Quantification of the Adenylate Cyclase Toxin of *Bordetella pertussis* *In Vitro* and during Respiratory Infection

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Whooping cough results from infection of the respiratory tract with *Bordetella pertussis*, and the secreted adenylate cyclase toxin (ACT) is essential for the bacterium to establish infection. Despite extensive study of the mechanism of ACT cytotoxicity and its effects over a range of concentrations *in vitro*, ACT has not been observed or quantified *in vivo*, and thus the concentration of ACT at the site of infection is unknown. The recently developed baboon model of infection mimics the prolonged cough and transmissibility of pertussis, and we hypothesized that measurement of ACT in nasopharyngeal washes (NPW) from baboons, combined with human and *in vitro* data, would provide an estimate of the ACT concentration in the airway during infection. NPW contained up to $\sim 10^8$ CFU/ml *B. pertussis* and 1 to 5 ng/ml ACT at the peak of infection. Nasal aspirate specimens from two human infants with pertussis contained bacterial concentrations similar to those in the baboons, with 12 to 20 ng/ml ACT. When $\sim 10^8$ CFU/ml of a laboratory strain of *B. pertussis* was cultured *in vitro*, ACT production was detected in 60 min and reached a plateau of ~ 60 ng/ml in 6 h. Furthermore, when bacteria were brought into close proximity to target cells by centrifugation, intoxication was increased 4-fold. Collectively, these data suggest that at the bacterium-target cell interface during infection of the respiratory tract, the concentration of ACT can exceed 100 ng/ml, providing a reference point for future studies of ACT and pertussis pathogenesis.

Pertussis, a highly transmissible infection of the respiratory tract with *Bordetella pertussis*, produces a characteristic coughing syndrome which can last for weeks to months. Whooping cough, the classical manifestation of pertussis, occurs primarily in unimmunized children and is characterized by a marked leukocytosis and paroxysms of coughing punctuated by inspiratory "whoops." In adults and adolescents, infection with *B. pertussis* may be subclinical but frequently results in several weeks of paroxysmal coughing (1–3). Complications such as apnea, seizures, pneumonia, pulmonary hypertension, and death occur most frequently in infants who are less than 6 months old (4–7), and an estimated 300,000 pertussis-related deaths occur annually worldwide (8).

No single virulence trait fully accounts for survival of the bacterium in the airway or for the symptoms of the disease, but the adenylate cyclase toxin (ACT) is necessary for causing lethal infection in mice (9–11). This 1,706-amino-acid single-polypeptide toxin is composed of an adenylate cyclase (AC) enzymatic domain and a cell-binding domain homologous to that of the repeats-in-toxins (RTX) family of bacterial toxins (12, 13). Upon binding of the toxin to a target cell, the enzymatic domain translocates across the cell membrane and, inside the cytoplasm, converts ATP to cyclic AMP (cAMP) in a high-turnover reaction that is activated by eukaryotic calmodulin (14, 15).

Early investigation into the toxicity of ACT showed that ACT-induced cAMP accumulation paralyzes the oxidative burst of human neutrophils (16, 17). Since then, ACT-induced, cAMP-dependent dysfunction of macrophages, dendritic cells, lymphocytes, and respiratory epithelial cells has been described (18–23). The following cAMP-mediated effects occur in some cells at low (ng/ml) concentrations of ACT: cell cycle arrest in J774 macrophages (24), chloride secretion from polarized epithelial cells (25),

and stimulation of Cox-2 expression (26). ACT also has effects, including induction of calcium influx, stimulation of potassium efflux, and formation of oligomeric membrane pores (27–29), that occur independently of cAMP, and these are generally detectable at µg/ml concentrations of ACT. These data demonstrate that the effects of ACT are concentration dependent and occur over a wide range of concentrations; however, the pathophysiologically relevant concentrations for *in vitro* studies are unknown.

Measurement of the ACT concentration at the interface between host cell and bacterium during infection is limited by multiple factors. Because fatal pertussis occurs most frequently in infants and most cases are nonfatal (30, 31), acquisition of respiratory tissue specimens from infected humans is not feasible. Nasopharyngeal aspirates and washes from humans are accessible as diagnostic specimens, but special handling is required in order to obtain a living culture of this fastidious bacterium, and serial sampling from humans can be traumatic to the airway. A variety of animals—mice, rats, guinea pigs, rabbits, and piglets—have been used for the study of pertussis; however, infection of most animals with *B. pertussis* does not result in characteristic coughing and transmission (32), limiting the ability to study pathogenesis with these models.

The recently characterized baboon model mimics human per-

tussis, with prolonged paroxysmal cough and transmission, and allows serial sample acquisition for following the course of infection (33, 34). We hypothesized that infected baboons could be used in conjunction with *in vitro* studies and human specimens to estimate the concentration of ACT present in the airway and at the bacterium-epithelial cell interface during pertussis. ACT was detectable in nasopharyngeal washings from infected baboons, and the levels of ACT (up to 5 ng/ml) paralleled the concentrations of bacteria over the course of infection. In nasopharyngeal specimens from two human infant cases of pertussis, ACT was measurable during infection, at concentrations of up to 20 ng/ml, and bacterial loads were similar to those found in baboons. Additionally, *in vitro* studies of ACT production by *B. pertussis* suggested that the concentration of ACT to which a target cell is exposed when in contact with bacteria is substantially greater than the 20 ng/ml detected in the infant nasopharyngeal specimens. Taken together, the baboon, human, and *in vitro* data suggest that ≥100 ng/ml of ACT may be present at some locations in the airway during infection and that one should use caution in considering the pathophysiologic relevance of studies in which >1,000 ng/ml of ACT is used.

