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Detection, Isolation, and Analysis of a Released *Bordetella pertussis* Product Toxic to Cultured Tracheal Cells

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Cultured hamster trachea epithelial cells were selected as an in vitro model system to study *Bordetella pertussis* pathogenesis in the respiratory tract. DNA synthesis by serum-stimulated tracheal cells, in contrast to other cell types tested, was inhibited by the supernatant from log-phase *B. pertussis* broth cultures. A sensitive microassay with these tracheal cells permitted the development of a chromatographic purification scheme based on aggregation of the biological activity under salt-free conditions. The active fraction from this first stage of purification caused a dose-dependent inhibition of DNA synthesis without a similar effect on RNA or protein synthesis. Organ cultures of hamster tracheal rings, when exposed to this partially purified fraction, developed epithelial cytopathology comparable to that seen during *B. pertussis* infection. Ciliary activity slowed and eventually ceased as ciliated cells were extruded from the ring, leaving an intact but mostly nonciliated epithelium. Further purification of this biological activity was achieved with preparative-scale high-voltage paper electrophoresis. Based on ninhydrin staining and the radioactive profile of material purified from radiolabeled *B. pertussis* cultures, four fractions were eluted from the paper by descending chromatography. Only component B caused a dose-dependent inhibition of cultured tracheal cell DNA synthesis and epithelial cytopathology in tracheal rings. Combination experiments also demonstrated enhanced inhibition by component B in the presence of component G (oxidized glutathione), a copurifying molecule from the growth medium. Amino acid analysis of component B revealed a composition of glutamic acid (five residues), alanine (five residues), glycine (two residues), cysteine (two residues), and diaminopimelic acid (one residue), as well as muramic acid and glucosamine.

Seventy years ago, Mallory and Hornor (18) histologically characterized whooping cough, or pertussis, as a noninvasive bacterial colonization of ciliated cells in the respiratory epithelium. Although much literature on *Bordetella pertussis* has accumulated since that study, remarkably little is known about the pathogenesis of the disease. A variety of *B. pertussis* components have been purified and characterized, and they cover an impressive range of biological activities. However, none has been demonstrated to be responsible for the primary lesion of pertussis—cytopathology in the respiratory tract.

Collier et al. (3) studied cellular pathogenesis of pertussis in an in vitro model of mammalian respiratory epithelium, the hamster trachea in organ culture. They observed the classical association of bacteria with ciliated cells, mimicking in vivo infection, followed by selective destruction of those colonized cells and their extrusion from the epithelium. Lost ciliated cells cannot be replaced in the tracheal organ culture system; therefore, *B. pertussis* infection results in an intact but nonciliated epithelium. In vivo, regenerative activity might be expected, but the rate and efficiency of epithelial repair during pertussis infection are unknown. Since ciliated cells provide the mechanism for transport and clearance of tracheobronchial mucus, the loss or slow recovery of ciliary activity could be responsible for congestion of the airways with accumulating mucous secretions and multiplying bacteria.

Although tracheal organ culture has proved to be an excellent model in reflecting morphological and ultrastructural cytopathology during *B. pertussis* infection (see also references 20 and 21), the system has revealed little concerning the molecular mechanisms of this distinctive pathogenesis. One of the limitations of tracheal rings is the difficulty in quantitatively evaluating cyto-

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pathology in terms of the metabolic activity of the affected cells. Radiolabeling studies are difficult to interpret because of the variety of cell types present in the respiratory epithelium. Furthermore, a single experiment often requires the rings from many separate animals, a complicating factor in any quantitative assay. Significant variations in ring size, cell number, and epithelial integrity can be expected not only among animals but even among rings of the same animal.

As an alternative model system, we have developed a reliable technique to isolate a homogeneous proliferating epithelial cell population from the hamster trachea (7). These cells can be cultured and manipulated much like any established cell line, but they are not transformed and are therefore a relevant system to study cells of the normal respiratory tract. Like goblet cells of the hamster trachea, they synthesize and secrete a mucus-like glycoprotein (8) and retain this differentiated function throughout their life-span in vitro; yet like ciliated cells, they allow attachment and colonization by virulent *B. pertussis* (W. E. Goldman and J. B. Baseman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, D53, p. 48); this is a property of other tissue culture cells as well (13). Since the cultured tracheal cells are able to proliferate readily in vitro, they also parallel the regenerative epithelial basal cell population in vivo (17). Thus, these hamster trachea epithelial (HTE) cells may function as a multipotential target for *B. pertussis*, since the biological characteristics of this homogeneous cell culture resemble those of three separate epithelial cell types in the respiratory tract.

Preliminary experiments indicated not only that *B. pertussis* could attach to HTE cells, but also that the supernatant from broth cultures of phase I (virulent) organisms could inhibit DNA synthesis in uninfected cells (W. E. Goldman and J. B. Baseman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, D71, p. 49). The release of this molecule in log-phase cultures and its unique toxicity for HTE cells and tracheal rings have prompted its designation as the *B. pertussis* tracheal cytotoxin (TCT), since it is biochemically unlike previously characterized *B. pertussis* components. This paper discusses the purification of TCT and its interaction with relevant target cells in vitro as a model for *B. pertussis* pathogenesis in vivo.

MATERIALS AND METHODS

Bacterial cultures. Virulent phase I *B. pertussis* BB114 (used in vaccine production) was obtained from C. R. Manclark, Bureau of Biologics, U.S. Food and Drug Administration, Bethesda, Md. Bacteria were stored in aliquots at −70°C until grown on agar plates made with modified Stainer-Scholte medium (SSM) (12, 25) and 10% sheep blood (in Alsever solution). Growth on solid medium continued for 3 days at 37°C in an atmosphere of 95% air–5% CO_2(3). Transfer of bacteria to liquid SSM was most successful and consistent if initial seed cultures were grown with 10% fetal bovine serum (FBS; GIBCO Laboratories, Grand Island, N.Y.) in SSM. Bacteria were inoculated into this serum-containing medium at a concentration of 5×10^8 organisms per ml, based on absorbance at 540 nm (3). After 2 h of incubation at 37°C in an atmosphere of 95% air–5% CO_2, the cultures were placed in a 37°C shaker bath for 16 h. Bacteria were removed by centrifugation at 12,000 × *g* for 10 min, washed twice with serum-free medium, and then suspended in fresh SSM without FBS. Inoculation density and growth conditions in SSM matched those for the serum-containing medium of the seed cultures. For experiments with radiolabeled TCT, L-[*G*-^{3}H]glutamic acid (Amersham, Arlington Heights, Ill.) was added to SSM at a concentration of 0.5 μCi/ml during the last 30 h of broth culture; glutamic acid serves as the primary carbon source for *B. pertussis* in SSM.

