

***Bordetella pertussis* tracheal cytotoxin and other muramyl peptides: Distinct structure–activity relationships for respiratory epithelial cytopathology**

(muramyl dipeptide/peptidoglycan/diaminopimelic acid/bacterial toxins/pertussis)

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ABSTRACT Tracheal cytotoxin (TCT) is a disaccharide-tetrapeptide released by *Bordetella pertussis*, the causative agent of pertussis (whooping cough). We have previously determined the structure of TCT to be GlcNAc-1,6-anhydro-MurNAc-L-Ala- γ -D-Glu-meso-A₂pm-D-Ala, where MurNAc = *N*-acetylmuramic acid and A₂pm = diaminopimelic acid. Purified TCT reproduces the respiratory cytopathology observed during pertussis, including ciliostasis and extrusion of ciliated cells. We have tested structural analogs of TCT for their ability to reproduce native TCT toxicity in explanted hamster tracheal tissue and hamster trachea epithelial (HTE) cell cultures. Other investigators have evaluated many of these analogs, which are muramyl or desmuramyl peptides, for muramyl peptide activities such as immunopotential, induction of slow-wave sleep, and pyrogenicity. Four desmuramyl peptides were produced in our laboratory from *B. pertussis* peptidoglycan or by chemical synthesis, including unusual peptides containing α -aminopimelic acid in place of A₂pm. Based on the relative ability of compounds to inhibit DNA synthesis in HTE cells, truncated analogs lacking A₂pm entirely or lacking only the side-chain amine or carboxyl group of A₂pm were less active than TCT by a factor of at least 1000. All active analogs included a native or near-native peptide moiety, independent of the presence, absence, or substitution of the sugar moiety. We conclude that the structural requirements for TCT toxicity differ considerably from those for most other muramyl peptide activities, in that the disaccharide moiety is irrelevant for toxicity and both the free amino and carboxyl groups of the A₂pm side chain are required for activity.

Tracheal cytotoxin (TCT) is a low molecular weight glycopeptide released during logarithmic-phase growth of *Bordetella pertussis*. It is the only *B. pertussis* product that reproduces the respiratory cytopathology observed during pertussis (whooping cough) (1). This pathology includes ciliostasis and specific extrusion of ciliated cells from the respiratory epithelium. In the absence of ciliary activity, coughing becomes the only way to clear the airways of accumulating mucus, bacteria, and inflammatory debris. Thus, TCT-mediated destruction of ciliated cells may trigger the violent coughing episodes symptomatic of pertussis. In addition, the absence of a ciliary clearance mechanism predisposes patients to secondary pulmonary infections, the primary cause of pertussis mortality (2).

We have previously reported the purification of TCT by reversed-phase HPLC (RP-HPLC) and determined its structure by fast atom bombardment (FAB)-MS to be GlcNAc-1,6-anhydro-MurNAc-L-Ala- γ -D-Glu-meso-A₂pm-D-Ala

(Fig. 1) (3), where MurNAc is *N*-acetylmuramic acid and A₂pm is diaminopimelic acid. TCT belongs to a family of compounds known as muramyl peptides, which have many biological activities including adjuvanticity, somnogenicity, and pyrogenicity (4–6). Naturally occurring muramyl peptides are of bacterial origin; they are fragments of peptidoglycan, the polymeric component that confers structural rigidity to the cell wall. Muramyl peptides are released during growth of *Bordetella* Spp. (7, 8) and *Neisseria gonorrhoeae* (9) and during the processing of whole bacteria by macrophages (10, 11). Normal bacterial flora are also the source of the slow-wave sleep-promoting factor FS₁₀, a muramyl peptide present in mammalian brain tissue and identical in structure to TCT (12–14). The most well-known muramyl peptide is a synthetic analog, muramyl dipeptide (MDP; MurNAc-L-Ala-D-Glu-NH₂) (Fig. 1), the minimal structure responsible for the immunopotential properties of Freund's complete adjuvant (15).