MATERIALS AND METHODS

Materials. All reagents, unless otherwise stated, were purchased from Sigma Chemical Co. (St. Louis, MO).

Strains and growth of *B. pertussis*. *B. pertussis* strain BP338 (wild type; Tohama I) was grown on Bordet-Gengou (BG) agar (Gibco) supplemented with 10% defibrinated sheep blood (Cocalico) for 48 to 72 h at 37°C. Bacteria were transferred to modified synthetic Stainer-Scholte liquid medium (SSM) (35) and grown for 16 to 20 h at 35.5°C to an optical density at 650 nm (OD_{650}) of 0.6 to 0.8. Bacteria were pelleted by centrifugation at 4,300 × *g* for 10 min, washed in SSM, and resuspended to an OD_{650} of 0.8, yielding approximately 2×10^9 to 4×10^9 CFU/ml. For *in vitro* experiments (see Fig. 4A and B and 5), the bacterial concentration was estimated based upon the OD and was measured by plating serial dilutions on BG agar plates, followed by determination of CFU/ml. For this reason, ranges of bacterial concentrations are provided for some data sets.

B. pertussis strain D420 (clinical isolate; Centers for Disease Control) was grown on BG agar supplemented with 1% proteose peptone (Becton Dickinson) and 15% defibrinated sheep blood.

Handling and infection of baboons. Animal procedures were performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, in accordance with protocols approved by the Center for Biologics Evaluation and Research Animal Care and Use Committee and the principles outlined in the *Guide for the Care and Use of Laboratory Animals* (36). Baboons were obtained from the Oklahoma Baboon Research Resource at the University of Oklahoma Health Sciences Center. The housing and biocontainment unit was designed for study of airborne transmission of pertussis, with unidirectional airflow from directly inoculated (index) subjects to naïve subjects, as previously described (33, 34). The walls of cages of naïve subjects were mesh on the ends facing index cases in order to allow free airflow. The walls of naïve subject cages facing other naïve subject cages were steel, preventing exposure between naïve subjects. Air flowing across naïve subjects exited the room through a perforated wall, was processed by high-efficiency particulate air (HEPA) filtration, and was returned to the end of the unit housing the index subjects.

Inocula were prepared from *B. pertussis* strain D420, which was resuspended in phosphate-buffered saline (PBS) to a concentration of 1×10^{10} bacteria/ml. Baboons were anesthetized with 10 to 15 mg of ketamine/kg of body weight, administered intramuscularly. For direct inoculation, the pharynx was swabbed with 2% lidocaine solution, and animals were in-

tubated using a 2- to 3-mm endotracheal tube to deliver a 1-ml inoculum to the top of the trachea. A 24-gauge, 3.2-cm intravenous (i.v.) catheter (Abbocath) was used to deliver a 0.5-ml inoculum to the back of each naris. Animals were placed in a sitting position for 3 min, returned to their cages, and observed until they recovered from anesthesia.

Prior to collection of blood and nasopharyngeal wash samples (NPW), baboons were anesthetized with ketamine. Whole blood was evaluated for the number of circulating white blood cells (WBC) by complete blood count (CBC). The back of each naris was flushed with 1 ml phosphate-buffered saline, using a 24-gauge, 3.2-cm i.v. catheter. The recovered nasopharyngeal washes from both nares were combined, and 100 μl of the recovered sample was divided and plated onto Regan-Lowe plates for determination of the number of CFU per plate after incubation at 37°C for 4 to 5 days. An additional NPW sample was frozen at −80°C for measurement of AC enzyme activity.

Production and purification of ACT. *Escherichia coli* XL-1 Blue cells (Stratagene, La Jolla, CA) containing pT7CACT1 were grown as previously described (37). Cultured bacteria were centrifuged and the resulting pellet resuspended in 50 mM Tris, pH 7.5, sonicated, and extracted with 8 M urea. Urea-extracted ACT was purified on a DEAE ion-exchange column and a calmodulin affinity column as described previously (38). ACT was stored at −80°C in 8 M urea, 10 mM Tricine, 0.5 mM EDTA, 0.5 mM EGTA, pH 8.0.

Recombinant ACT produced in *E. coli* exhibits catalytic (AC enzyme) and invasive (ability to raise intracellular cAMP) activities equivalent to those of ACT produced by *B. pertussis*. Expression of ACT in *E. coli* does result in reduced hemolytic activity due to differential posttranslational fatty acylation of the RTX domain (39), but this did not affect the measurements of ACT activity used in the studies presented here.