Cell and organ cultures. HTE cells were isolated and cultured from frozen stocks as described previously (7). HEp-2 human epithelial cells and baby hamster kidney (BHK) cells were obtained from the American Type Culture Collection, Rockville, Md. Growth medium was usually nutrient mixture F-12 (GIBCO) with 10% FBS and with 100 U of penicillin per ml and 100 μg of streptomycin per ml. Tracheal rings were prepared (2) from male Syrian golden hamsters (Engle Laboratory Animals, Inc., Farmerburg, Ind.) between 6 weeks and 4 months of age. In experiments to evaluate cytopathology, the rings were cultured in minimum essential medium (MEM; GIBCO) containing antibiotics, with FBS added to a final concentration of 10% after a 4-h preexposure to TCT or buffer controls. The same effects were observed in experiments in which tracheal rings were cultured in the absence of FBS.

Microassay for HTE cell inhibition. Nearly confluent monolayers of HTE cells were washed twice with phosphate-buffered saline (PBS) and then incubated for 10 min with 0.02% EDTA in PBS, pH 8.0. Trypsin was added to a final concentration of 0.05%, and rapid pipetting of this enzyme solution dissociated the cells from the plastic surface. Bovine serum albumin was then added to a final concentration of 0.05%, and the cell suspension was centrifuged for 5 min at 400 × *g*. The pellet was resuspended in MEM plus 2.5% FBS at a concentration of 25,000 cells per ml, as determined by hemacytometer counts. Microtiter plates (Falcon Plastics, Oxnard, Calif.) were seeded with 0.2 ml of the cell suspension in each well and agitated frequently for 10 min to avoid irregular distribution of cells. Wells on the outside rows were generally filled with distilled water to reduce the evaporation of the medium during incubation. After 48 h of incubation in this low concentration of serum, medium was removed from each well by gentle suction and replaced with 0.15 ml of MEM. Test samples, dissolved in PBS, were then added to triplicate or quadruplicate wells; controls received PBS alone. Sample wells were supplemented 4 h later with FBS at a final concentration of 10%. At 8 h after serum stimulation, [*methyl*-^{3}H]thymidine (Schwarz/Mann, Orangeburg, N.Y.; 50 to 60 Ci/mmol), was added at 2.5 μCi/ml of medium. The total volume in each well was not greater than 0.2 ml after all addi-

tions. At 26 h after stimulation, samples were harvested with cotton-tipped applicators, precipitated with trichloroacetic acid, and washed in ethanol (28). After the addition of 10 ml of Omnifluor (New England Nuclear Corp., Boston, Mass.) dissolved in toluene, samples were counted for radioactivity in a Packard liquid scintillation spectrometer. In assays measuring RNA or protein synthesis, the precursors were [5,6-^{3}H]uridine (Schwarz/Mann; 30 Ci/mmol), or L-[G-^{3}H]serine (New England Nuclear; 2.76 Ci/mmol), respectively. Additions were made 4 h after serum stimulation, also at a concentration of 2.5 μCi/ml of medium. Incubation during the assay was at 37°C in an atmosphere of 95% air–5% CO_2.

Some revisions were made in the DNA synthesis assay to minimize test sample amounts in larger-scale experiments. In this assay, samples were dissolved in MEM rather than in PBS, and the solutions were pH and temperature equilibrated for 30 min in 95% air–5% CO_2 at 37°C. Aliquots (50 μl) of these samples were added directly to each well after the removal of the 48-h preincubation medium. Even distribution of this small volume of sample was achieved by placing the microtiter plate on a Nutator rotating rocker platform (Clay Adams, Parsippany, N.J.). After 4 h of such incubation in 95% air–5% CO_2 at 37°C, 100 μl of MEM with 15% FBS was added to each well; subsequent steps in the protocol remained unchanged. Preliminary experiments indicated that this threefold dilution after a 4-h exposure did not significantly reduce the inhibitory effect compared to the previous assay. Because less than one-third as much sample was required, we chose the revised assay for the series of combination experiments described in Tables 3 and 4.

Purification of *B. pertussis* TCT. Thirty-hour *B. pertussis* cultures in SSM were centrifuged in 200-ml-capacity bottles at 12,000 × *g* for 15 min (see Fig. 1). The supernatant was then filtered twice with 0.2-μm Nalgene filter units (Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.) to remove any remaining bacteria. The pH was adjusted to 7.3 with 6 N HCl, and 600 ml of sterile culture supernatant was then placed in a 2-liter-capacity ultrafiltration cell (Amicon Corp., Lexington, Mass.) with a UM2 membrane (molecular weight exclusion limit estimated at 1,000). Subsequent ultrafiltration and column chromatography steps were performed at 4°C. After ultrafiltration at 35 lb/in^2 to approximately 80 ml, the retained material and PBS membrane washings were transferred to a smaller Amicon stirred cell for further concentration. Ultrafiltration was continued until the final volume was 1/100 of the original supernatant. The pH was readjusted to 7.3 with 1 N HCl, and after filtration through a 0.2-μm filter, the concentrated culture supernatant was frozen in 1-ml aliquots at −70°C. A Bio-Gel A-1.5 m agarose column (80 by 1.5 cm; Bio-Rad Laboratories, Richmond, Calif.), with a molecular weight exclusion limit of 1.5 × 10^6, was coupled with a 280-nm absorbance monitor (ISCO, Lincoln, Nebr.) for chromatography. The eluent buffer was PBS, pH 7.3, supplemented with 0.5 mM $MgCl_2$ and 0.14 mM $CaCl_2$; the flow rate was 10 ml/h, and 1-ml fractions were collected. After chromatography of 1 ml of concentrated culture supernatant, the low-molecular-weight peak was collected and placed in a 200-ml-capacity ultrafiltration cell with a UM2 membrane. Salts and other small molecules were removed by "diafiltration," a process in which ultrafiltrate leaving the cell is continuously replaced by salt-free liquid from a pressurized reservoir. This reservoir solution was 5% glycerol in water, which significantly reduced binding to the membrane during diafiltration; it was also used routinely in a 2- to 3-h ultrafiltration pretreatment of the membranes. After 950 to 1,000 ml of ultrafiltrate had been collected (approximately 48 h), the sample was concentrated to 3 to 4 ml and removed from the stirred cell with several 5% glycerol washes of the membrane. This solution was further concentrated by lyophilization, and the glycerol was removed by rechromatography on the same agarose column, this time preequilibrated for 48 h with deionized water. The major peak, apparently an aggregate formed in the absence of salts, eluted just after the void volume. Those fractions were pooled, filter sterilized, lyophilized in aliquots, and stored at −20°C in a desiccator. SSM controls were processed identically and tested for biological activity after each purification step.

