Epithelial autotoxicity of nitric oxide: Role in the respiratory cytopathology of pertussis

(Bordetella pertussis/tracheal cytotoxin/muramyl peptide/bacterial toxins/interleukin 1)

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ABSTRACT   Bordetella pertussis releases a specific peptidoglycan fragment known as tracheal cytotoxin (TCT) that reproduces the respiratory epithelial cytopathology of whooping cough (pertussis). In vitro, TCT inhibits DNA synthesis in hamster trachea epithelial cells and causes specific destruction of ciliated cells in explants of human and hamster respiratory epithelium. We have recently demonstrated that TCT triggers production of intracellular interleukin 1 by respiratory epithelial cells, and this cytokine may act as an intermediate signal in the generation of TCT toxicity. Here we report the identification of a subsequent critical step in this pathway: induction of nitric oxide synthesis in the respiratory epithelium. The toxic effects of nitric oxide are consistent with spectroscopic evidence of the formation of iron–dinitrosyl–dithiolate complexes in TCT-treated cells. Aconitase, with its iron–sulfur center, is one expected target of nitric oxide, and TCT inhibited 80% of the activity of this enzyme in respiratory epithelial cells. The deleterious effects of TCT and interleukin 1 were dramatically attenuated by the nitric oxide synthase inhibitors N^G-monomethyl-L-arginine and aminoguanidine. These results indicate that nitric oxide mediates the toxicity of TCT for the respiratory epithelium, thus implicating a central role for nitric oxide in the pathogenesis of pertussis.

Colonization of the human respiratory tract by Bordetella pertussis results in a syndrome characterized by violent, debilitating coughing episodes (1). The coughing spasms, which serve as the primary mode of disease transmission, are a response to loss of the normal ciliary transport mechanism for clearing airways of mucus, bacteria, and inflammatory debris. Our earlier studies have shown that B. pertussis tracheal cytotoxin (TCT) causes ciliostasis and ciliated cell damage consistent with in vivo pertussis pathology and symptomatology. In vitro, TCT generates this cytopathology in explanted human or hamster respiratory epithelium and also inhibits the proliferation of cultured hamster trachea epithelial (HTE) cells (2–4).

TCT is a 921-Da disaccharide tetrapeptide (5) that is a member of a large family of molecules known as muramyl peptides, which are fragments of bacterial cell wall peptidoglycan. Released during logarithmic-phase growth of Bordetella spp. (6, 7), TCT is identical to a muramyl peptide released by Neisseria gonorrhoeae (8) and responsible for destruction of ciliated cells in human fallopian tube epithelium (9). Muramyl peptides have also been shown to have adjuvant, somnogenic, arthritogenic, and pyrogenic activities, which appear to share a common mediator, the cytokine interleukin 1 (IL-1; refs. 10–13). As with these other muramyl peptide actions, the cytotoxic actions of TCT have been correlated with the production of IL-1, which itself reproduces pertussis pathology (14).

Cytokines such as IL-1 can trigger the synthesis of a number of cytotoxic effector molecules, including the free radical nitric oxide (15). Nitric oxide is generated enzymatically from L-arginine (16) through the actions of at least three isoforms of nitric oxide synthase. Two distinct constitutive isoforms (17, 18) generate the low levels of nitric oxide responsible for endothelium-dependent relaxation and neural transmission, while an inducible isoform (19–21) generates the high levels of nitric oxide that mediate the antitumor and antimicrobial actions of cytokine-activated macrophages. The cytotoxic and cytostatic effects of nitric oxide result from its ability to deplete intracellular iron and inhibit the activities of a number of iron-containing enzymes important for mitochondrial respiration and DNA synthesis (reviewed in ref. 22).

Since the initial identification of a cytokine-inducible nitric oxide synthase activity in macrophages, IL-1 has been shown to induce nitric oxide synthase in a variety of other cell types, including epithelial cells (23–25). Given the correlations between TCT, IL-1, and pertussis pathology, we have investigated the possibility that TCT induces nitric oxide synthase in respiratory epithelial cells and that the nitric oxide formed is involved in the pathogenesis of pertussis.

MATERIALS AND METHODS

Reagents. TCT was purified from B. pertussis culture supernatant as described (4). Recombinant murine IL-1β was a gift from K. Hogquist and D. Chaplin (Washington University School of Medicine). N^G-monomethyl-L-arginine (NMMA) was purchased from Sigma and aminoguanidine (AG) was purchased from TCI America (Portland, OR).