In this study, we have defined several structural requirements for TCT toxicity in respiratory epithelium. Our results show that the sugar moiety of TCT is irrelevant for toxicity and that both the amino and carboxyl groups of the A₂pm side chain are necessary for toxicity. These findings differ markedly from structure–activity correlations for muramyl peptides in studies of slow-wave sleep enhancement, pyrogenicity, and immunoactivity (see *Discussion*).

EXPERIMENTAL PROCEDURES

Preparation of TCT. TCT was purified from the culture supernatant of *B. pertussis* strain Tohama I or III as described (18). Briefly, a crude TCT-containing fraction of culture supernatant was obtained by sequential solid-phase extractions. This material was subjected to RP-HPLC with an Aquapore octyl column and gradient elution with MeOH in a triethylamine/acetate-buffered mobile phase. The fraction containing TCT was further purified by RP-HPLC on the same type of column, and gradient elution was with acetonitrile in a CF₃COOH-buffered mobile phase. TCT was identified, quantified, and shown to be free of peptidic contaminants by analysis of amino acid and amino sugar

Abbreviations: TCT, tracheal cytotoxin; A₂pm, diaminopimelic acid; α Apm, α -aminopimelic acid; GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid; HTE cells, hamster trachea epithelial cells; IL-1, interleukin 1; RP-HPLC, reversed-phase HPLC; FAB-MS, fast atom bombardment MS; MDP, muramyl dipeptide (MurNAc-L-Ala-D-Glu-NH₂); GMDP, glucosylmuramyl dipeptide (GlcNAc-MurNAc-L-Ala-D-Glu-NH₂); LacAEA₂pmA, D-lactyl-L-Ala- γ -D-Glu-meso-A₂pm-D-Ala; LacAEKA, D-lactyl-L-Ala- γ -D-Glu-L-Lys-D-Ala; LacAE(L) α ApmA or LacAE(D) α ApmA, D-Lac-L-Ala- γ -D-Glu-(L or D)- α Apm-D-Ala.

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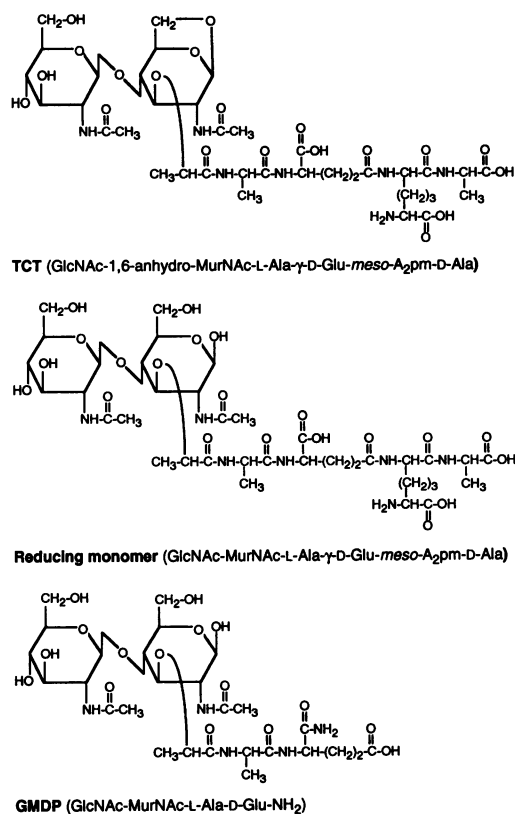


FIG. 1. TCT and analogs containing sugar moieties.

content. Preparation of TCT in this manner has been shown to yield material that has no detectable endotoxin.

Synthesis of D-Lactyl-L-Ala- γ -D-Glu-*meso*-A₂pm-D-Ala (LacAEA₂pmA). Avirulent *B. pertussis* Tohama III was harvested from cultures by centrifugation. Crude peptidoglycan was prepared from the bacteria and digested with lysozyme to produce reducing monomer (Fig. 1) (19). Lysozyme was removed from the solution by reversed-phase extraction as for TCT (18), and the eluate was evaporated and treated with ammonium hydroxide to eliminate the sugar moiety (20). The resulting lactyl tetrapeptide was purified by RP-HPLC under the same conditions as for TCT. The product was confirmed by FAB-MS ($M+H = 534$) and amino acid analysis.