Measurement of AC enzymatic activity. AC enzymatic activity was measured by conversion of [α-^{32}P]ATP to [^{32}P]cAMP in a cell-free assay as described previously (14). Briefly, the reaction was carried out at 30°C and continued for 20 min, with a final reaction volume of 60 μl. Each assay mixture contained 60 mM Tricine, pH 8.0, 10 mM $MgCl_2$, 2 mM ATP (with 2×10^5 to 5×10^5 cpm of [α-^{32}P]ATP), and 1 μM calmodulin. The reaction was terminated by the addition of 100 μl of a solution containing 1% sodium dodecyl sulfate (SDS), 20 mM ATP, and 6.25 mM cAMP (including 15,000 to 20,000 cpm of [^{3}H]cAMP per tube for calculation of recovery). Cyclic AMP was quantified by the double-column method of Salomon et al. (40).

The AC enzymatic assay was used to calculate the ACT concentrations in NPW and bacterial growth media. AC activities of 6 concentrations of purified, soluble ACT (ranging from 0.064 to 80 ng/ml) were measured, linear regression was performed using GraphPad Prism 4, and ACT concentrations were determined based upon the measured AC activity in the NPW or bacterial medium samples. A similar concentration curve for purified, soluble ACT was constructed for each experiment, and an example is shown in Fig. 1.

Cell culture. J774 cells, a murine macrophage cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Gibco) and 10% heat-inactivated fetal bovine serum (FBS-HI). Cells were grown at 37°C in 5% CO_2.

Measurement of intracellular cAMP. J774 cells were plated the day before the experiment at 40,000/well in DMEM plus 10% FBS-HI. ACT, bacteria, or NPW solution (from infant 2) was added directly to the cells and incubated at 37°C for the indicated times. Cells were washed and lysed, and cAMP was measured using a Tropix enzyme-linked immunosorbent assay (ELISA)-based cAMP assay kit (Applied Biosystems). Cell protein was measured, and data are expressed as pmol cAMP/mg cell protein. Under the conditions used for these experiments, there was no cell death.

Acquisition of clinical specimens. Samples from humans were obtained from the University of Virginia Clinical Laboratory as discarded specimens and are referred to as NPW, although the technique for collection was not controlled by the investigators. Fluid from human NPW was
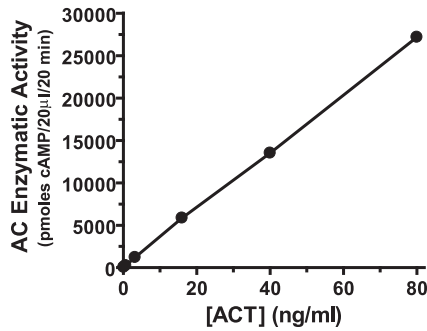
FIG 1 Enzymatic activity of serial dilutions of purified ACT for determination of concentrations of ACT in NPW and bacterial growth media. The AC enzymatic activities of 6 concentrations of purified ACT (ranging from 0.064 to 80 ng/ml) were measured, linear regression was performed, and ACT concentrations in NPW or bacterial medium samples were determined based upon the measured AC activities in the samples. A similar concentration curve for purified, soluble ACT was constructed for each experiment. Shown is a representative graph with means and standard deviations for duplicates from one set of samples ($r^2 = 0.9993$) (error bars are not visible, and the lowest point is obscured due to the linear y axis scale).

cultured on BG agar supplemented with cephalexin (40 µg/ml), and the remaining fluid was stored at −80°C until testing for AC enzyme activity and ACT invasive activity (intracellular cAMP generation). The sample from infant 1 was refrigerated for 3 days prior to culture and freezing. The sample from infant 2 was cultured and frozen on the same day as acquisition of the sample.

PCR. For calculation of bacterial concentrations (CFU/ml) by linear regression (see Fig. 6), serial dilutions of BP338 were made in SSM. For extraction of DNA, 200 µl of the specimen of interest was combined with an equal volume of Bartels ViraTrans medium and heated to 100°C, followed by extraction using a bioMérieux easyMAG instrument. Real-time PCR primers (based upon the *B. pertussis* IS*481* element) and reagents were provided by Luminex (41, 42), and PCR was performed on a Cepheid SmartCycler instrument in accordance with clinical laboratory standards at the University of Virginia Health System.

Histology. Formalin-fixed, paraffin-embedded lung tissues, obtained at autopsy from 3 infants with fatal, laboratory-confirmed *B. pertussis* infection, were evaluated by using an immuno-alkaline phosphatase technique with a rabbit polyclonal anti-ACT antibody (25) (produced by Covance Laboratory and purified by GammaBind Plus Sepharose [GE Healthcare Life Sciences]) diluted 1/100 or monoclonal antibody clone IH2, isotype IgG2a, specific to *B. pertussis* lipooligosaccharide A, as described previously (43).