UV light absorbance of this partially purified *B. pertussis* TCT dissolved in PBS was scanned from 190 to 360 nm with a Beckman model 25 recording spectrophotometer. Absorbance at 204 nm (A_{204}) of TCT was compared to a standard curve constructed for oxidized glutathione.

HVPE. Chromatographically purified TCT was analyzed and further purified by high-voltage paper electrophoresis (HVPE) (15) in a pH 1.9 buffer of formic acid-acetic acid-water (1:4:50). Samples were spotted onto Whatman 3MM paper and electrophoresed for 30 min at 3,000 V. Electropherograms were stained with 1% ninhydrin in acetone or with silver nitrate (16). For preparative HVPE, the TCT sample was dissolved in 2% 2-mercaptoethanol in water and streaked over an 8 to 12-cm horizontal line. Cool air was used to dry the streak before electrophoresis and to dry the paper afterward. A vertical guide strip was cut and stained with ninhydrin as a reference for cutting horizontal strips corresponding to the electrophoretically separated fractions. Material was eluted from the paper by overnight descending chromatography with pH 1.9 electrophoresis buffer as the eluting solvent. "Blank" strips were also cut and eluted for buffer controls. Eluted fractions were lyophilized and then tested on HTE cells and tracheal rings or analyzed for amino acids. In experiments with radiolabeled TCT, strips (10 by 50 mm) were placed into scintillation vials and soaked with 1 ml of water to elute radioactive components from the paper. Samples were incubated overnight at 37°C with 10 ml of Aquasol-2 (New England Nuclear), cooled to 4°C, and counted in a Packard liquid scintillation spectrometer.

Amino acid analysis. HVPE-purified TCT fractions were hydrolyzed under vacuum in 6 N HCl for 24 h at 110°C. Basic amino acids separated on a column (3.2 by 150 mm) with Aminex A-9 resin (Bio-Rad); neutral and acidic amino acids were separated on the same resin in a longer column (3.2 by 250 mm). Performic acid oxidation (24) before hydrolysis allowed for the quantitative recovery of cysteine and cystine as cysteic acid. Molar ratios were calculated by comparison to a standard containing 5 nmol of each amino acid. Oxidized glutathione, for amino acid analysis and for other experiments, was purchased from Sigma Chemical Co., St. Louis, Mo.

Histopathology of tracheal organ cultures. Treated and control tracheal rings were fixed at various time points and processed for light microscopy as described previously (3), except that staining was with toluidine blue. Sections were examined, and representative fields were photographed with a Leitz Ortholux II microscope.

RESULTS

Detection of toxic activity in culture supernatant. As a means for evaluating the cytopathic effect of *B. pertussis*, we developed a microassay to monitor DNA synthesis in serum-stimulated monolayers of mammalian cells. Initially, *B. pertussis* broth culture supernatant was assayed to determine whether the organisms released an inhibitory factor during growth. In SSM under the growth conditions described above, cultures harvested 30 h after inoculation represented late-log-phase organisms according to time course absorbance readings at 540 nm. Therefore, the appearance of bacterial products in the culture supernatant was probably not due to extensive lysis (as might be expected in the stationary growth phase). To assay this cell-free supernatant for inhibitory activity, we first diafiltered it extensively with water and a UM2 membrane to remove small molecules that might compete with radiolabeled thymidine. After lyophilization, the diafiltered culture supernatant was redissolved in PBS and tested on all three cell types. When compared with SSM processed in the same manner, this material caused a dose-dependent inhibition of DNA synthesis in HTE cells (Table 1); yet at the same dilutions, BHK and HEp-2 cells were not significantly affected.

Partial purification of TCT. During the purification of TCT from the culture supernatant, cytotoxicity was monitored as the presence of a reproducible, statistically significant ($P < 0.05$ in the Student's t test), dose-dependent inhibition of HTE cell DNA synthesis. Dose-response curves tended to differ in shape at various stages of purification, therefore, estimates of activity and yield from each step were rather qualitative and are not included here. The rationale for the procedure outlined in Fig. 1 was an apparent aggregation of the toxic activity in the absence of salt. High-molecular-weight contaminants were eliminated during the first column run with a PBS eluent. Salts and some small molecules were then removed by extensive diafiltration, and other low-molecular-weight contaminants were discarded in the second (salt-free) column run. The TCT was detected in the major peak eluting just after the void volume, indicating an apparent molecular weight of near 1.5×10^6 for the aggregate. Other experiments demonstrated that when the aggregated fraction was redissolved in PBS or even in much weaker ionic solutions, the activity once again appeared in a low-molecular-weight peak.

TABLE 1. Cell specificity of inhibition by *B. pertussis* culture supernatant

Cell type	% Inhibition of DNA synthesis[a]		
	1:1[b]	1:4[b]	1:16[b]
HTE	40.0	26.4	12.8
BHK	7.0	3.2	<0
HEp-2	8.1	<0	1.9

[a] Based on comparisons between triplicates or quadruplicate samples with standard deviations of <10% of the mean. DNA synthesis was measured as the incorporation of [^{3}H]thymidine by serum-stimulated monolayer cell cultures. HTE control values generally ranged from 3.5×10^4 to 4×10^4 cpm per well in this microassay.

[b] Relative dilutions of diafiltered and lyophilized supernatant test samples and controls; these do not reflect actual concentrations of culture supernatant.

The effectiveness of this purification scheme was initially evaluated by protein analysis on sodium dodecyl sulfate-polyacrylamide gels of different porosities. Purification resulted in the complete disappearance of all protein bands in the crude culture supernatant (data not shown), even when examined with highly cross-linked gels (26). However, we were able to detect the purified material after HVPE and ninhydrin staining (Fig. 2). The pattern seen in Fig. 2b was quite consistent for different preparations. None of the spots stained with silver nitrate, implying that the ninhydrin reactivity is probably due to amino acids rather than free amino sugars.

Further comparison of crude culture supernatant with this partially purified TCT is shown in UV light absorbance spectra (Fig. 3). Since the purified TCT had a single absorbance peak at 204 nm, we used this wavelength to roughly quantitate the amounts of material in different TCT preparations (see below). The concentrations reported here are based on a comparison of the A_{204} of TCT and oxidized glutathione (50 μg/ml = 0.85 A_{204}), the major component of this chromatographically purified fraction (see below). The low A_{280} not only suggests the absence of aromatic amino acids in the purified preparation, but also indicates that the elution profiles in Fig. 1 are probably misleading for quantitation.

