INFECTION AND IMMUNITY, Sept. 1987, p. 2117–2120
0019-9567/87/092117-04$02.00/0
Copyright © 1987, American Society for Microbiology
Vol. 55, No. 9

Major Fragment of Soluble Peptidoglycan Released from Growing *Bordetella pertussis* Is Tracheal Cytotoxin

RAOUL S. ROSENTHAL,[1]* WALLACE NOGAMI,[2] BRAD T. COOKSON,[3] WILLIAM E. GOLDMAN,[3] AND W. JOSEPH FOLKENING[1]

Departments of Microbiology and Immunology[1] and Surgery,[2] Indiana University School of Medicine, Indianapolis, Indiana 46223, and Department of Microbiology and Immunology, Washington University School of Medicine, St. Louis, Missouri 63110[3]

Received 6 April 1987/Accepted 6 June 1987

***Bordetella pertussis* is known to release a factor which promotes the loss of ciliated respiratory epithelium and copurifies with a soluble peptidoglycan (PG) fragment termed tracheal cytotoxin (TCT). The objective of this study was to determine whether pertussis organisms turn over and release PG derivatives in addition to TCT. *B. pertussis* Tohama (phase III) was grown in liquid Stainer-Scholte medium containing [^{3}H]diaminopimelic acid (DAP) to label PG specifically, washed to remove free label, and suspended in fresh medium without [^{3}H]DAP. Molecular sieve chromatography of supernatants obtained from such cultures revealed a single included peak of ^{3}H, the elution volume of which corresponded roughly to a disaccharide peptide monomer standard (ca. 10^3 daltons). This material (i) contained [^{3}H]DAP in acid-hydrolyzable linkage, (ii) comigrated with 1,6-anhydro-*N*-acetylmuramic acid-containing disaccharide peptides on paper chromatography, (iii) was resistant to degradation by mild alkali, and (iv) was indistinguishable from authentic TCT by high-voltage paper electrophoresis and two reversed-phase high-performance liquid chromatography systems. Together, the data suggest that *B. pertussis* releases a markedly homogeneous set of PG fragments, consisting principally of TCT, and that TCT is possibly a nonreducing, anhydromuramic acid-containing fragment or a cyclic PG derivative.**

Although once regarded as merely a biologically inert macromolecule required for the structural integrity of bacteria, peptidoglycan (PG) and PG fragments are now generating considerable interest because of their likely roles in disease and health. Among several model systems available to explore the biological relevance of PG, *Neisseria gonorrhoeae* has been exploited by one of our laboratories as a model bacterium in which PG-host interactions are probably extensive during natural infections. We have devoted considerable attention to identifying and isolating physiologically realistic gonococcal PG fragments (8, 12–17, 19) and have found that, collectively, these compounds do trigger diverse biological reactions, e.g., complement activation (10) and arthritogenicity (2), consistent with the notion that PG does indeed contribute to gonococcal pathogenesis.

Of particular interest to this study was the previous collaborative finding with Melly and McGee (9) that gonococcal PG fragments damage human fallopian tube mucosa. For example, reducing disaccharide peptide monomers, as well as nonreducing, 1,6-anhydro-*N*-acetylmuramic acid-containing disaccharide peptide monomers, were found to promote loss of ciliated cells from the epithelial surface of fallopian tube pieces in organ culture. A remarkably analogous story was provided by Goldman and colleagues (5–7), who found that a purified tracheal cytotoxin (TCT), released from *Bordetella pertussis* and capable of promoting loss of ciliated cells from hamster tracheal epithelium, was a low-molecular-mass PG derivative (about 1,400 daltons). Whether or not other PG fragments also were released from pertussis organisms was not determined. Together, these findings raise the intriguing hypothesis that release of cytotoxic PG fragments might be a common denominator among some bacteria for which pathogenesis depends on colonization of ciliated mucosal surfaces.

Our aim is to define the structural relationships which govern these functional similarities between gonococcal and pertussis PG. This study examined culture supernatants of [^{3}H]diaminopimelic acid (DAP)-labeled *B. pertussis* to determine the extent of PG release and to characterize comprehensively the soluble PG fragments released.

* Corresponding author.