RESULTS

Measurement of *B. pertussis* concentrations and adenylate cyclase toxin levels in baboons after infection by direct inoculation or airborne transmission. Infection of nonprimates with *B. pertussis* does not result in the prolonged, paroxysmal coughing characteristic of pertussis. Infection of weanling baboons, however, elicits a clinical syndrome similar to typical human pertussis, with cough and leukocytosis followed by recovery after 2 to 3 weeks (34). On day zero, baboons were inoculated with 1×10^{10} bacteria, half intratracheally and half intranasally, and NPW were collected serially (34). The concentration of ACT in NPW was determined as described in Materials and Methods (Fig. 1). As shown in Fig. 2, ACT concentrations reached 1 to 5 ng/ml in the NPW from the three baboons tested. Because secreted ACT is both adherent to the bacterial surface and released (44), AC activity in NPW was measured before and after centrifugation for removal of
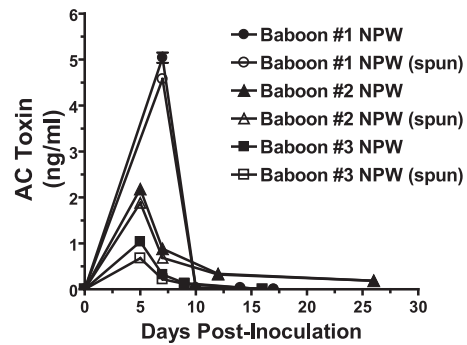

FIG 2 Secreted ACT concentrations in NPW from baboons infected with *B. pertussis*. On day zero, baboons were inoculated with ~10^{10} live bacteria, half intratracheally and half intranasally. NPW were obtained at intervals, and the concentration of ACT was determined (as described in the legend to Fig. 1) on samples without and with centrifugation (spun; $15,000 \times g$ for 15 min) to remove bacteria.

whole bacteria. There was virtually no difference in ACT concentrations whether bacteria were present or not (Fig. 2), indicating that almost all of the ACT detected in the samples was released from the bacteria.

Because baboons infected with *B. pertussis* transmit infection (33), we sought to determine whether transmitted infection results in similar levels of ACT to those in infection caused by direct inoculation. With the baboon housing unit configured for airborne transmission as described in Materials and Methods (33), the index subjects were directly inoculated with *B. pertussis*, and NPW were taken serially from the naïve, exposed baboons at the indicated time points. These NPW were analyzed separately for CFU/ml and secreted ACT (Fig. 3A). Concentrations of ACT were measured after centrifugation to remove bacteria. *B. pertussis* was first detected in NPW on day 11 after exposure for baboon 6 (40 CFU/ml), day 15 for baboon 5 (160 CFU/ml), and day 25 for baboon 4 (300 CFU/ml). NPW from baboon 4 were culture positive for the longest duration, with the highest peak concentrations of bacteria (~1×10^8 CFU/ml) and ACT (1.5 ng/ml). In addition, the increase in peripheral WBC count corresponded to the increases in CFU/ml and ACT concentration in each subject, with a maximum of 26,800 cells/µl in baboon 4 (Fig. 3C). The WBC count serves as an objective indicator of disease severity in unimmunized baboons and parallels the cough frequency as determined from video recordings (34). Figure 3B shows the correlation between ACT concentrations and bacterial levels in all NPW with detectable ACT, indicating a threshold for detection of ACT at ~4.5×10^6 CFU/ml of bacteria.

ACT production by *B. pertussis* in vitro. During prototypical infection of the respiratory tract with *B. pertussis*, bacteria are in close proximity to ciliated epithelial cells (43, 45–48). Respiratory mucus production and adherence of bacteria may impair the ability for saline washes to remove bacteria and toxin from the respiratory lining (45). Therefore, it is difficult to know whether the amount of ACT in the NPW reflects the concentration at or near the bacterium-epithelium interface in the nasopharynx. For this reason, we asked whether the amounts of bacteria found in NPW could produce levels of ACT in vitro comparable to those in NPW. From work previously performed in our lab, we knew that released ACT, rather than bacterial surface-associated toxin, is re-
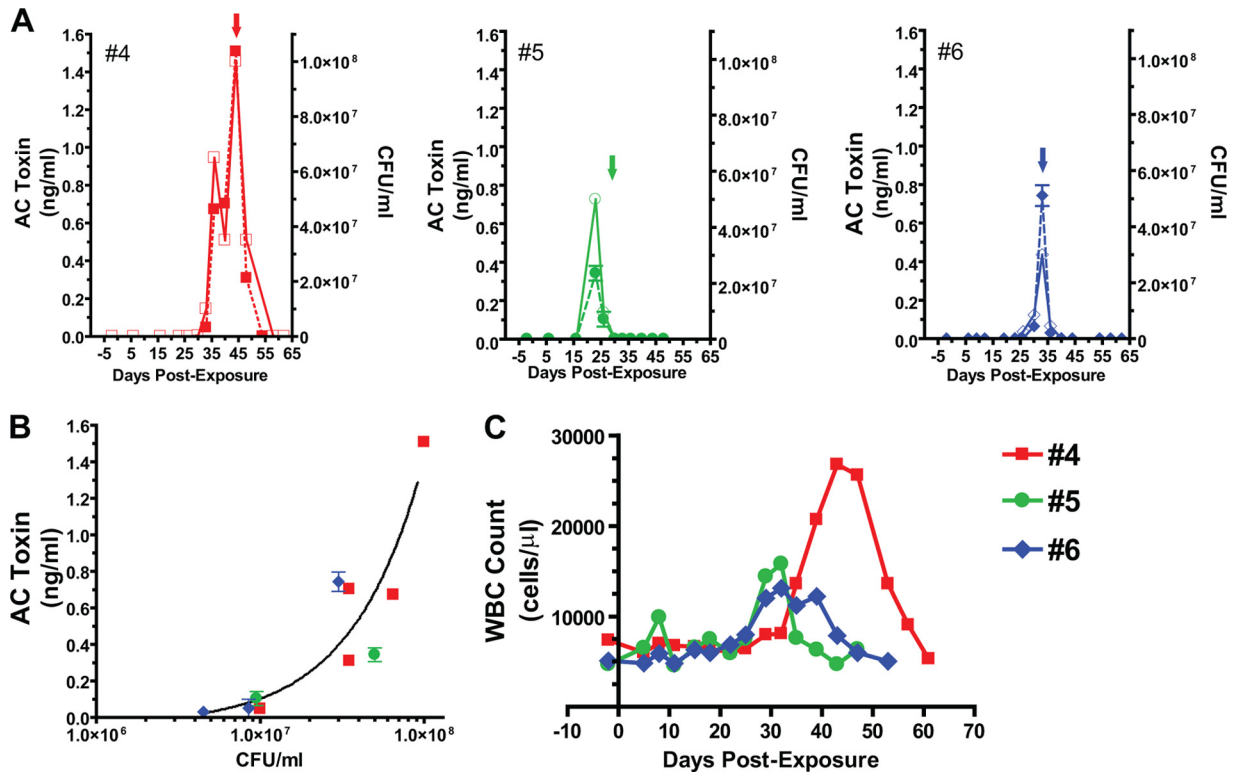
FIG 3 ACT concentrations in NPW from baboons infected with *B. pertussis* by airborne transmission. (A) NPW from 3 secondarily infected baboons (housed with index baboons that were directly inoculated with pertussis on day zero) were collected at intervals, and CFU/ml (open symbols) were determined. The samples were then centrifuged to remove bacteria, enzymatic activity was measured, and the concentration of ACT (closed symbols) was determined (as described in the legend to Fig. 1). Arrows show the day of peak WBC count, corresponding to panel C. (B) Correlation between ACT concentrations and bacterial numbers (CFU/ml) for the data presented in panel A. Symbol colors correspond to the baboon numbers (red for baboon 4, green for baboon 5, and blue for baboon 6). (C) WBC counts were determined over the course of infection for the 3 baboons in panel A. The day on which the WBC counts peaked for each baboon is indicated as an arrow in panel A.