As estimated by A_{204} comparison with oxidized glutathione, the yield from this purification procedure was 0.9 to 1.3 mg from 100 ml of *B. pertussis* culture supernatant. Since this material has not been completely purified by HVPE (see below), the estimated concentrations reported in the biological assays do not reflect the actual concentrations of TCT. However, the chromatographically purified preparations are very stable during storage at −20°C compared

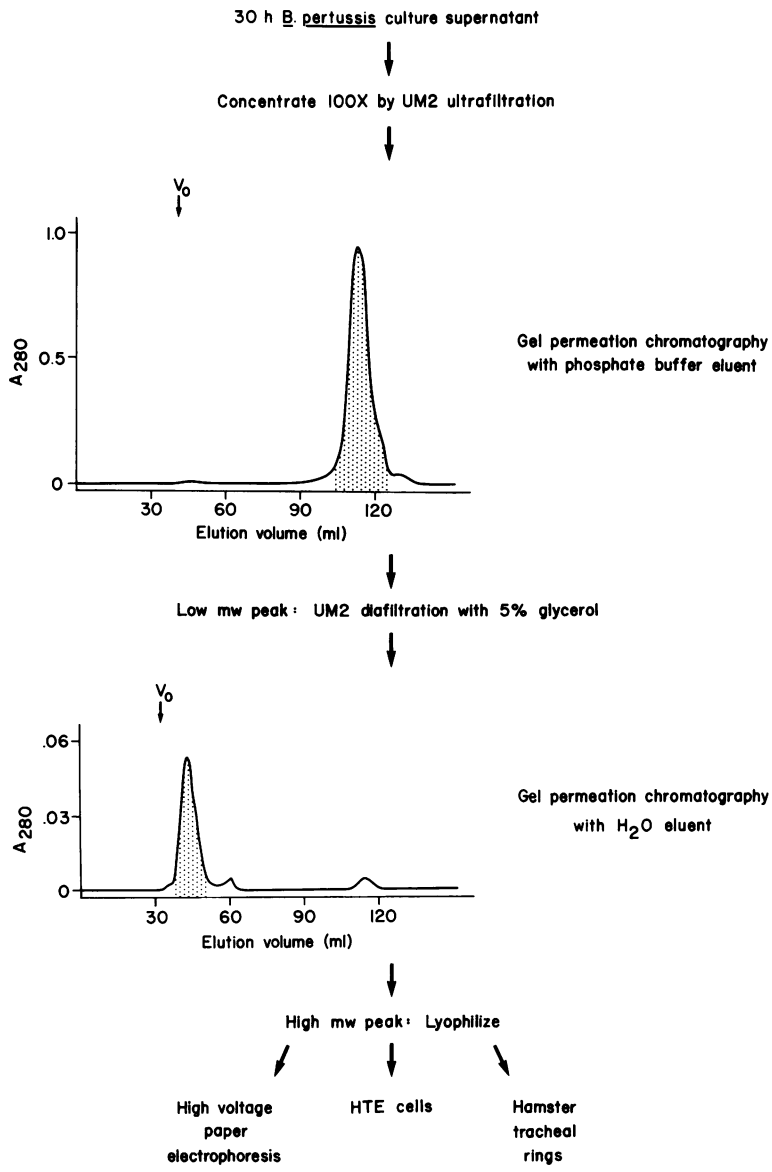

FIG. 1. Chromatographic purification of *B. pertussis* tracheal cytotoxin from the culture supernatant. Bio-Gel A-1.5 m agarose was used for column chromatography; molecular weight (mw) exclusion limit = 1.5×10^6.

with the active fraction from further purification steps; highly purified TCT loses biological activity within a few weeks. The biological experiments discussed below were therefore performed with only partially purified TCT, and expressed values for TCT are only for comparison among experiments.

Analysis of biological activity. Different purified preparations consistently generated an HTE cell dose-response curve like that in Fig. 4. The correlation coefficient for this regression line as calculated from four different purified TCT preparations is 0.97. Though this partially purified TCT produced a concentration-dependent inhibition of DNA synthesis, there was no similar dose-related inhibition of RNA and protein syntheses (Fig. 5). Only at levels of 100 μg/ml or greater did inhibition of RNA synthesis become statistically significant; protein synthesis remained virtually unaffected even at this high dose.

Other experiments with the HTE cell microas-

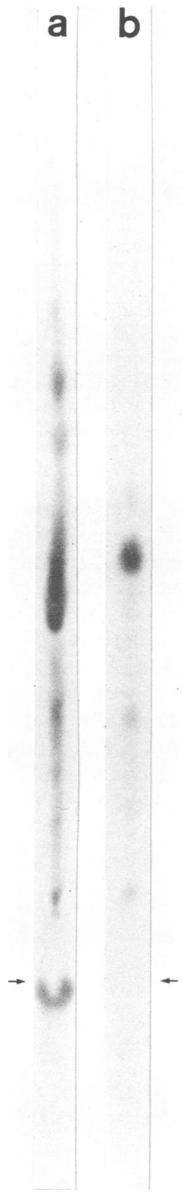

FIG. 2. HVPE of TCT: (a) 30-h culture supernatant after diafiltration with UM2 membrane and (b) after purification as in Fig. 1. Origin at arrow; cathode end of paper at the top; stained with ninhydrin after electrophoresis for 30 min at 3,000 V, pH 1.9.

say have shown that TCT, in contrast to *B. pertussis* endotoxin (6), lost most of its activity after treatment for 1 h at 100°C (data not shown). The difference between the inhibition caused by heat-treated and untreated TCT was statistically significant ($P < 0.01$) in a nondirectional Student's t test. Dose-response comparison of the inhibitory values indicates that the effective TCT concentration is reduced by approximately 75% as a result of heat treatment.