MATERIALS AND METHODS

Bacteria and growth media. *B. pertussis* Tohama phase III was stored at −70°C in modified Stainer-Scholte medium (SSM; 18) plus skim milk and passaged on SSM agar containing sheep blood as described previously (3). Liquid medium was modified SSM containing 0.1% (wt/vol) glutamic acid (3). *B. pertussis* Tohama phase III, similar to its phase I counterparts and other *Bordetella* species so far tested, is known to release TCT during growth (5).

Turnover and release of PG. Experiments to label cultures and examine the turnover and release of PG used a pulse-chase type of design. *B. pertussis* was inoculated at a starting density of approximately 10^8 bacteria per ml into 300-ml nephaloculture flasks containing 20 ml of liquid SSM and incubated at 36.5°C on a rotary shaker. After about one-half generation, [^{3}H]DAP, a specific precursor of PG in *B. pertussis* (3), was added to a concentration of 12.5 μCi/ml. The culture was incubated for two to three additional generations (generation time, ca. 5 h), washed extensively to remove free extracellular label, and inoculated into liquid SSM lacking DAP. After 5 h, the culture supernatant was obtained by centrifugation, filtered through a 0.45-μm (pore size) membrane, heated at 80°C for 1 h, concentrated by

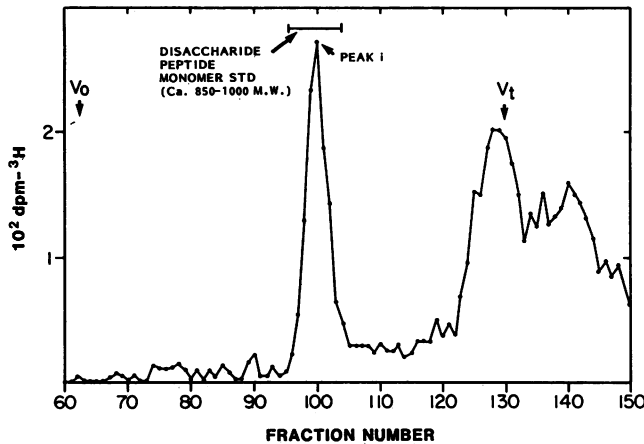

FIG. 1. Gel filtration on connected columns of Sephadex G-50 and G-25 of [^{3}H]DAP-labeled material released by growing *B. pertussis*. The elution volume of the disaccharide peptide monomer standard (STD) derived from gonococcal PG, the voided volume (V_0), and the total volume (V_t) are indicated. M.W., molecular weight.

lyophilization, and used to characterize soluble PG fragments released into the medium. In addition, samples were taken at various times during the chase to measure turnover of sodium dodecyl sulfate-insoluble PG (12). Preliminary experiments demonstrated that, at the beginning of the chase period, all of the [^{3}H]DAP had already been incorporated into trichloroacetic acid-insoluble macromolecules exclusively, indicating that any soluble PG fragments found in the medium likely would have been derived from the insoluble PG matrix.

Analytical procedures. The following procedures were performed as previously described: molecular sieve high-performance liquid chromatography (HPLC) and gel filtration with connected columns of TSK 3000SW and 2000SW and Sephadex G-50 and G-25, respectively (15, 19); paper chromatography in butanol-acetic acid-water (4:1:5; upper phase) of acid-hydrolyzed samples or soluble PG fragments, e.g., disaccharide peptide monomers (12, 15, 19); high-voltage paper electrophoresis in formic acid-acetic acid-water (1:4:50) at pH 1.9 (7); β elimination of reducing PG fragments by mild alkali treatment (16, 20), and quantitation of radioactivity (3, 12). PG standards (see below) and experimental samples were subjected to reversed-phase HPLC with a C_8 column (Rainin Instrument Co., Inc., Woburn, Mass.) eluted with a triethylamine- or trifluoroacetic acid-containing solvent system. These HPLC systems proved capable of resolving the numerous, i.e., 7 to 13, components making up the various classes of gonococcal PG monomers, as did the previously reported system (8) using a C_{18} column.

PG standards. Partially purified [^{3}H]TCT was obtained, as previously described (6), from supernatants of *B. pertussis* cultures grown in the presence of [^{3}H]DAP. Purified TCT standard was isolated by reversed-phase HPLC with a C_8 column. Additional PG standards obtained from *N. gonorrhoeae* or *B. pertussis* were prepared as previously described (3, 9, 12, 15, 16, 19).