Synthesis of D-Lactyl-L-Ala- γ -D-Glu-L-Lys-D-Ala (LacA-EKA). Boc-D-Ala (Boc *tert*-butoxycarbonyl) was coupled to Merrifield resin (Sigma) in dimethylformamide (DMF) by using KF (21). In subsequent coupling cycles, 4 equivalents of *N*^α-Boc-*N*^ε-(2-chlorocarbonyl)-L-Lys, Boc-D-Glu α -benzyl ester, Boc-L-Ala, and D-lactic acid were coupled in DMF by using either diisopropylcarbodiimide and hydroxybenzotriazole or benzotriazolyl-*N*-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP; Sigma) as the linking agent, with excess diisopropylethylamine. The peptide was simultaneously removed from the resin and deprotected by treatment at 0°C with HF containing 10% (vol/vol) anisole. Purification was by RP-HPLC on a Beckman Ultrasphere PTH column with a CF₃COOH-based mobile phase. The purified product was confirmed to be LacA-EKA by FAB-MS ($M+H = 490$) and amino acid analysis.

Synthesis of α -Aminopimelic Acid (α Apm) Derivatives D-Lactyl-L-Ala- γ -D-Glu-L- α Apm-D-Ala and D-Lactyl-L-Ala- γ -D-Glu-D- α Apm-D-Ala [LacAE(L) α ApmA and LacAE(D)- α ApmA]. DL- α Apm ϵ -benzyl ester [NMR in (CH₃)₂SO-*d*₆: δ _H 4.1, 5.3, 7.8; m.p. = 197°C (decomposes)] was prepared from DL- α Apm and benzyl bromide (22). Treatment with Boc-pyrocyanate yielded Boc-DL- α Apm ϵ -benzyl ester, which was used as the racemic oil. The peptide was synthesized as

above, except [2-(1H-benzotriazol-1-yl)]-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU; Richelieu Biotechnologies, Quebec, Canada) was the coupling agent. Following cleavage from the resin, the peptides were purified by RP-HPLC under conditions similar to those used for the purification of TCT. The products were confirmed by FAB-MS ($M+H = 519$) and amino acid analysis.

Amino Acid Analyses. Triplicate peptide samples of ≈ 1 nmol were hydrolyzed with gaseous HCl in a sealed chamber for 24 hr at 110°C. Triplicate amino acid standards of 1.0 nmol each were prepared by using amino acid standard H (Pierce); triplicate A₂pm and α Apm standards were prepared separately. Dry samples and standards were neutralized and derivatized with phenylisothiocyanate (Pierce). Derivatized amino acids were analyzed by RP-HPLC on a Beckman Ultrasphere PTH column. Quantitation of peptides was based on comparison of average peak areas for each amino acid of the sample and standards.

Chiral Analyses. The synthetic peptides LacA-EKA, LacAE(L) α ApmA, and LacAE(D) α ApmA were confirmed to be free of diastereomeric peptides within the limits of detection of the following analysis. Peptide samples of ≈ 5 nmol were hydrolyzed for 4 hr as for amino acid analysis. Hydrolyzed samples and standards of enantiomerically pure amino acids were derivatized with Marfey's reagent [1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Pierce)], with modifications to the published procedure (23) to accommodate the small sample size. Analysis was by RP-HPLC on a Beckman Ultrasphere PTH column with MeOH/MeCN/0.02 M NaOAc, 2:1:7 (vol/vol), pH 4.0 (24) using a MeOH gradient. As previously reported (24), all D-amino acids were observed to be retained longer than their L counterparts; since no standards were available, L- and D- α Apm were assumed to follow this pattern.