sponsible for intoxicating cells (49), but the kinetics of secretion had not been characterized under these conditions.

ACT production *in vitro* was examined using concentrations of bacteria similar to those found in the NPW during baboon infection. Wild-type *B. pertussis* (BP338) was grown in SSM, washed, and added to DMEM plus 10% FBS-HI at $\sim 2 \times 10^8$ (1.6×10^8 to 2.5×10^8) CFU/ml. AC activity was measured at the indicated time points (Fig. 4A), and the value at time zero (1.2 ± 0.4 ng/ml, which represents ACT associated with the bacterial surface) was subtracted from the total to determine the amount of newly se-
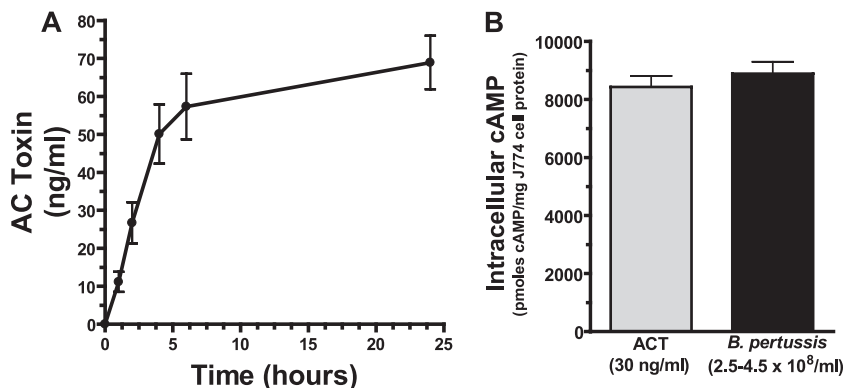

FIG 4 ACT production by *B. pertussis* in vitro. (A) *B. pertussis* was grown in SSM and washed, and *B. pertussis* cells (1.6×10^8 to 2.5×10^8/ml) were incubated in DMEM plus 10% FBS-HI. AC enzymatic activity was measured at the indicated time points, and the concentration of ACT was determined (as described in the legend to Fig. 1). The concentration of ACT at time zero was subtracted to give the amount of newly secreted ACT. Data represent the means and standard deviations for 3 individual experiments, each performed in duplicate. (B) *B. pertussis* and ACT at the indicated concentrations were added to J774 cells and incubated for 2 h at 37°C, and intracellular cAMP was measured. Data represent the means and standard deviations for 3 individual experiments, each performed in triplicate.