RESULTS

Turnover and release of *B. pertussis* PG. Molecular sieve HPLC or gel filtration (Fig. 1) of soluble products released from growing *B. pertussis* revealed only a single included peak (peak i) containing radiolabel added as [^{3}H]DAP. This material had an elution volume that corresponded to that of PG monomer standard, suggesting a molecular mass on the order of 10^3 daltons. In addition to peak i, the culture supernatant also contained labeled material eluting at the total volume (V_t), but this material migrated on paper chromatography as free [^{3}H]DAP (with or without prior acid hydrolysis) and thus probably represented residual unincorporated DAP (Fig. 2). Paper chromatography of acid hydrolysates of peak i indicated that the ^{3}H in peak i was present as DAP exclusively (Fig. 2), confirming that peak i contained labeled PG only and not other substances which conceivably might contain metabolic products derived from [^{3}H]DAP.

Whereas soluble PG released from pertussis organisms was surprisingly uniform in size, the total amount of PG detected in supernatants was a very small fraction (less than 1%) of the macromolecular PG available in intact PG. This was consistent with turnover experiments (data not shown) which indicated that loss of sodium dodecyl sulfate-insoluble PG was undetectable at a sensitivity that could have detected a turnover rate of as little as 8% of existing PG per generation.

Radiochemical analysis of [^{3}H]DAP-labeled peak i. Paper chromatography of isolated ^{3}H-peak i (Fig. 3) indicated that most (>90%) of the radioactivity comigrated with an internal standard of ^{14}C-labeled, nonreducing anhydromuramic acid-containing monomer. The remainder eluted at positions corresponding to hydrated (reducing) disaccharide peptides. Furthermore, peak i was resistant to β elimination by mild alkali treatment (Fig. 4) under conditions in which authentic, reducing PG monomers were degraded. The NaOH resistance of peak i is consistent with the possibility that the principal soluble PG fragment released by *B. pertussis* is a compound containing a 1,6-anhydromuramic acid end. The data do not rule out the possibility that peak i is a cyclic PG derivative.

High-voltage paper electrophoresis (Fig. 5) confirmed that peak i was homogeneous on the basis of charge and also revealed that the electrophoretic mobility of peak i was identical to that of purified TCT. Peak i and TCT were also indistinguishable on the basis of retention times as determined by two different reversed-phase HPLC systems, one

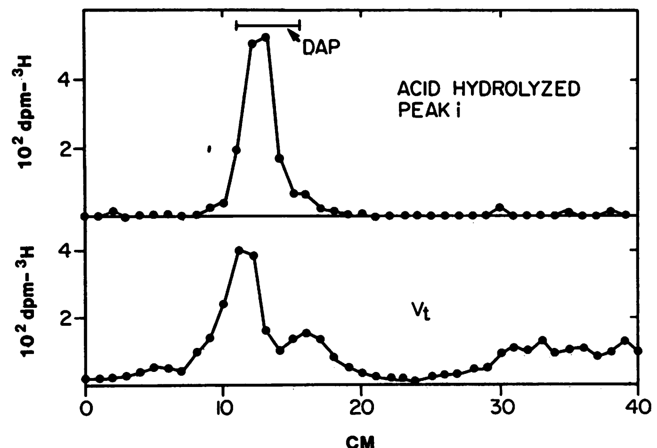

FIG. 2. Paper chromatography for 16 h in butanol-acetic acid-water (4:1:5; upper phase) of material eluting at V_t and of acid-hydrolyzed peak i derived from supernatants of [^{3}H]DAP-labeled *B. pertussis*. Migration of the DAP standard is indicated by the bar.