HTE Cell Culture and Toxicity Assay. HTE cells were isolated from a Syrian Golden hamster as described (26) and cultured in F-12 tissue culture medium (GIBCO) containing 10% fetal bovine serum (FBS; HyClone), 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 2 mM L-glutamine. Cells were maintained in 95% air/5% CO_2 at 37°C.

Inhibition of DNA synthesis by HTE cells was assessed in the microtiter plate assay described (14). Briefly, HTE cells resuspended in minimum essential medium (GIBCO) containing 2.5% FBS were plated in 96-well culture plates at a density of 6000–12,000 cells per well. Incubation for 24 hr synchronized these cells in the nonproliferative phase of the cell cycle. The nonproliferative cultures were incubated for 4 hr with sample additions in serum-free minimum essential medium (containing 600 μM L-arginine). Cells were then

Abbreviations: TCT, tracheal cytotoxin; HTE, hamster trachea epithelial; IL-1, interleukin 1; AG, aminoguanidine; NMMA, N^G-monomethyl-L-arginine; EPR, electron paramagnetic resonance.
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stimulated with 15% FBS, pulsed with [^{3}H]thymidine, and allowed to incubate for 26 hr. The cells were harvested on cotton-tipped applicators, DNA was precipitated with trichloroacetic acid, and incorporation of radiolabel was determined. The relative inhibition of DNA synthesis was calculated by comparing incorporated cpm of treated cells to untreated controls.

Hamster Tracheal Ring Toxicity Assay. Damage to hamster tracheal epithelium was evaluated as described (2). Briefly, tracheas from male Syrian Golden hamsters (Charles River Breeding Laboratories) at least 90 days old were dissected and sectioned into rings. Test samples were coded and added to tracheal rings cultured in F-12 medium in an atmosphere of 95% air/5% CO_2 at 37°C. Tracheal rings were screened for ciliated cell damage daily by light microscopy over the course of 144 hr. Toxicity was defined as ciliostasis and extrusion of ciliated cells from the tracheal epithelium after exposure to test compound for 4–5 days.

Nitrite Determination. One hundred microliters of each culture supernatant, obtained from either HTE cells or hamster tracheal rings in the assays described above, was mixed with 100 μl of Griess reagent (27). Nitrite production was quantitated by measuring absorbance at 562 nm using a microplate reader.

Aconitase Activity. HTE cells seeded at a density of 2 × 10^6 cells per 150-cm^2 tissue culture flask were synchronized, exposed to test compounds, and serum stimulated as described for the toxicity assay. After an 18-hr incubation, the cells were harvested by trypsin treatment, pelleted by centrifugation (800 × g; 4°C), and resuspended in 5 ml of buffer (250 mM sucrose/20 mM Hepes/10 mM MgCl$_2$/2 mM KH$_2$PO$_4$/1 mM EGTA, pH 7.4). The cells were permeabilized with 0.007% digitonin (5 min on ice), pelleted as described above, and lysed with 0.2% Triton X-100/30 mM NaCl/30 mM Tris·HCl, pH 7.4. After centrifugation of the lysate (5000 × g; 4°C; 15 min), the supernatant was immediately assayed for aconitase activity. Aconitase activity was followed spectrophotometrically at 340 nm using a coupled enzyme assay measuring the formation of NADPH from NADP$^+$ by isocitrate dehydrogenase (28).

Electron Paramagnetic Resonance (EPR) Spectroscopy. HTE cells seeded at a density of 2 × 10^6 cells per 150-cm^2 tissue culture flask were synchronized, treated with test compounds, and serum stimulated as described for the toxicity assay. After a 24-hr incubation, cells were harvested and frozen at −70°C. EPR spectroscopy was performed at 77 K, using a Bruker 300 spectrometer. The power was 1 mW, modulation amplitude was 6.3 G, and microwave frequency was 9.44 GHz.

RESULTS AND DISCUSSION

To examine the possibility that nitric oxide mediates the cytotoxic activities of TCT, we initially tested the ability of TCT to stimulate the production of nitric oxide by respiratory epithelial cells. The presence of nitrite, a stable oxidation product of nitric oxide and therefore an indicator of nitric oxide synthase activity (16, 29), was assessed in supernatants from HTE cells cultured in the presence of TCT. TCT induced the formation of 248 nmol of nitrite per 10^6 HTE cells, while untreated HTE cells produced little detectable nitrite (Table 1). TCT-stimulated nitrite production was decreased by 88% when cells were incubated in arginine-free medium. Formation of nitrite triggered by TCT was blocked by the nitric oxide synthase inhibitor AG (30, 31), and that inhibition was reversed with addition of excess L-arginine but not D-arginine. Similar results were obtained with another inhibitor of nitric oxide synthase, NMMA (data not shown).