The chirality of lactic acid in lactyl peptides was confirmed by using lactic dehydrogenase (Sigma), which converts lactic acid to pyruvate with concomitant reduction of NAD to NADH, detectable at 340 nm. Peptide samples (20 nmol) were hydrolyzed in 3 M HCl for 8 hr at 100°C (20) and then evaporated and neutralized with MeOH/H₂O/triethylamine, 2:2:1 (vol/vol). Reactions were done as described in the Sigma enzymatic assay for L-(+)-lactic acid. Parallel samples were assayed with L- and D-lactic acid dehydrogenase to determine the presence of each enantiomer.

FAB-MS. Mass spectra were obtained on a VG 40-250T triple quadrupole spectrometer fitted with an 8-kV Xenon neutral atom gun. One microgram or less of the sample was deposited on the probe tip along with 1–2 μ l of glycerol/thioglycerol/heptafluorobutyric acid, 1:1:5 (vol/vol), matrix. Single-scan spectra were obtained on all samples. Multichannel analysis techniques were used to increase signal intensities on selected samples.

Determination of Toxicity by DNA Synthesis Inhibition in Hamster Trachea Epithelial (HTE) Cells. HTE cells (25), which are a proliferating, nontransformed, homogeneous cell culture, were used in this assay (18). HTE cells in microtiter wells were synchronized in G₀ by serum starvation for 24 hr, exposed to a range of concentrations of the test compound for 4 hr in the presence of bovine serum albumin as a protein protectant/carrier, and then serum-stimulated in the presence of radiolabeled thymidine. After 26 hr, DNA from the cells was precipitated onto cotton swabs with CCl₃COOH, and the incorporation of radiolabel was determined. Toxicities of TCT analogs are reported relative to TCT in a given experiment.

We have recently improved toxicity assays in HTE cells by culturing the cells in medium containing epidermal growth factor (EGF). HTE cells are grown in the presence of 10 ng of EGF per ml and were serum-starved in the presence of 0.01 ng of EGF per ml. Under these conditions, HTE cells exhibit

half-maximal DNA synthesis inhibition at TCT concentrations 1/10th to 1/100th those previously reported (18). The mechanism of this increased sensitivity is not known.

Determination of Toxicity in Hamster Tracheal Rings. Assays of toxicity in hamster tracheal rings were performed as described (18). Briefly, tracheas were dissected from male Golden Syrian hamsters and were sectioned into rings. Tracheal rings were cultured in nutrient mixture F-12 (GIBCO) with 5 mM Hepes (pH 7.3) at 37°C. Test compounds were added to this culture medium at several concentrations from 1.0 to 30 μ M and examined daily by light microscopy for ciliated cell damage, which appears as arcs devoid of ciliary activity. Toxicity is defined as the ability to damage ciliated cells within 5 days of exposure.

Sources of Material. FK-156 and FK-565 were a gift from Masashi Hashimoto (Fujisawa Pharmaceutical, Ibaraki, Japan). GlcNAc-MurNAc-L-Ala- γ -D-Glu-*meso*-A₂pm-D-Ala (reducing monomer) was a gift from Raoul S. Rosenthal (Indiana University School of Medicine). MDP was purchased from Sigma. GlcNAc-MurNAc-L-Ala-D-Glu-NH₂ [glucosylmuramyl dipeptide (GMDP), where Glu-NH₂ is isoglutamine] was purchased from C-C Biotech (Poway, CA).

RESULTS

TCT was originally isolated as the component of *B. pertussis* culture supernatant responsible for the destruction of the ciliated cells in respiratory epithelium (1). This activity, when assayed in hamster tracheal organ cultures, appears as ciliostasis and the extrusion of ciliated cells from the epithelium. A less dramatic but more quantitative evaluation of TCT activity is possible with HTE cells, a proliferating monolayer culture derived from hamster tracheal tissue (25). We have suggested (1) that HTE cell growth inhibition by TCT may reflect a secondary *in vivo* effect on basal cells, which normally divide and differentiate to replace lost ciliated cells. The response by HTE cells is measured as inhibition of DNA synthesis and is dose-dependent. Half-maximal inhibition occurs at 3.5 to 35 nM TCT under the current cell-culture conditions.