The most dramatic biological effects of TCT were seen in longer time course experiments with organ cultures of hamster tracheal rings (Fig. 6). When incubated in a TCT concentration of 50 μg/ml, living or sectioned tissue showed no noticeable changes for the first 60 h. Starting at 60 h, however, extrusion of ciliated cells from the epithelium was observed in the cultured rings. By 72 h, ciliary activity had significantly declined and many more cells had been expelled. Stained sections showed a reduced number of ciliated cells; some were extruded and most appeared less elongated and rounder than ciliated cells in control rings. At 96 h, ciliary activity was totally absent, most of the remaining epithelium was nonciliated, and the relatively few ciliated cells that remained were balloon-shaped and constricted at the apical end. In sharp contrast, control rings at 96 h still had typically elongated ciliated cells with excellent ciliary activity. Furthermore, the depth of the epithelium in TCT-treated tissue was much shallower than that of control rings, presumably

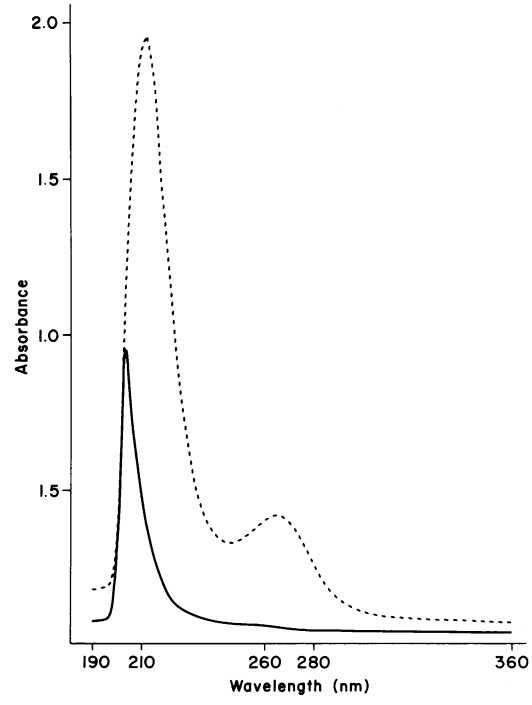

FIG. 3. Absorbance of TCT by the UV spectrum. (---) Crude culture supernatant (after concentration by UM2 ultrafiltration); (—), UV scan of TCT purified as in Fig. 1.

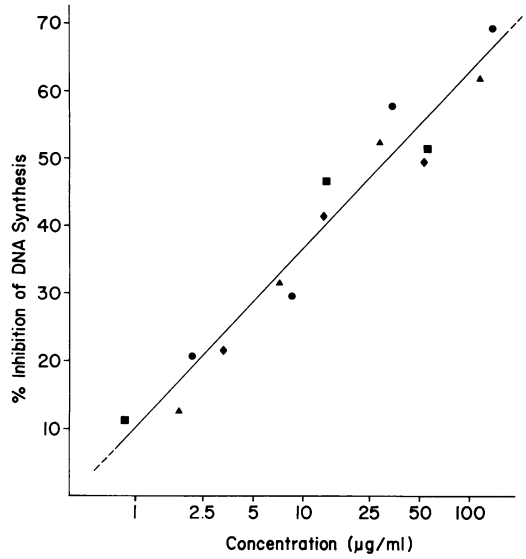

FIG. 4. Dose-response analysis of TCT: effect of the concentration of purified TCT on DNA synthesis in serum-stimulated HTE cells. Each symbol represents a different purification attempt assayed in separate experiments. All points are calculated from a comparison of triplicate samples with standard deviations of <10% of the mean. The concentration is based on comparative peptide A_{204} (see text), where 50 μg/ml = 0.85 A_{204}.

because nonciliated cells migrated to fill the gaps left by the extruded ciliated cells. Preliminary studies with transmission electron microscopy showed distorted, ballooning mitochondria in the few ciliated cells remaining after 4 to 5 days of exposure to TCT; nonciliated cells, however, had normal ultrastructure.

Tracheal rings exposed to lower doses of TCT showed the same characteristic pattern of cytopathology, but the time course of events was somewhat slower. For example, 5 μg of TCT per ml required 5 days to completely shut down ciliary activity as compared with 4 days with 50 μg/ml. The specific tracheal cell damage mediated by this toxin from *B. pertussis* culture supernatant is comparable to the cytopathology observed in *B. pertussis*-infected tracheal rings (3).

Electrophoretic purification of TCT. For the identification of the biologically active fraction in this partially purified TCT, a final step was necessary to isolate the chromatographically distinct components. Ion-exchange resins proved to be ineffective, so we employed preparative-scale HVPE and were able to elute fractions by descending chromatography. When *B. pertussis* was grown in the presence of [^{3}H]glutamic acid, analysis of the electrophoretic fractions revealed three radioactive components in the TCT preparation (Fig. 7 and Table 2). Radioactive fraction A aligned with the major component identified by ninhydrin staining. However, reduction with 2-mercaptoethanol shifted the migration of this ninhydrin-positive spot but did not largely alter the radioactive profile. A ninhydrin-negative spot must therefore exist at the zone marked A and is separated from the nonradioactive spot G only under reducing conditions. Another nonstaining fraction, labeled C, was also identified by the presence of radiolabel. A third spot (B) stained weakly with

TABLE 2. HVPE fraction: incorporation of radioactivity from [^{3}H]glutamic acid for HVPE fractions

Fraction	% of total radioactivity[a]	
	Unreduced	Reduced with 2-mercaptoethanol
A	22.6[b]	18.7
G		Background
B	6.1	5.5
C	42.8	49.4

[a] Percentage of total counts per minute (over background radiation) in all HVPE fractions. The remaining 25 to 30% of the radioactivity was distributed over unstained areas of the electropherogram and was only marginally above background levels.

[b] Components A and G were not separated in the absence of 2-mercaptoethanol (Fig. 7).

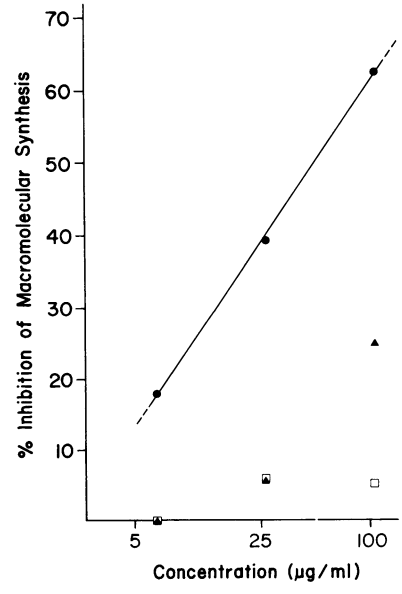

FIG. 5. Effect of TCT on DNA (●), RNA (▲), and protein (□) syntheses by serum-stimulated HTE cells. Calculations of percent inhibition and concentration are as described in the legend to Fig. 4.