The cytotoxic effects of nitric oxide primarily result from its inhibition of iron-containing enzymes through formation

Table 1. Induction of respiratory epithelial cell nitric oxide synthase activity by TCT

Treatment*	Nitrite, nmol per 10^6 HTE cells†
Untreated	6 ± 2
TCT (3 μM)	248 ± 12
TCT (3 μM) + AG (500 μM)	37 ± 2
TCT (3 μM) + AG (500 μM) + L-Arg (20 mM)	225 ± 9
TCT (3 μM) + AG (500 μM) + D-Arg (20 mM)	24 ± 3
TCT (3 μM) in Arg-free medium	31 ± 3

*HTE cells (6000 cells per well) were exposed to the samples indicated for 30 hr, as described for the toxicity assay.
†Data shown are means ± SD for quadruplicate samples.

of iron–nitrosyl complexes (32). EPR spectroscopy was used to assess the presence of iron–nitrosyl complexes in TCT-treated cells. EPR spectroscopy performed on HTE cells incubated with TCT detected an axial signal at g = 2.04 (Fig. 1). An EPR signal at g = 2.04 has been ascribed to the iron–dinitrosyl–dithiolate complexes formed when nitric oxide binds to iron-containing enzymes (32). Detection of the iron–nitrosyl species by EPR correlated with the production of nitrite by cells treated with TCT. Exposure of HTE cells to TCT in the presence of 1 mM AG prevented formation of the g = 2.04 signal. This signal was also absent in untreated HTE cells or in cells incubated with AG alone (data not shown).

Iron-containing enzymes that are the targets of inhibition by nitric oxide include aconitase (33), complexes I and II of the mitochondrial respiratory chain (29, 34), and ribonucleotide reductase (35). We measured aconitase activity to assess the degree to which TCT inhibits the activities of enzymes containing catalytically active nonheme iron. Treatment of HTE cells with TCT resulted in an 80% reduction in aconitase activity compared to untreated cells (Fig. 2). NMMA prevented inhibition of aconitase seen in cells exposed to TCT. These results suggest that nitric oxide produced by TCT-treated respiratory epithelial cells causes damage to these cells by inactivating iron-containing enzymes.

To evaluate the role of nitric oxide in the cytopathology generated by TCT, we examined the ability of nitric oxide synthase inhibitors to prevent respiratory epithelial cell damage by TCT. Exposure of HTE cells to TCT in the presence of AG resulted in a dramatic reduction of the level of toxicity, measured as inhibition of DNA synthesis (Fig. 3). Similar results were obtained with NMMA (data not shown), which was also tested on intact respiratory epithelium. NMMA inhibited TCT-induced nitrite production in hamster tracheal
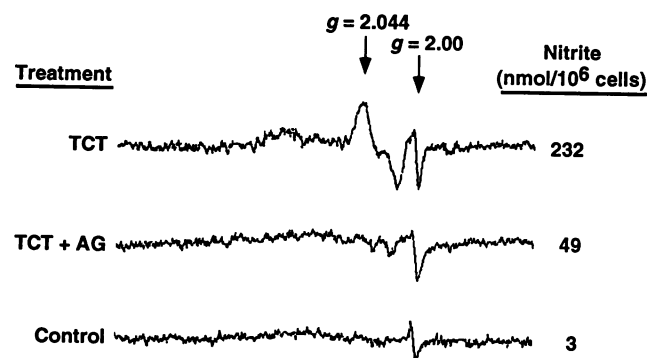