To determine which portions of TCT are responsible for activity, we obtained or produced a number of TCT analogs. Because some of these analogs have been evaluated for biological activities in other systems, we were able to determine whether structure-activity relationships derived in respiratory epithelial cells differed from those derived for other muramyl peptide activities. We reasoned that the most probable structures to be recognized by a putative TCT receptor/target site might be the two components of TCT that are unique to peptidoglycan: muramic acid and A₂pm. Consequently, these moieties were the major targets of our structural investigation. The tested TCT analogs may be divided into four structural categories: (i) those that contain a sugar moiety and a full or truncated peptide, (ii) peptide-only analogs that contain A₂pm, (iii) peptide-only analogs with substitutions for A₂pm, and (iv) the free amino acid A₂pm.

Analog Containing Sugars (Table 1, Fig. 1). The reducing monomer differs from TCT only in the muramic acid moiety, which lacks the dehydration between carbons 1 and 6 of the sugar ring. This structural difference did not alter toxicity in HTE cells or tracheal rings. GMDP has a disaccharide moiety identical to the reducing monomer but contains a truncated peptide moiety. MDP is similar to GMDP but lacks GlcNAc. Both GMDP and MDP were less toxic than TCT by at least a factor of 500 in DNA synthesis-inhibition assays with HTE cells. MDP was also nontoxic for hamster tracheal rings at concentrations up to 30 μ M, 30 times the lowest toxic dose of TCT. Data from these analogs indicated that the anhydro or reducing state of muramic acid was irrelevant for toxicity and that some portion of the peptide that is lacking in GMDP (and also in MDP) was needed for activity.

Table 1. Toxicity of muramyl peptide analogs of TCT

Test compound	Bioassay system	
	HTE cells	Tracheal rings
TCT	1	+
Reducing monomer	1	+
GMDP	<0.001	ND
MDP	0.002	-

Toxicity of compounds was assessed in HTE cells and in hamster tracheal rings. Toxicity of analogs for HTE cells is calculated as a ratio relative to TCT, based on the concentrations eliciting half-maximal toxicity in a representative experiment. For toxicity in hamster tracheal rings, + indicates ciliostasis and extrusion of ciliated cells, and - denotes no difference from control. ND, not determined. Tracheal rings were treated with \leq 30 μ M test compound. See Fig. 1 for structures.

Peptide Analogs Containing A₂pm (Table 2, Fig. 2). These analogs contain full-length peptide moieties including A₂pm and differ from TCT primarily in the N-terminal capping group. Two analogs, LacAEA₂pmA and FK-156, lack the disaccharide while retaining the D-lactyl group from MurNAc. In the third analog, FK-565, a heptanoyl group takes the place of the lactyl group. Each of these peptides reproduced TCT toxicity in HTE cells within experimental error. LacAEA₂pmA toxicity for tracheal rings was also indistinguishable from that of TCT. Our results indicate that these analogs contain the structural features of TCT that are sufficient for full toxicity. Comparison with the results from GMDP suggested that some elements of the C-terminal dipeptide might be required for activity.

Peptide Analogs with Substitutions for A₂pm (Table 3, Fig. 3). We suspected that A₂pm, unique to bacteria, might be a key feature for recognition of TCT and that alteration of the side chain might affect toxicity. To test this hypothesis, we synthesized LacAEKA and LacAE α ApmA (two diastereomers), which contain amino acid substitutions for A₂pm. These substitutions result in peptides that differ from LacAEA₂pmA only in the absence of either the amine or carboxyl of the A₂pm side chain. Each of these analogs were less toxic for HTE cells by at least a factor of 1000 than TCT. Fig. 4 shows the dramatic difference in activity between the native lactyl tetrapeptide LacAEA₂pmA and LacAEKA. In tracheal ring assays, 30 μ M LacAEKA was also inactive under conditions in which 1.0 μ M TCT was toxic. To assess whether these analogs might be binding nonproductively to the TCT binding site of a putative TCT receptor, we compared TCT dose-response curves obtained in the presence and absence of the inactive analogs. For both LacAEKA and LacAE(L) α ApmA, TCT toxicity in HTE cells was unaffected by the presence of 50 μ M analog, suggesting that these peptides do not compete for binding to the putative TCT receptor.