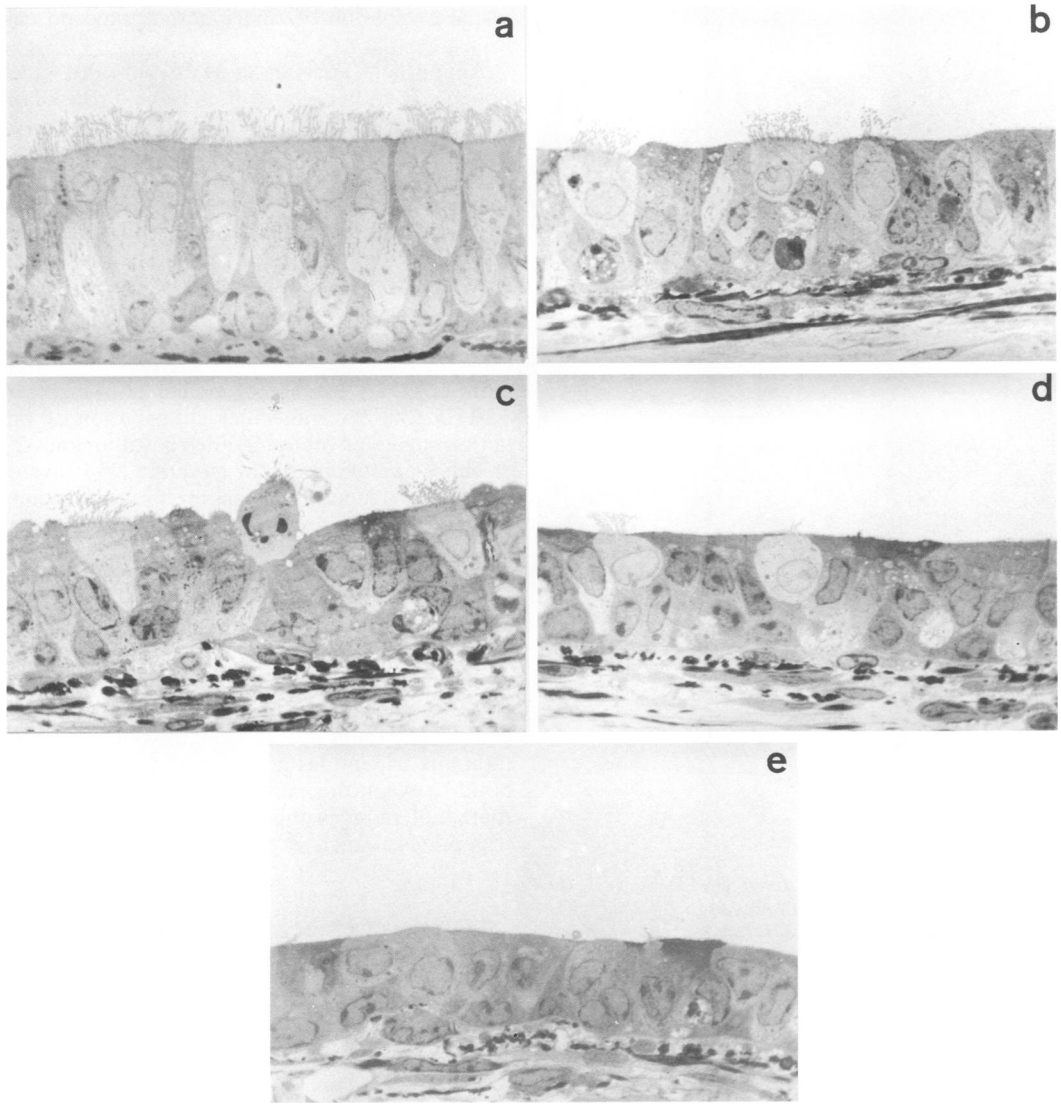

FIG. 6. Effect of TCT on hamster tracheal rings in organ culture. (a) Control after 96 hours. (b and c) Treated rings (50 μg/ml) after 72 h; ciliated cells appear more rounded, and some are seen extruding from the epithelium. (d and e) Treated ring after 96 h; occasional balloon-shaped ciliated cells can still be found, but most of the epithelium is nonciliated, as in (e). Epon 812 sections (0.5 μm), stained with toluidine blue, all at ×569.

ninhydrin and was poorly radiolabeled, though consistently well above background levels.

Preparative HVPE was also employed to determine whether the biological activity of TCT could be assigned to any of the radioactive fractions or to the nonradioactive spot G. In the HTE cell microassay, values for DNA synthesis were compared with similarly diluted controls in which buffer had been eluted from blank strips of paper. In preliminary experiments, we first determined a dose at which a 1:1:1:1 combination of all four fractions caused a statistically significant inhibition. Fractions were then tested alone or in other combinations, always with consistent concentrations of each individual fraction. Table 3 summarizes the dose-response analyses compiled from a series of experiments in which a "positive" combination is defined as a reproducible, dose-dependent, statistically significant inhibition. Fraction B, alone or combined with other fractions, was responsible for the biological activity of *B. pertussis* TCT. Furthermore, this purified component also caused the characteristic TCT cytopathology in organ

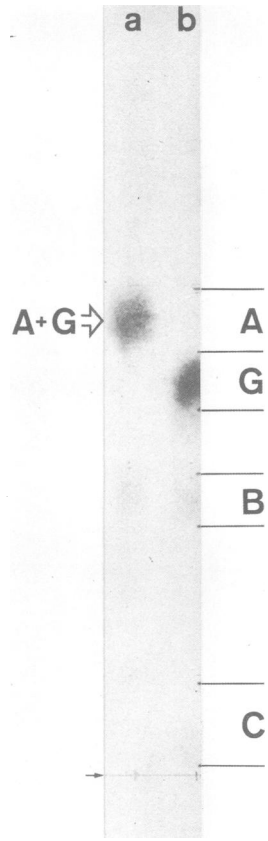

FIG. 7. Guide strip cut from HVPE of TCT (a) dissolved in water, and (b) dissolved in 2% 2-mercaptoethanol. Note that A and G no longer comigrate under reducing conditions (see Table 2 for labeling of spots). Origin at small arrow; cathode end of paper at the top; stained with ninhydrin after electrophoresis for 30 min at 3,000 V, pH 1.9.

cultures of tracheal rings, with loss of ciliary activity and extrusion of ciliated cells.

In the combination experiments with HTE cells, the presence of both fractions B and G was more inhibitory to DNA synthesis than was fraction B alone (Table 4). Since fraction G had no inhibitory activity by itself, its effect was to enhance the HTE cytotoxicity of fraction B. These data suggest that a three- to fourfold increase in the concentration of TCT would be required to match the level of inhibition observed after adding component G to component B. Interestingly, hamster tracheal organ cultures did not show noticeably increased damage when component B was supplemented with component G. It is difficult to quantitate the effect on tracheal epithelium, however, since our observations in that system are based only on the visual evaluation of ciliary activity and on cell loss.