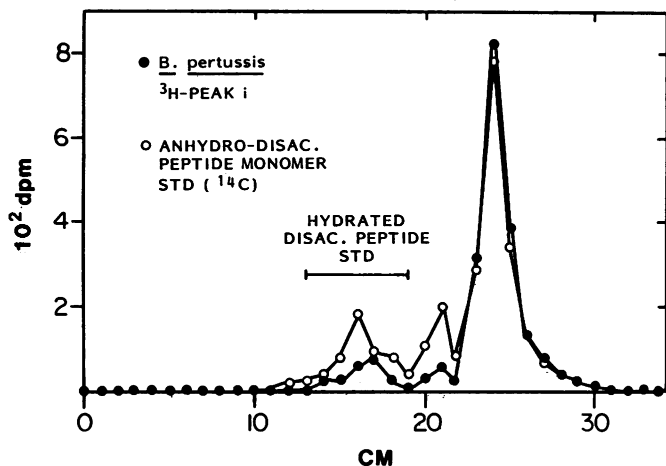

FIG. 3. Paper chromatography of [^{3}H]DAP-labeled peak i for 65 h in butanol-acetic acid-water (4:1:5; upper phase). Migrations of the hydrated disaccharide (DISAC.) peptide monomer standard (STD) and the ^{14}C-anhydromuramic acid-containing monomer (internal standard) are indicated.

of which is shown in Fig. 6. Together, these data provide strong evidence that peak i and TCT are identical.

DISCUSSION

This report documents the facts that *B. pertussis* releases a markedly homogeneous set of soluble PG products and that the predominant fragment appears structurally identical to the cytotoxic PG derivative (TCT) previously described by Goldman et al. (5–7). Thus, TCT appears to be virtually the sole PG-derived product released by *B. pertussis* in vitro.

On the basis of what is currently known regarding the chemical structure of TCT (this manuscript and references 5 through 7), it seems likely that generation of TCT depends on the hydrolysis, i.e., turnover, of PG fragments already incorporated into the existing PG matrix rather than on premature release of cytoplasmic uridine diphosphate-containing PG precursors. Nevertheless, despite the ease of detecting soluble PG with chemical properties similar to those of TCT directly in *B. pertussis* supernatants, we found no evidence for concurrent turnover of macromolecular PG. However, the total amount of soluble PG present in supernatants under our experimental conditions accounted for

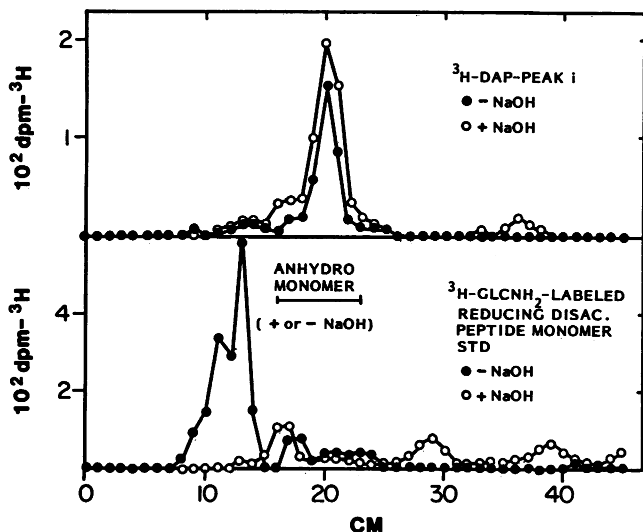

FIG. 4. Paper chromatography in butanol-acetic acid-water (4:1:5; upper phase) for 65 h of NaOH-treated (and control) peak i (top) and PG monomer standards (bottom). PG samples were treated with mild alkali to cause β elimination of peptides from PG fragments, e.g., the [^{3}H]glucosamine ($GlcNH_2$)-labeled reducing disaccharide (DISAC.) monomer standard (STD), that possess a reducing muramic acid end.

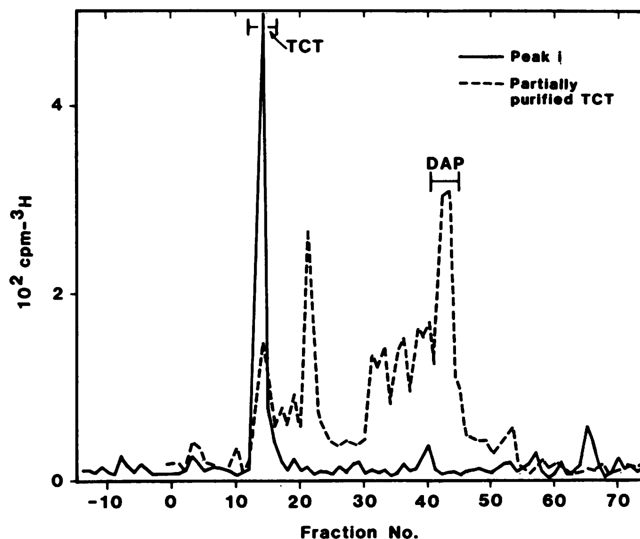

FIG. 5. High-voltage paper electrophoresis (pH 1.9) of isolated [^{3}H]DAP-labeled peak i, partially purified [^{3}H]DAP-labeled TCT, purified TCT (detected with ninhydrin), and the DAP standard (detected with ninhydrin).