FIG. 1. TCT-induced formation of iron–nitrosyl complexes in respiratory epithelial cells. HTE cells were treated with 3 μM TCT, both 3 μM TCT and 1 mM AG, or medium alone. Cells were harvested and EPR spectroscopy was performed at 77 K. Nitrite was determined on aliquots of culture supernatants.
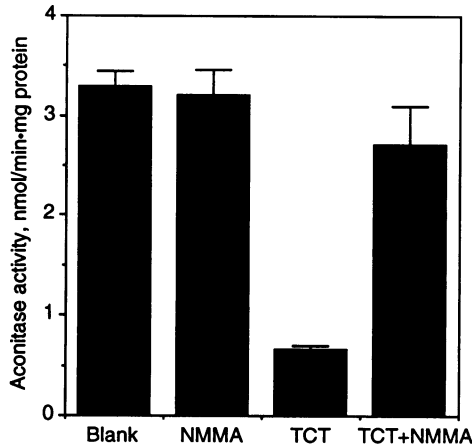
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FIG. 2. Inhibition of aconitase activity in respiratory epithelial cells exposed to TCT. HTE cells were exposed to 3 μM TCT, 1 mM NMMA, both TCT and NMMA, or blank medium. Extracts of HTE cells were assayed for aconitase activity. Aconitase activity is presented as nmol of NADPH formed per min per mg of protein in the supernatant of HTE cell lysates. Data shown are means ± SD for triplicate samples.

organ cultures and prevented ciliostasis and destruction of ciliated cells (Fig. 4).

We have previously reported that exposure of respiratory epithelial cells to TCT stimulates production of cell-associated IL-1α, which may act as an intracellular mediator of pertussis cytopathology (14). The link of IL-1 to TCT toxicity is reinforced by the observation that recombinant IL-1 can mimic the effects of TCT on HTE cells and on tracheal organ cultures (14). To explore further the correlation between TCT and IL-1 toxicity, we examined the effects of recombinant IL-1 on nitrite production by respiratory
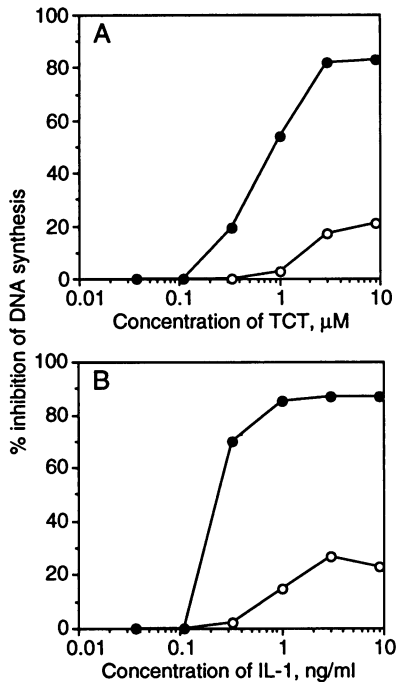

FIG. 3. Effect of nitric oxide synthase inhibitors on toxicity of TCT and IL-1 for respiratory epithelial cells. HTE cells (12,000 cells per well) were treated with the indicated concentrations of TCT without (●) or with (○) 1 mM AG (A), or recombinant IL-1β without (●) or with (○) 1 mM AG (B). Relative inhibition of DNA synthesis was calculated by comparing mean incorporated cpm of treated cells to untreated controls (quadruplicate samples).
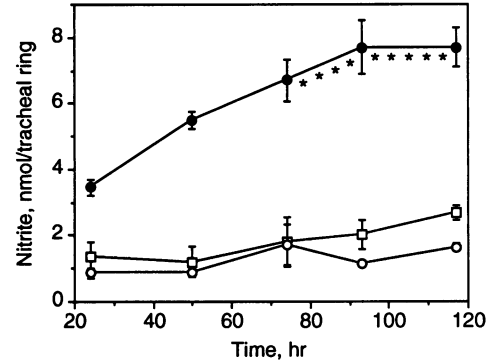

FIG. 4. Effect of nitric oxide synthase inhibitors on TCT-induced formation of nitrite and ciliostasis in hamster tracheal organ cultures. Sectioned hamster tracheas were incubated with 3 μM TCT (●), 3 μM TCT and 3 mM NMMA (○), or F-12 medium alone (□). At the times indicated, culture supernatants were removed and nitrite was quantitated. Data shown are means ± SD for triplicate samples. Tracheal rings were also screened daily for ciliated cell damage by light microscopy, which revealed ciliostasis and extrusion of ciliated cells as indicated with asterisks.

epithelial cells. At a concentration (10 ng/ml) that completely inhibits DNA synthesis in HTE cells, IL-1 stimulated production of nitrite, which reached levels similar to those obtained when cells were treated with 3 μM TCT (Fig. 5). The accumulation of nitrite over a period of 64 hr for HTE cells treated with TCT or IL-1 was virtually the same. To determine whether nitric oxide was also responsible for the toxic effects of IL-1, the ability of nitric oxide synthase inhibitors to prevent IL-1 damage to respiratory epithelial cells was assessed. AG markedly attenuated the level of DNA synthesis inhibition seen in IL-1-treated HTE cells (Fig. 3), and NMMA inhibited IL-1-induced destruction of ciliated cells in hamster tracheal organ culture (data not shown). Therefore IL-1, like TCT, relies on the synthesis of nitric oxide to damage target cells.