Stereoisomers of A₂pm. Because A₂pm seemed to be necessary for toxicity of TCT analogs, we tested A₂pm as a free amino acid, both as a mixture of three stereoisomers and as pure *meso*-A₂pm. Neither of these produced any measurable toxicity in HTE cell assays up to 50 μ M (more than 1000 times the concentration of TCT eliciting half-maximal toxicity). *meso*-A₂pm was also inactive in tracheal rings at 30 μ M,

Table 2. Toxicity of desmuramyl peptides containing *meso*-A₂pm

Test compound	Bioassay system	
	HTE cells	Tracheal rings
LacAEA ₂ pmA	0.5	+
FK-156	0.3	ND
FK-565	1	ND

See Table 1 legend and see Fig. 2 for structures of test compounds. ND, not determined.

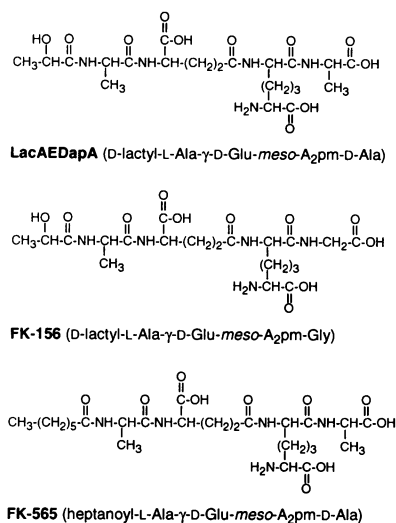


FIG. 2. Desmuramyl peptide analogs of TCT containing *meso*-A₂pm. Based on the invariant features of Gram-negative peptidoglycan (26), the L stereocenter of *meso*-A₂pm is in the peptide chain; the side chain contains a D stereocenter.

while 1.0 μ M TCT was toxic. Consequently, A₂pm was eliminated as the sole required substructure of TCT.

DISCUSSION

A large number of muramyl peptide analogs have been generated previously, particularly in attempts to enhance adjuvanticity and to understand and distinguish between various muramyl peptide activities in the immune and central nervous systems. Those studies have focused mainly on analogs containing at least one sugar and a dipeptide, MDP being the archetype. Desmuramyl peptides, muramyl peptide analogs having no sugar moiety, have been of some interest for their immunoactivity, but few studies of muramyl peptide structure-function relations have included desmuramyl peptides (for review, see ref. 5). By generating new peptidic analogs for TCT, we have been able to demonstrate that the peptide portion of TCT is sufficient for respiratory epithelial toxicity; furthermore, we have established that A₂pm plays a critical role in this activity. The evidence for these findings is discussed below, with comparisons to the structure-activity relations for muramyl and desmuramyl peptides in other systems.

We first showed that altering or removing the sugars of TCT had no apparent effect on toxicity for respiratory epithelium. The reducing monomer of *B. pertussis* peptidoglycan is as toxic as TCT in our assays. This indicates that the dehydration at carbons 1 and 6 in the muramic acid moiety of TCT is not important in toxicity. In contrast, the reducing monomer is less potent than TCT by at least a factor of 10 in slow-wave sleep enhancement (27).