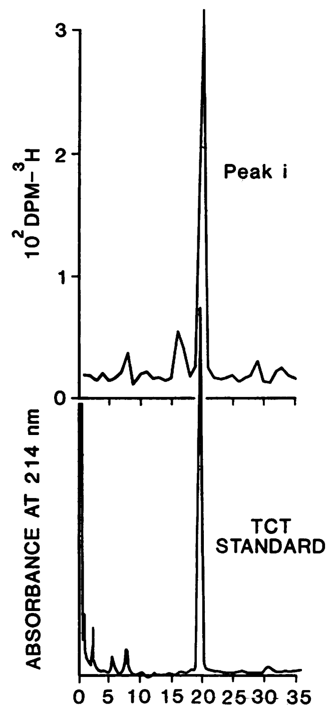

FIG. 6. Reversed-phase HPLC of isolated [^{3}H]DAP-labeled peak i and purified TCT with a trifluoroacetic acid-buffered mobile phase and gradient elution with acetonitrile.

less than 1% of macromolecular PG available in intact bacteria, a value considerably less than the capacity of our turnover experiments to detect. In fact, if the results of turnover experiments per se were the sole predictor of the biological relevance of extracellular PG, then the pathobiological potential of soluble *B. pertussis* PG (5, 7) may have gone unrecognized. We think this may be a valuable lesson in general because it makes us consider that PG might conceivably alter host-bacterium interactions, even when derived from bacteria in which the rate of PG turnover in vitro appears trivial by most standards.

The homogeneity of soluble PG (peak i) detected in *B. pertussis* supernatants, although not predicted on the basis of structural heterogeneity of PG subunits found in other gram-negative PGs (1, 4, 8, 11), is consistent with the marked homogeneity of monomer subunits in intact *B. pertussis* PG (3). However, the principal monomeric fragment making up intact *B. pertussis* PG was identified unambiguously by tandem mass spectrometry (3) as *N*-acetylglucosaminyl-*N*-acetylmuramyl-alanyl-glutamyl-DAP-alanine (NAG-NAM-Ala-Glu-DAP-Ala; 939 daltons), whereas TCT might be a somewhat larger fragment (about 1,400 daltons) containing an additional residue of the muramyl dipeptide *N*-acetylmuramyl-alanyl-glutamic acid (5). To excise such a product from intact PG would require endogenous glycan- and peptide-splitting PG hydrolases in addition to that required to release NAG-NAM-Ala-Glu-DAP-Ala from native PG. Together with previous observations (3, 5, 7), current findings such as the alkali resistance of peak i, i.e., TCT, further suggest that TCT is an anhydromuramic acid-containing fragment or a cyclic PG derivative. Future studies should define the complete structure of TCT and determine whether cytotoxic PG fragments in general may play a greater role in the pathogenesis of infections at mucosal surfaces than is currently appreciated.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grants R01 AI-14826, P01 AI-20110 (to R.S.R.), and R01 AI-22243 (to W.E.G.) and training grants GM-07200 and AI-07172 (to Washington University) from the National Institutes of Health.

We are grateful to Sandra Wilson and the Medical Illustrations Department of the Indiana University School of Medicine for valuable assistance in preparation of the manuscript and to Warner S. Wegener for critical review of the manuscript.