FIG 5 Increased ability of *B. pertussis* to intoxicate J774 cells when centrifuged onto cells. *B. pertussis* was added to J774 cells and either centrifuged (spun) onto the cells (700 × *g*) or not (unspun) for 10 min at room temperature. Cells were then incubated for 1 h at 37°C, and intracellular cAMP was measured. Data represent the means and standard deviations for 3 individual experiments, each performed in triplicate.
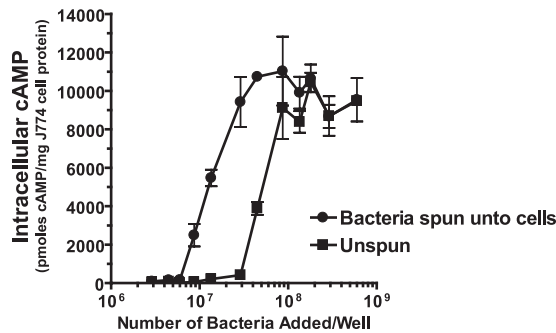
creted ACT. The concentration of secreted ACT increased linearly with time, reaching 21 to 34 ng/ml at 2 h, a plateau at 4 to 6 h, and 60 to 77 ng/ml after 24 h.

To test whether ACT secreted from the bacteria was able to increase intracellular cAMP in target cells under these conditions, we added *B. pertussis* (2.5×10^8 to 4.5×10^8 CFU/ml) to J774 cells for 2 h and found that the level of intracellular cAMP was equivalent to that generated by 30 ng/ml purified ACT (Fig. 4B), a concentration of purified ACT similar to the amount of ACT secreted by this concentration of bacteria in 2 h (Fig. 4A). These data indicate that the amount of ACT measured in bacterial supernatants is active and that activity is not affected substantially by other virulence factors present on the whole bacteria *in vitro*.

We hypothesize that there is a gradient of toxin concentrations, with the concentration decreasing with distance from the bacterium, and that because newly secreted toxin is responsible for generating cAMP in target cells (49), a bacterium closer to a target cell is more effective at raising intracellular cAMP than a more distant one. To address this hypothesis, we centrifuged bacteria onto adherent J774 cells to bring them into close proximity prior to incubation at 37°C. As shown in Fig. 5, when bacteria were centrifuged onto J774 cells, higher levels of intracellular cAMP were generated than when bacteria were not centrifuged onto cells (Fig. 5). By comparing points along the linear segments of the concentration-cAMP curves (Fig. 5), we determined that centrifugation increased cAMP generation approximately 4-fold. As expected, centrifugation of J774 cells in the presence of purified, soluble ACT rather than whole bacteria did not alter cAMP generation under these conditions (data not shown). In summary, these data describe the kinetics of toxin secretion and suggest that the toxin concentration at the bacterium-cell interface *in vivo* is greater than the concentration measured in medium or NPW.

Adenylate cyclase toxin in NPW and tissue specimens from infants with severe pertussis. Many adult patients with pertussis present to physicians after several weeks of symptoms, at which point the residual bacterial burden is believed to be low (1, 5, 50, 51). In children, however, the number of bacteria is generally larger than that in adults at the time of diagnosis, possibly due to earlier presentation and testing, immunization status, or other undefined factors (52–55). The data from baboons indicate that levels of ACT in NPW are proportional to the bacterial concen-

tration and that $\sim 4.5 \times 10^6$ CFU/ml of bacteria is the threshold for detection of ACT in NPW (Fig. 3B). In order to relate these data to human disease, we measured ACT concentrations and bacterial concentrations in NPW from two infants with pertussis.

Infant 1 was a 17-day-old female admitted to the pediatric intensive care unit in respiratory distress after 5 days of coughing with cyanosis and posttussive vomiting. Several older household contacts also had coughs. On admission, the patient was afebrile, the respiratory rate was 68 (normal is <60), oxygen saturation was 97% on room air (normal is >95%), and she exhibited mild suprasternal retractions. Her peripheral WBC count was 53,000 per μl (normal is 5 to 15,000 per μl), and her chest radiograph showed right upper lobe consolidation. She was started empirically on azithromycin. Her WBC count rose to a peak of 102,000 per μl, and she rapidly progressed to severe respiratory distress, requiring mechanical ventilation and extracorporeal membrane oxygenation. She died on hospital day 25 of multisystem organ failure, polymicrobial pneumonia, bacteremia with *Enterobacter cloacae*, and fungemia with *Candida lusitaniae*. Nasopharyngeal fluid obtained on admission was positive for *B. pertussis* by PCR, with a cycle threshold (C_T) value of 17.4. We determined that the specimen contained 12.1 ± 0.1 ng/ml of ACT. No bacteria grew from the specimen, which had not been cultured by the clinical laboratory.

Infant 2 was a 34-day-old female presenting to the pediatrician's office after 1 week of irritability followed by 5 days of afebrile cough, gasping at night, and posttussive emesis. The patient's aunt was a teacher at a school where there were suspected cases of pertussis, and she herself had a cough. The patient was treated empirically with azithromycin, and a nasopharyngeal aspirate was tested for pertussis by PCR. The following day, the patient was admitted to the hospital with respiratory distress. She spent 30 days in the pediatric intensive care unit and was subsequently discharged to home. The PCR C_T value was 19.2, and *B. pertussis* grew on a BG isolation plate. The ACT concentration was 20.1 ± 0.4 ng/ml, and when the sample was centrifuged to remove bacteria, the concentration was 18.4 ± 0.3 ng/ml. Because of the quantity of this NPW which was available, we were able to measure ACT activity (ability to raise intracellular cAMP) by incubating an NPW solution with J774 cells for 30 min at 37°C and measuring intracellular cAMP. Using linear regression, we determined that the ability of purified, soluble ACT to raise cAMP in J774 cells is linear across the range of 3 to 100 ng/ml ($r^2 = 0.9923$) (graph not shown). From this regression analysis, we determined that the NPW sample raised cAMP in J774 cells equivalently to 12.2 ± 1.4 ng/ml of purified ACT. Thus, calculation from the AC activity in the NPW showed 18.4 ng/ml of ACT, of which 12.2 ng/ml was active when added directly to J774 cells.