TCT has been previously identified as the *B. pertussis* virulence factor that reproduces the respiratory cytopathology of pertussis (2). Our initial studies concerning the mechanism of TCT action suggest that IL-1α, produced by epithelial cells in response to TCT, may act as a mediator of TCT toxicity (14). This report demonstrates that in addition to producing IL-1, respiratory epithelial cells exposed to TCT synthesize nitric oxide. It remains to be established whether the induction of nitric oxide synthase activity is a direct effect
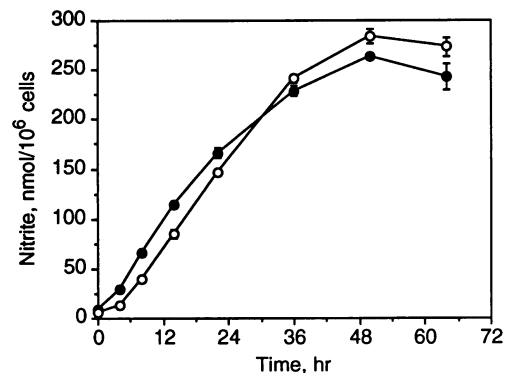

FIG. 5. Kinetics of nitrite production by respiratory epithelial cells exposed to TCT or IL-1. HTE cells (6000 cells per well) were seeded and incubated with 3 μM TCT (●) or 10 ng of IL-1 per ml (○) in serum-free medium as described for the toxicity assay. At the indicated time after serum stimulation, supernatants were removed and nitrite was quantitated. Data shown are means ± SD for quadruplicate samples.

of TCT or is secondary to TCT-stimulated IL-1 production. However, the kinetics and levels of nitric oxide production are consistent with the hypothesis that TCT acts via IL-1 to trigger production of the cytokine-inducible isoform of nitric oxide synthase. Although we have not determined whether nitric oxide is sufficient alone for toxicity, our *in vitro* models clearly implicate nitric oxide as a necessary effector molecule for TCT damage to respiratory epithelial cells. Our evidence suggests that the damage generated by nitric oxide results from the inhibition of iron-containing enzymes through destruction of catalytically active iron–sulfur groups, although we have not ruled out the possibility of other toxic nitric oxide effects.

These results provide a mechanistic explanation for the respiratory pathology of pertussis as well as a causal link between nitric oxide and the role of a bacterial exotoxin in disease. This finding may also have implications for the pathogenesis of *N. gonorrhoeae*, which elaborates a muramyl peptide identical to TCT that causes similar ciliated cell-specific pathology in human fallopian tubes (8, 9). Endotoxin (bacterial lipopolysaccharide) is another bacterial component known to trigger nitric oxide-mediated damage of mammalian target cells through either macrophage or target cell production of nitric oxide. However, endotoxin-stimulated nitric oxide production in target cells generally requires the presence of a macrophage- or lymphocyte-derived cytokine as a cofactor (36–38). TCT is unique in eliciting cytokine and nitric oxide production in the same cells that suffer the subsequent deleterious effects.

Our models of the respiratory epithelium suggest that in pertussis, ciliated cells may not be the only targets of nitric oxide. We have previously speculated that HTE cells *in vitro* represent the regenerative basal cell population of the epithelium in the large airways. Inhibition of HTE cell proliferation by TCT may reflect a profound secondary effect of nitric oxide *in vivo*: slowed replacement of the destroyed ciliated cells by blocking division (through inactivation of ribonucleotide reductase) and differentiation of the underlying basal cells. Nitric oxide has been shown to have either cytotoxic (23, 33, 39) or cytostatic (40) effects on a variety of cells, indicating that sensitivities to nitric oxide may differ between cells. Such varying sensitivities may result from the relative importance in different cells of iron-containing enzymes. Thus, the high energy requirements of ciliated cells could explain their exquisite susceptibility to irreversible damage by nitric oxide, which targets a number of enzymes involved in ATP synthesis. In contrast, the nitric oxide effect on basal cells does not result in their destruction and may even be reversible. Selective inhibitors of the inducible nitric oxide synthase (such as AG) may therefore have a unique therapeutic application in pertussis, where regeneration of a functional ciliated epithelium is the key to restoring normal airway function.

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