LITERATURE CITED

1. **Dougherty, T. J.** 1985. Analysis of *Neisseria gonorrhoeae* peptidoglycan by reverse-phase, high-pressure liquid chromatography. J. Bacteriol. **163:**69–74.
2. **Fleming, T. J., D. E. Wallsmith, and R. S. Rosenthal.** 1986. Arthropathic properties of gonococcal peptidoglycan fragments: implications for the pathogenesis of disseminated gonococcal disease. Infect. Immun. **52:**600–608.
3. **Folkening, W. J., W. Nogami, S. A. Martin, and R. S. Rosenthal.** 1987. Structure of *Bordetella pertussis* peptidoglycan. J. Bacteriol. **169:**4223–4227.
4. **Glauner, B., and U. Schwarz.** 1983. The analysis of murein composition with high-pressure-liquid-chromatography, p. 29–34. *In* R. Hakenbeck, J.-V. Holtje, and H. Labischinski (ed.), The target of penicillin. W. de Gruyter, Berlin.
5. **Goldman, W. E.** 1986. *Bordetella pertussis* tracheal cytotoxin: damage to the respiratory epithelium, p. 65–69. *In* L. Leive, P. F. Bonventre, J. A. Morello, S. D. Silver, and H. C. Wu (ed.), Microbiology—1986. American Society for Microbiology, Washington, D.C.
6. **Goldman, W. E., and L. A. Herwaldt.** 1985. *Bordetella pertussis* tracheal cytotoxin. Dev. Biol. Stand. **61:**103–111.
7. **Goldman, W. E., D. G. Klapper, and J. B. Baseman.** 1982. Detection, isolation, and analysis of a released *Bordetella pertussis* product toxic to cultured tracheal cells. Infect. Immun. **36:**782–794.
8. **Martin, S. A., R. S. Rosenthal, and K. Biemann.** 1987. Fast atom bombardment mass spectrometry and tandem mass spectrometry of biologically active peptidoglycan monomers from *Neisseria gonorrhoeae*. J. Biol. Chem. **262:**7514–7522.
9. **Melly, M. A., Z. A. McGee, and R. S. Rosenthal.** 1984. Ability of monomeric peptidoglycan fragments from *Neisseria gonorrhoeae* to damage human fallopian-tube mucosa. J. Infect. Dis. **149:**378–386.
10. **Petersen, B. H., and R. S. Rosenthal.** 1982. Complement consumption by gonococcal peptidoglycan. Infect. Immun. **35:**442–448.
11. **Pisabarro, A. G., M. A. de Pedro, and D. Vázquez.** 1985. Structural modifications in the peptidoglycan of *Escherichia coli* associated with changes in the state of growth of the culture. J. Bacteriol. **161:**238–242.
12. **Rosenthal, R. S.** 1979. Release of soluble peptidoglycan from growing gonococci: hexaminidase and amidase activities. Infect. Immun. **24:**869–878.
13. **Rosenthal, R. S., W. J. Folkening, D. R. Miller, and S. C. Swim.** 1983. Resistance of *O*-acetylated gonococcal peptidoglycan to human peptidoglycan-degrading enzymes. Infect. Immun. **40:**903–911.
14. **Rosenthal, R. S., M. A. Gfell, and W. J. Folkening.** 1985. Influence of protein synthesis inhibitors on regulation of extent of *O*-acetylation of gonococcal peptidoglycan. Infect. Immun. **49:**7–13.
15. **Rosenthal, R. S., R. M. Wright, and R. K. Sinha.** 1980. Extent of peptide cross-linking in the peptidoglycan of *Neisseria gonorrhoeae*. Infect. Immun. **28:**867–875.
16. **Sinha, R. K., and R. S. Rosenthal.** 1980. Release of soluble peptidoglycan from growing gonococci: demonstration of anhydro-muramyl-containing fragments. Infect. Immun. **29:**914–925.
17. **Sinha, R. K., and R. S. Rosenthal.** 1981. Effect of penicillin G on release of peptidoglycan fragments by *Neisseria gonorrhoeae*: characterization of extracellular products. Antimicrob. Agents Chemother. **20:**98–103.
18. **Stainer, D. W., and M. J. Scholte.** 1971. A simple chemically defined medium for the production of phase I *Bordetella pertussis*. J. Gen. Microbiol. **63:**211–220.
19. **Swim, S. C., M. A. Gfell, C. E. Wilde III, and R. S. Rosenthal.** 1983. Strain distribution in extents of lysozyme resistance and *O*-acetylation of gonococcal peptidoglycan determined by high-performance liquid chromatography. Infect. Immun. **42:**446–452.
20. **Tipper, D. J.** 1968. Alkali-catalyzed elimination of D-lactic acid from muramic acid and its derivatives and the determination of muramic acid. Biochemistry **7:**1441–1449.