To estimate the bacterial burdens present during infection of these patients, we correlated quantitative PCR values with CFU of *B. pertussis*. Serial dilutions of bacteria from 4×10^4 CFU/ml to 4×10^9 CFU/ml were made with a laboratory strain of *B. pertussis* (BP338) and tested using the clinical PCR diagnostic test (Fig. 6). By regression analysis, infant 1's C_T value of 17.4 corresponds to 1×10^8 CFU/ml, and infant 2's value of 19.2 corresponds to 7.5×10^7 CFU/ml. These values fall within the range of CFU/ml found in the nasal washings at the peak of infection for baboons 4 to 6, which acquired infection through airborne transmission (Fig. 3). Table 1 summarizes the results of bacteriologic and ACT testing of baboon and human infant nasopharyngeal washes.
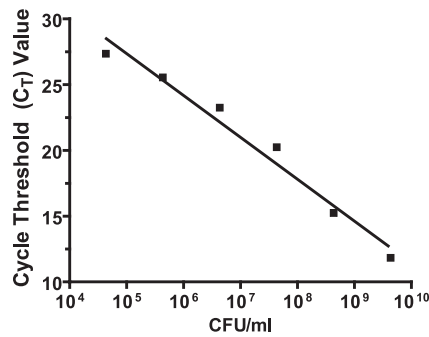
FIG 6 Correlation between *B. pertussis* CFU/ml and *B. pertussis* PCR C_T values for calculation of *B. pertussis* concentrations in infant pertussis cases. The C_T values were determined for a series of dilutions of *B. pertussis* organisms (Tohama I strain BP338) ranging from 4×10^4 to 4×10^9 CFU/ml. Linear regression was performed ($r^2 = 0.972$), and CFU/ml were calculated from the C_T values for the NPW from infants 1 (17.4) and 2 (19.2), which equaled 1×10^8 CFU/ml and 7.5×10^7 CFU/ml, respectively.

Washings from baboons and humans capture material from the nasopharynx, and past studies of pathology from patients with pertussis indicate that the majority of *B. pertussis* bacteria are located in the lumen of the respiratory tract during infection (43, 56). Because ACT has not previously been visualized within the airway, we used an immunohistochemical technique to determine the localization of ACT within the respiratory tracts of three infants who died of laboratory-confirmed pertussis. Shown in Fig. 7 is a representative tissue section from the lower airway of one of these infants. This 1-month-old girl presented with several days of paroxysmal cough, wheezing, and vomiting. She was hospitalized and found to have a peripheral WBC count of 81,100 per µl. Direct fluorescent-antibody testing for pertussis was positive. Therapy with azithromycin was initiated, but she developed respiratory failure, hypotension, and pulmonary hypertension and died approximately 9 days after the onset of her illness. An autopsy revealed bilateral bronchopneumonia, and PCR evaluation of a nasopharyngeal swab and lung tissue was positive for *B. pertussis*. Serial sections of lung tissue were stained by using an immunohistochemical technique for lipooligosaccharide of *B. pertussis* and for ACT, and they demonstrate nearly identical distributions of bacterial antigens and toxin within alveoli and bronchioles. Similar observations were made in the airways and airspaces of two other infants with fatal *B. pertussis* infection (not shown).

DISCUSSION

Difficulty in determining the concentration of a bacterial toxin at its target tissue derives from multiple factors, including the limited number of animal infection models that reflect human dis-