Amino acid analysis of HVPE-purified components B and G is shown in Table 5. Based on this comparison with amino acid standards, we have estimated the composition of toxic fraction B as glutamic acid (five residues), alanine (five residues), glycine (two residues), cysteine (two residues), and diaminopimelic acid (one residue). The identification of diaminopimelic acid was also confirmed by HVPE. Some amino sugars consistently survived acid hydrolysis, and those fractions have been identified as muramic acid and glucosamine. The peptide portion alone has an estimated molecular weight of 1,511.5, but the molecular contribution of the amino sugars or other moieties is still unknown.

The equimolar ratio of glutamic acid, cysteine, and glycine for fraction G corresponds exactly to the amino acid composition of glutathione (γ-glutamylcysteinylglycine), a component of the medium in which the bacteria were grown. A comparison of the amino acids in component G with commercially available glutathione is also shown in Table 5. The migration of component G during HVPE corresponded exactly to the migration of the oxidized form of glutathione (data not shown), in which two glutathione tripeptides are linked by a disulfide bond between the cysteine residues. 2-Mercaptoethanol reduces this molecule to glutathione tripeptides, which is less basic at this pH and

TABLE 3. Inhibitory activity of HVPE-purified fractions on HTE cells

Fraction(s) assayed[a]	Inhibition of DNA synthesis[b]
A	−
B	+
C	−
G	−
A + B	+
A + C	−
B + C	+
A + G	−
B + G	++
C + G	−
A + B + C	+
A + B + C + G	++

[a] The concentration of fractions, individually or when combined with others, was the same in all test samples.

[b] Summary of results from five separate experiments (involving three different purification attempts). Symbols: +, reproducible, dose-dependent, statistically significant ($P < 0.05$ in non-directional Student's t test) inhibition of [^{3}H]thymidine incorporation, as determined by triplicate or quadruplicate samples; −, no inhibition ($P \geq 0.05$) of DNA synthesis; ++, enhanced inhibition (Table 4).

TABLE 4. Effect of fraction G on HTE cell inhibition by fraction B

HTE cells exposed to[a]:	Incorporation of [^{3}H]thymidine[b]	% Inhibition of DNA synthesis	Dose equivalent for unfractionated TCT (μg/ml)[c]
Fraction B	25,475 ± 556	27.3	4.4
Buffer elution control	35,047 ± 3,074		
Fractions B + G	21,547 ± 1,220	42.1	16.1
Buffer elution control + fraction G	37,204 ± 3,263		

[a] The concentration of component B is constant, but too low to measure by A_{204}; the concentration of component G = 70 μg/ml.

[b] Mean ± 1 standard deviation from the mean, based on triplicate or quadruplicate samples (representative of three experiments).

[c] Corresponding concentration of unfractionated (pre-HVPE) preparation required to cause this level of inhibition.

migrates more slowly during HVPE (as in Fig. 7). The absence of radiolabel incorporation into spot G also confirms that its origin must be the reduced glutathione in SSM; apparently component G is oxidized from the reduced form during broth culture growth. Commercially prepared oxidized glutathione also substituted for component G in combination experiments that demonstrated enhancement of cytotoxicity by component B.

DISCUSSION

The previously described biologically active components of *B. pertussis* can be readily distinguished from TCT by a number of criteria. For example, TCT was purified from 30-h log-phase cultures of *B. pertussis* BB114 in SSM. In contrast, Cowell et al. (4) demonstrated that dermonecrotic toxin from this same strain grown in identical medium is undetectable in the culture supernatant even at 72 h, after bacteria have entered the stationary growth phase. Second, most, if not all, of the inhibitory activity of TCT was lost when heated at 100°C for 1 h, whereas *B. pertussis* endotoxin has been shown to be stable under those conditions (6). In addition, the molecular weight of the peptide portion of TCT was calculated at about 1,500 from amino acid analysis. This differs substantially from molecular weight estimates of other *B. pertussis* components, such as 95,000 to 130,000 for the fimbrial hemagglutinin (14, 23), which is believed to be important in bacterial attachment; 70,000 for adenylate cyclase (12), which is also released by log-phase cultures (11); and 77,000 to 108,000 for pertussigen (see reference 19), a molecule responsible for a wide range of systemic effects.

TABLE 5. Amino acid analysis of HVPE-purified components B and G

Amino acid	Amino acid composition[a] of component:		
	B (compared to amino acid standards)	G (compared to amino acid standards)	G (compared to oxidized glutathione)
Glutamic acid	4.9	1.0	1.0
Alanine	5.1		
Glycine	2.1	0.9	1.2
Cysteine[b]	2.0	0.8	1.0
Diaminopimelic acid	1.0		

[a] Number of residues based on diaminopimelic acid (for component B) or glutamic acid (for component G) being defined as 1.0.

[b] Determined as cysteic acid after oxidation by performic acid.

Besides the biological and physical discrepancies, an important difference is that none of these previously characterized molecules has ever been demonstrated to have a toxic effect on cells of the respiratory tract. Dermonecrotic toxin (obtained from J. L. Cowell, Bureau of Biologics, U.S. Food and Drug Administration), adenylate cyclase (obtained from E. L. Hewlett, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases), and pertussigen (obtained from J. J. Munoz, National Institute of Allergy and Infectious Diseases) have been tested in this laboratory on HTE cells or hamster tracheal rings. No cytopathological effects were observed at concentrations that were biologically active in other test systems. TCT appears to be unique in its ability to mimic the pathogenesis of *B. pertussis* organisms on the respiratory epithelium.

Though the cytopathic effect of TCT on tracheal rings is dramatic, a microassay with serum-stimulated HTE cells has been most useful in monitoring activity during and after purification. Because so little material was required to inhibit DNA synthesis significantly in these microtiter-well monolayers, samples could be run in triplicate or quadruplicate and were thus amenable to statistical analysis. Given enough flexibility to assay many samples in a single

experiment, we were able to develop a purification scheme based on the behavior of TCT in an agarose gel filtration column. When salt was removed by extensive diafiltration, the low-molecular-weight activity eluted as a high-molecular-weight aggregate when chromatographed with a salt-free eluent. No apparent loss of inhibitory activity occurred with this aggregation procedure, and disaggregation took place as soon as the lyophilized TCT was dissolved in an ionic solution.

This protocol effectively removed many high- and low-molecular-weight contaminants, as determined by UV light absorbance and by HVPE. The absorbance maximum for this partially purified preparation was 204 nm, which matched the wavelength for the maximum absorbance of peptide bonds. By comparing the A_{204} with that of known concentrations of oxidized glutathione (later determined to be the major component in this preparation), we were able to roughly estimate the concentration of the partially purified TCT. Standard protein assays, such as A_{280} or colormetric reactions, were not applicable since those estimates vary greatly with the amino acid compositions of small peptides. The highest concentration values were obtained with the A_{204} comparison, so we chose that method as an admittedly crude quantitation rather than underestimating with one of the other techniques. The dose-response curves typically showed a high correlation between concentration and inhibitory activity of different purified preparations, implying that the A_{204} estimate of concentration was at least a reliable means of standardization between experiments in the same laboratory.