We produced the lactyl peptide analog LacAEA₂pmA, which corresponds to the peptide portion of TCT, to test whether any part of the disaccharide might be important for toxicity. Removal of the sugars from the reducing monomer

Table 3. Toxicity of desmuramyl peptides containing substitutions for *meso*-A₂pm

Test compound	Bioassay system	
	HTE cells	Tracheal rings
LacAEKA	<0.001	—
LacAE(L) α ApmA	<0.002	ND
LacAE(D) α ApmA	<0.001	ND

See Table 1 legend and see Fig. 3 for structures of test compounds. ND, not determined.

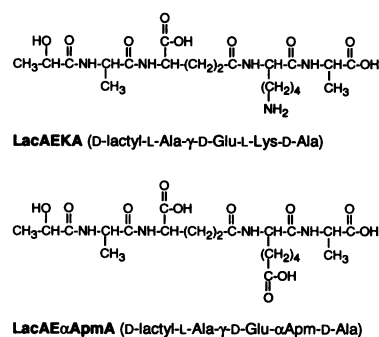


FIG. 3. Desmuramyl peptide analogs of TCT with amino acid substitutions for A₂pm. These analogs are identical to LacAEA₂pmA (see Fig. 2) except that they lack the side-chain amine or carboxyl group of A₂pm. LacAE(D) α ApmA also differs in the chirality of the α -carbon of the penultimate residue.

by base-catalyzed elimination left D-lactic acid capping the N terminus of the peptide. This analog exhibited toxicity identical to TCT in our assays. To confirm the expendability of the disaccharide, we also tested desmuramyl peptides FK-156 and FK-565. These analogs are similar to LacAEA₂pmA, except FK-156 lacks the methyl group of the C-terminal alanine and FK-565 contains heptanoic acid rather than lactic acid at its N terminus. Each of these analogs were similar to TCT in toxicity, confirming that the disaccharide moiety is irrelevant for toxicity. The activity of FK-565 also indicated that the lactyl moiety is not crucial for activity. FK-156 and FK-565 are also known to be active in immunopotentiality (28), induction of IL-1 and inhibition of IL-2 release (29), and pyrogenicity (30). Although FK-156 has also been reported to enhance slow-wave sleep (30), its activity is comparable to that of MDP, which is 1/50th as active as TCT (FS₅₀) in those assays (31). That FK-156 and MDP are both less somnogenic than TCT is further evidence that the sugar moiety plays a role in slow-wave sleep induction, even though this portion is unimportant for respiratory-tract damage.

Several TCT analogs provide evidence that the C-terminal dipeptide of TCT plays a crucial role in toxicity for the respiratory epithelium. GMDP is \approx 1/1000th as toxic as the reducing monomer for HTE cells; since the sugar portion of these two analogs is identical, this result must be due to truncation of the peptide in GMDP to only the first two amino acids. The inactivity of MDP is also consistent with the requirement for C-terminal peptide elements. Therefore,

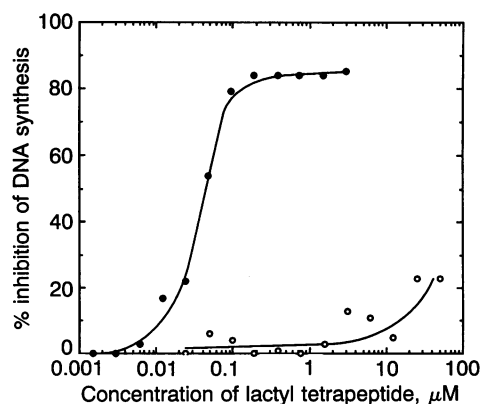


FIG. 4. Representative dose-response curves for LacAEA₂pmA and LacAEKA. Toxicity of LacAEA₂pmA (●) and LacAEKA (○) for HTE cells (for structures, see Figs. 2 and 3). Similar data were obtained with TCT and other analogs; these dose-response curves fell into two potency classes (see Tables 1–3), represented by the two dose-response curves illustrated here.

some portions of A₂pm or the C-terminal alanine, or both, must be necessary for pertussis-related toxicity.

