ester confirms the presence of one amino, three carboxyl, and three hydroxyl functional groups on the TCT molecule. Importantly, derivative analysis not only determined the number of functional groups present on TCT but, in doing so, verified that the structure diagrammed in Figure 3 was correct.

Derivative formation not only reveals information about the number of functional groups, but it can also support fragment ion analysis. Methylation identifies available carboxyl moieties and can therefore aid in identifying C-terminal fragment ions containing –$COOCH_3$ groups because of an observed mass increase of 14 daltons. By analogy, acetylation can identify available amino groups and the corresponding N-terminal fragment ions. It is interesting that the methylation of TCT was not complete: mono- and dimethyl products were observed in addition to the trimethyl ester. Since this observation has also been made when subjecting a variety of peptidoglycan molecules to similar reaction conditions (Martin et al., 1984), we sought to study the matter further. In particular, the dimethyl TCT ester was the predominant methylation product (Table I). By investigating the fragment ions in the FAB mass spectrum of this product (data not shown), we determined that most of the dimethyl species consisted of TCT esterified at the glutamic acid and the terminal alanine carboxyl moieties. The C-terminal fragment ions of TCT at m/z 747, 719, 446, and 391 (Figure 3) exhibited an increase in mass of 28 daltons in the TCT dimethyl ester. This indicates that methylation occurred at two locations in the peptide portion of the molecule distal to the N-terminal alanine. A 14-dalton mass increase was observed for the N-terminal TCT fragment ions m/z 606, 678, and 850 as well as a 28-dalton observed mass increase for m/z 877 in the mass spectrum of the TCT dimethyl ester. This implies that the carboxyls of alanine and glutamic acid were preferentially methylated over the terminal side-chain carboxyl of diaminopimelic acid. To some extent this may be explained by the unique chemical environment of the diaminopimelic acid –COOH group, which may have reduced reactivity due to the proximity of an amino function (see Figure 3). These data are consistent with the previous observation that the dimethyl ester is the predominant peptidoglycan methylation product (Martin et al., 1984).

Discussion

The primary structure of TCT is *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramylalanyl-γ-glutamyldiaminopimelylalanine. Previously we determined that TCT is a single molecule produced by *B. pertussis* and causes ciliated cell-specific pathology in respiratory epithelium (Cookson et al., 1989). In this paper we report the unambiguous structure of the molecule and in so doing firmly establish its relationship to peptidoglycan. The internal 1,6-glycoside is unusual, but it is not unique to TCT. Anhydromuramic acid has been demonstrated to be a minor constituent of the macromolecular peptidoglycan of Gram-negative organisms such as *N. gonorrhoeae* (Blundell & Perkins, 1985; Dougherty, 1985) and *E. coli* (Glauner & Schwarz, 1983; Pisabarro et al., 1985), as well as *B. pertussis* (Folkening et al., 1987). Among these organisms, however, the macromolecular peptidoglycan of *B. pertussis* is significantly less complex. More than 95% of the *B. pertussis* peptidoglycan monomer subunits are the disaccharide–tetrapeptide: *N*-acetylglucosaminyl-*N*-acetylmuramylalanylglutamyldiaminopimelylalanine (Folkening et al., 1987). Therefore, the peptidoglycan of pertussis would seem to be a particularly well-suited substrate for TCT production.

Anhydromuramic acid containing peptidoglycan fragments are liberated by *Escherichia coli* undergoing lytic phenomena induced either by bacteriophage (Taylor et al., 1975) or by β-lactam antibiotics (Kitano et al., 1986), but not by normally growing *E. coli* (Goodell & Schwarz, 1985; our unpublished observations). Growing *N. gonorrhoeae* rapidly turn over their macromolecular peptidoglycan (Hebeler & Young, 1976), and as a result they release a diversity of peptidoglycan fragments, some containing anhydromuramic acid (Sinha & Rosenthal, 1980). In contrast to these two organisms, growing *B. pertussis* has very low peptidoglycan turnover, and greater than

95% of its released peptidoglycan is TCT (Rosenthal et al., 1987). This suggests that *B. pertussis* may be uniquely evolved to produce TCT.