The biological activities of this partially purified TCT were examined with tracheal cells from two in vitro systems. Treatment of cultured HTE cells caused a highly reproducible dose-dependent inhibition of DNA synthesis, whereas RNA synthesis and protein synthesis remained largely unaffected. The most visible cytopathic effects were seen with hamster tracheal organ cultures, where ciliary activity of the respiratory epithelium was totally lost within 96 h of exposure to TCT. Stained sections of these rings in a time course study revealed both the abnormal morphology and the eventual extrusion of ciliated cells, leaving an almost totally nonciliated epithelial cell layer. Parallels to virulent *B. pertussis* infection of tracheal organ cultures (3, 20) are clear. Avirulent strains do not attach to or damage ciliated cells (21), though it is not known whether such strains produce TCT. Even if TCT is released, however, the inability of these avirulent organisms to colonize cells and establish a respiratory tract infection precludes the possibility of delivering the toxic molecule to its target cell.

Chromatographically purified TCT was further fractionated on the basis of radioactivity incorporation, with [^{3}H]glutamic acid as a precursor in the *B. pertussis* culture medium. By running preparative-scale HVPE under reducing conditions, we purified fractions corresponding to all three radioactive components (A, B, and C), as well as the major ninhydrin-positive (but nonradioactive) fraction (G). Amino acid analysis and comparative HVPE identified fraction G as oxidized glutathione, a component apparently originating from the growth medium. When fraction G and the other individual fractions were tested on HTE cells in various combinations, it was clear that component B was the toxic component of *B. pertussis* TCT. This was confirmed when hamster tracheal organ cultures exposed to component B showed typical TCT pathogenesis.

Amino acid analysis of HVPE-purified component B revealed a composition of glutamic acid (five residues), alanine (five residues), glycine (two residues), cysteine (two residues), and diaminopimelic acid (one residue). Including only this peptide portion, the molecular weight of component B is 1,511.5. Muramic acid and glucosamine were also detected, but the correct molar ratios are still unknown due to a partial loss of these amino sugars during acid hydrolysis. The presence of both diaminopimelic acid and muramic acid strongly implies that at least part of this molecule originates from the *B. pertussis* peptidoglycan (5). The release of soluble peptidoglycan fragments from exponentially growing gram-negative organisms has precedent in studies with *Neisseria gonorrhoeae* (22). However, the large numbers of alanine and glutamic acid residues suggest that component B consists of more than a peptidoglycan subunit; in fact, glycine and cysteine have never been demonstrated in the peptidoglycan of gram-negative bacteria. How these additional amino acids are linked to or associated with the peptidoglycan fragment remains unknown. They are not simply the result of contamination by glutathione, as indicated by the absence of a glutathione spot after repeated HVPE of purified component B.

The low yield of component B, its weak ninhydrin staining, and its low radiolabeling efficiency suggest that it is present in only very small amounts compared with oxidized glutathione. The inhibitory concentrations of TCT estimated in Figs. 4 through 6 are based primarily on the amount of oxidized glutathione and therefore are far in excess of the actual toxic levels of component B. Based on yield (determined from amino acid analysis) after HVPE, we estimate that TCT may be as much as 15 times more "potent" than estimated in those experiments.

Although fraction B is certainly the toxic fraction, glutathione may be more than a fortuitous contaminant from the culture medium. Component B seems to be tightly bound to oxidized glutathione, with the two consistently behaving like a single molecule on gel filtration and ion-exchange columns; only a strong electrical field at a low pH separates the two components. Furthermore, oxidized glutathione enhanced the effect of component B on HTE cells to a level of inhibition corresponding to a three- to fourfold increase in TCT concentration. If oxidized glutathione indeed participates in the toxicity of TCT in vitro, might it also assume a role during *B. pertussis* infection in vivo?

Glutathione is a major constituent inside mammalian cells, although *B. pertussis* infection takes place extracellularly. However, recent studies with mice (9) as well as with lymphoid cells (10) and fibroblasts (1) have shown that cells normally translocate glutathione across the plasma membrane. Once outside the cell, glutathione may be oxidized by a specific membrane-bound glutathione oxidase (27) or perhaps by *B. pertussis*, which probably oxidized glutathione during broth culture growth. Thus, the cells lining the respiratory tract provide an extracellular source of oxidized glutathione that may associate with component B released from the organisms. The enhancement of component B cytotoxicity in the presence of oxidized glutathione was not visualized in tracheal organ cultures, but those cultures may be inappropriate for the observation of a glutathione effect. In addition to difficulties in quantitative evaluation, leakage of glutathione from the cells damaged by trachea slicing may mask the effect of exogenously supplied oxidized glutathione. HTE cells probably serve as a cleaner model to analyze the relationship of oxidized glutathione to toxic component B (though glutathione may simply increase the stability of component B in solution and during storage).

The relationship of DNA synthesis inhibition in HTE cells to the cytopathology seen in tracheal ring cultures is not yet understood. However, the effect on DNA synthesis occurred quite early (within 30 h after exposure) compared with the first visible effects on ciliated cells, which required at least twice as much time. The DNA synthesis inhibition of HTE cells may therefore reflect an early stage in the overall cytopathology that is seen later in organ cultures of tracheal rings. It may also indicate a toxic activity that cannot be measured with tracheal organ cultures—an effect on regeneration. Once damaged, the respiratory epithelium does not repair itself in vitro; in vivo, damaged or extruded cells are typically replaced by an underlying layer of basal cells, which divide and differentiate to the required specialized cell type (17). It therefore seems possible that *B. pertussis* TCT not only destroys the ciliated cell population but also partially or completely inhibits the normal replacement of epithelial cells.

The small size of TCT, and therefore its loss during many protein purification techniques, can easily explain why it has not been detected previously. More importantly, the activity may have been overlooked because of its specificity; even other cultured cells, such as HEp-2 and BHK, which allow the attachment of *B. pertussis*, were unaffected in terms of DNA synthesis inhibition. The most significant factor in the discovery and purification of this toxin was the availability of an appropriate cellular model of the respiratory tract. Animal models will also be an important subsequent step in understanding the role of *B. pertussis* TCT in vivo and in determining how well the pathogenesis correlates with in vitro systems.

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