With the TCT analogs LacAEKA and LacAE α ApmA, we have probed the importance of each of the functional groups of the A₂pm side chain by substituting L-Lys or L- α Apm for A₂pm. These analogs differ from LacAEA₂pmA by the absence of either the carboxyl or amino group of the side chain. The lysine-containing analog is biologically relevant, since it corresponds to the lactyl peptide moiety of peptidoglycan from Gram-positive bacteria. Each of these analogs has drastically reduced toxicity (<1/1000th as active as LacAEA₂pmA), indicating that the amino and carboxyl groups of the A₂pm side chain play a critical role in toxicity for respiratory epithelium. However, despite the importance of A₂pm in TCT activity, the free amino acid does not produce any measurable toxicity.

Although alterations in A₂pm sometimes produce changes in activity, A₂pm is generally not required for activity of muramyl peptides. This point is illustrated by the adjuvanticity, pyrogenicity, and somnogenicity of MDP (6, 13, 15). In contrast, the dipeptide γ -D-Glu-*meso*-A₂pm is the smallest active fragment of desmuramyl peptide FK-156 as assessed by phagocytic activity in DDY mice, stimulation of delayed-type hypersensitivity, and protective effect against *Escherichia coli* infection in mice (32). These results indicate that there are at least two different sets of structure-activity relationships to be described for muramyl and desmuramyl peptides. Since analogs with truncated peptides such as GMDP failed to reproduce TCT toxicity while peptide-only analogs containing A₂pm were fully active, our data correlate well with studies of desmuramyl peptides; this suggests that γ -D-Glu-*meso*-A₂pm may also prove to be the smallest active fragment for TCT toxicity.

In addition to the Bordetellae, one other organism, *N. gonorrhoeae*, has been shown to release TCT in significant quantities *in vitro* (9). Purified TCT damages human fallopian tubes in organ culture, causing loss of ciliated cells from the mucosa that closely resembles damage resulting from gonococcal infection (33). In addition, the reducing monomer of peptidoglycan from *N. gonorrhoeae*, identical to the reducing monomer from *B. pertussis*, reproduces this toxicity. Neither MDP nor GlcNAc were able to elicit any toxicity in this system. Thus, it appears that two pathogenic bacterial genera that colonize and destroy different ciliated epithelia have evolved identical mechanisms: the production and release of muramyl peptide toxins. In addition, the similarity between the specificity of cellular damage in the respiratory tract and fallopian tube and the consistent structure-activity correlations derived in these systems suggest that the toxins function through a common pathway.

Although our studies do not prove the presence of a receptor for TCT in the respiratory epithelium, the rigid requirements for structure imply that TCT activity is dependent on some specific host cell-recognition mechanism. Furthermore, the putative TCT receptor/target site in HTE cells and intact tracheal tissue appears to be the same, based on identical structure-activity relationships derived from these assays. This is consistent with the use of HTE cells, despite their lack of cilia, as a model system for studying the intracellular events triggered by TCT *in vivo*.

It is interesting that compounds containing muramic acid or A₂pm, or both, which are moieties of strictly bacterial origin, are recognized by a variety of eukaryotic cell types. Although cells of the immune system and epithelial cells might be expected to be responsive to bacterial products, it is more surprising to find receptors recognizing these products in brain tissue. That muramyl peptides may act through a family of related receptors is suggested by evidence that several muramyl peptide and desmuramyl peptide activities share a common mediator, the cytokine IL-1. Slow-wave sleep is

enhanced and brain temperature is increased by cerebral intraventricular infusion of IL-1 (17). MDP and several other adjuvant-active analogs induce high levels of membrane-bound IL-1 in mouse peritoneal macrophages; in contrast, adjuvant-inactive analogs, such as MDP containing D-Ala, are unable to trigger IL-1 production (16). FK-565 and FK-156 also induce the release of IL-1 from lipopolysaccharide-stimulated rat peritoneal exudate cells (29). In keeping with this pattern, we also have preliminary evidence that IL-1 may also mediate TCT toxicity in respiratory epithelial cells (34).

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