The structure of TCT, particularly the 1,6-anhydromuramic acid, indicates that it is a product of a muramidase-like enzyme acting upon the macromolecular peptidoglycan of *B. pertussis* organisms. Indeed, proteins defined as murein:murein transglycosylases have been purified from both membrane and cytosolic fractions of *E. coli* (Holtje et al., 1975; Mett et al., 1980). These enzymes cleave glycosidic bonds found in peptidoglycan between *N*-acetylmuramic acid and *N*-acetylglucosamine to form intramolecular 1,6-anhydro-*N*-acetylmuramyl bonds. Furthermore, partially purified preparations of this enzyme activity are capable of degrading purified macromolecular peptidoglycan from *N. gonorrhoeae* into subunits identical with TCT (Martin et al., 1987). This indicates a role for a transglycosylase function in TCT production by *B. pertussis*. Considering the observations mentioned above, we suggest that the peptidoglycan of *B. pertussis* probably performs normal cell wall type structural roles for the bacteria but is also an unusually appropriate substrate for producing its ciliated cell toxin, TCT. In addition, the murein degradation machinery of *B. pertussis* is capable of producing toxin in an economical fashion compatible with, or even as a function of, bacterial replication and cell growth. Because the elaboration of this toxin would seem to rely upon basic yet important cell processes and functions, TCT may represent an ancestral pathogenic mechanism—a virulence factor much simpler in structure and synthesis that conventional toxins.

In broader terms, this suggests that TCT or TCT-like molecules may participate in causing pathology associated with infections by various mucosal pathogens. For example, anhydromuramic acid containing disaccharide peptides released by *N. gonorrhoeae* (Sinha & Rosenthal, 1980; Rosenthal, 1979) cause a TCT-like destruction of ciliated cells in Fallopian tube epithelium (Melly et al., 1984). Because the active preparation contained predominantly (80%) disaccharide–tetrapeptide identical with TCT, this observation supports the role that TCT plays in damaging epithelium. We have also examined the three other members of the *Bordetella* genus for the ability to produce TCT. The three species each infect different warm-blooded hosts, causing respiratory illnesses very similar to pertussis and virtually identical ciliated cell-specific pathology. Representative strains from all members of the *Bordetella* genus produce TCT (Cookson & Goldman, 1987; Gentry-Weeks et al., 1988), suggesting that TCT is an evolutionarily conserved virulence determinant of these organisms. Currently we are investigating other mucosal pathogens for the ability to produce TCT as well as analyzing the structural basis of its toxicity for ciliated epithelium.

Elucidation of the primary structure of TCT not only reveals interesting information about *B. pertussis* physiology, but it also imparts a number of implications concerning biological activity of the molecule. Because the structural features define it as a glycopeptide specifically containing muramic acid, TCT joins a group of related muramyl peptides in forming a family of molecules with diverse biological activities. A large body of work in various biological systems describe the activities of these molecules which include adjuvanticity, arthritogenicity, stimulation of leukocytes to produce IL-1, and somnogenicity [for reviews see Kotani et al. (1986), Adam and Lederer (1984), and Chedid (1983)]. Among the interesting activities of muramyl peptides, some possess neurological activity in their ability to induce slow-wave sleep when inoculated into a lateral cerebral ventricle of experimental animals (Krueger et al., 1984). Active material was first purified from human urine and called FS_u or factor S (Krueger et al., 1982). Using HPLC and FABMS, the primary component of the active preparation was identified as a disaccharide–tetrapeptide identical with TCT (Martin et al., 1984). In addition, muramyl peptides identical with TCT produced by enzymatic digestion of purified *N. gonorrhoeae* peptidoglycan confirm the neuromodulating ability of the molecule when tested in the same system (Krueger et al., 1987). Because FS_u (TCT) accumulates in the cerebrospinal fluid of sleep-deprived animals (Pappenheimer et al., 1975), it has been postulated that muramyl peptides of bacterial origin play a role in normal regulation of mammalian sleep (Karnovsky, 1986).

It is our conclusion that TCT has the capacity to display varied biological activities depending upon the host cell environment in which it is presented. Accordingly, the clinical symptomatology of gonococcal infections does not resemble whooping cough because different organ systems harbor the infectious agents. However, since the toxin is delivered at similar mucosal surfaces, the destructive capacity of the molecule causes similar ciliated cell-specific pathology. Because the work of our group and the work of others have shown that TCT not only affects host cell activity in vitro but is actually present in host tissue in vivo, it is tempting to speculate that this toxin may have as yet undiscovered roles in active pertussis infections.

It is also noteworthy that pertussis vaccination is associated with the development of various forms of neuropathy, sometimes with devastating sequelae (Miller et al., 1981). Since the vaccine is a whole cell type vaccine, the presence of TCT in these preparations would not be unexpected. As mentioned above, muramyl peptides have been shown to have immunomodulating capacity, particularly in their ability to act as adjuvants. Furthermore, a recent hypothesis by Westall and Root-Bernstein suggests that postvaccinal neuropathies may be the result of a dual-antigen-induced autoimmune response (Westall & Root-Bernstein, 1986). According to the theory, one of those antigens must be structurally complementary to a component of the nervous system. Muramyl peptides are molecularly complementary to a portion of myelin basic protein, a central nervous system component (Westall & Root-Bernstein, 1983). Therefore, the structure of TCT implicates it as a potent factor in this model of autoimmune complications to vaccination. We are presently exploring the possibility that TCT, a rather ubiquitous molecule capable of inducing diverse responses from eukaryotic cells, is an active component of pertussis vaccine.

Registry No. TCT, 118013-66-4.

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Molecular Cloning of cDNA for Sarcocystatin A and Analysis of the Expression of the Sarcocystatin A Gene during Development of *Sarcophaga peregrina*[†,‡]

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ABSTRACT: Sarcocystatin A is a cysteine proteinase inhibitor purified from the hemolymph of *Sarcophaga peregrina* larvae [Suzuki, T., & Natori, S. (1985) *J. Biol. Chem. 260*, 5115–5120]. We isolated a cDNA clone for sarcocystatin A and analyzed the structure and expression of the sarcocystatin A gene. Sarcocystatin A consists of 102 amino acid residues. Significant homology was found between amino acid sequences of sarcocystatin A and other mammalian cystatins, and highly conserved sequences among mammalian cystatins were also found in sarcocystatin A. Using cloned cDNA as a probe, we investigated expression of the sarcocystatin A gene during the development of *Sarcophaga*. Results showed that this gene was transiently activated in the very early embryonic stage and in the pupal stage, suggesting that sarcocystatin A participates in morphogenesis of larval and adult structures of *Sarcophaga*.

Low molecular mass cysteine proteinase inhibitors, termed cystatins, have been found in various mammalian tissues and sera (Brzin et al., 1983, 1984; Green et al., 1984; Isemura et al., 1984; Kominami et al., 1982). The physiological roles of cystatins are not clear, but these inhibitors are expected to regulate the activity of cysteine proteinases in several intracellular and extracellular biological reactions. Chicken egg white was also shown to contain a cystatin (Barrett, 1981), suggesting that cystatin plays a role in embryogenesis during animal development.

Previously we reported the purification and characterization of a low molecular mass cysteine proteinase inhibitor named sarcocystatin A from the hemolymph of *Sarcophaga peregrina* (flesh fly) larvae (Suzuki & Natori, 1985). Sarcocystatin A was found to be a mixture of sarcocystatin A_α and A_β in a

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