TABLE 1 Summary of baboon and human infant nasopharyngeal specimen data

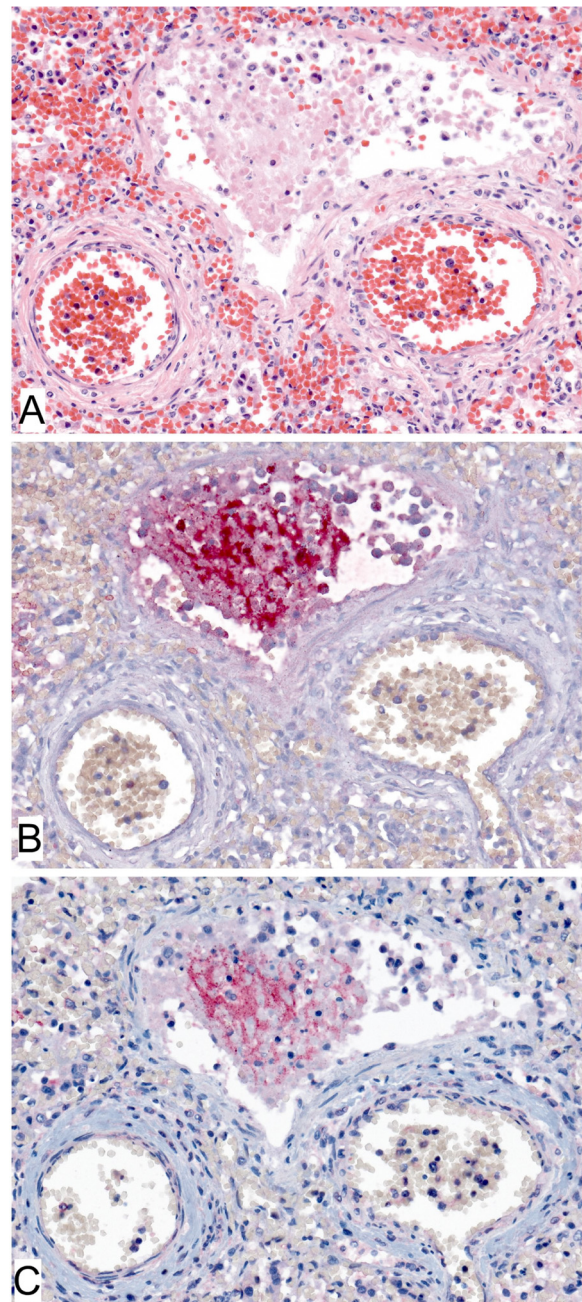| Baboon or infant	*B. pertussis* PCR C_T	Actual CFU/ml	Calculated CFU/ml	ACT concn (ng/ml)
Baboon 4		1×10^8		1.50
Baboon 5		5×10^7		0.34
Baboon 6		3×10^7		0.74
Infant 1	17.4		1.0×10^8	12.1
Infant 2	19.2		7.5×10^7	18.4

FIG 7 Serial sections of lung tissue from an infant with fatal pertussis. Each panel shows a bronchiole (center, top) with two arterioles (below and adjacent to the bronchiole). (A) Hematoxylin and eosin stain. (B) Immuno-alkaline phosphatase technique using a monoclonal antibody to *B. pertussis* lipooligosaccharide (crimson). (C) Immuno-alkaline phosphatase technique using a polyclonal antibody to ACT (crimson). Original magnifications, ×50.

ease, poor access to human tissues, and a paucity of information on the kinetics of toxin secretion. In this study, we set out to determine the concentration of ACT at target tissue by using complementary techniques that address these limitations. The baboon model recapitulates the paroxysmal cough and transmissibility of pertussis and permits sampling from the nasopharynx to document the proliferation of bacteria from the time of acquisition to the time of disease resolution. The data from baboons indicate

that ACT is detectable in NPW, with a threshold at $\sim4.5 \times 10^6$ CFU/ml, and that the concentration of ACT correlates with the bacterial concentration. Measurements of bacterial concentrations in human samples acquired during acute disease showed similar concentrations of bacteria to those in baboon specimens. Production of ACT by this quantity of *B. pertussis in vitro* plateaued at 6 h, at ~60 ng/ml, a concentration similar to that found in the NPW from infant 2 (~20 ng/ml). Considering human and baboon data together with measurements of ACT secretion *in vitro*, we estimate that ACT reaches levels higher than the 20 ng/ml detected in the airway of infant 2. Given the finding that centrifugation of bacteria onto target cells increases cAMP generation relative to that seen without centrifugation, the concentration of ACT at the bacterium-cell interface likely reaches at least 100 ng/ml and may be ≥100 ng/ml in areas of high bacterial concentration. Given that the effects of ACT are concentration dependent and that the concentration of ACT *in vivo* has until now been entirely unknown, this estimated concentration range can serve as a reference point for future *in vitro* studies and suggests that the pathophysiologic relevance of studies of ACT which are performed at concentrations of >1,000 ng/ml should be interpreted with caution.

While the findings in the infants are complementary to the baboon and *in vitro* data, they are clearly limited and thus highlight the importance of the baboon model for investigating pertussis pathogenesis. With the infants, bacterial colony counts were not determined for the clinical specimens, so analysis of quantitative PCR values was used to estimate bacterial concentrations. Nakamura et al. used PCR for measurement of bacterial burdens during pertussis and found that the outcome did not depend upon the strains of pertussis detected in their study population (52), suggesting that variations in the copy number of IS*481* (41, 42, 57–60), the target sequence for the PCR primers, do not substantially affect estimations of bacterial loads. Although there were comparable bacterial and ACT concentrations between these human infants and baboons, it is interesting that these infants progressed to respiratory distress, whereas the baboons recovered uneventfully. Importantly, the samples available in each of these infant cases were taken at the time of presentation, prior to respiratory decompensation, whereas the baboon samples that showed *B. pertussis* levels comparable to those of the infants were taken at the peak of infection. Since only a single sample was available from each of these infant cases, we hypothesize that samples taken later during the course of *B. pertussis* infection would show substantially higher bacterial and ACT concentrations. In addition, the samples from both infants and baboons were taken only from the nasopharynx, and the time of onset of *B. pertussis* pneumonitis in the infants is not known. For this reason, the baboon model is invaluable for future exploration of host and pathogen factors that contribute to disease progression.

There are limitations to *in vitro* studies that are overcome by *in vivo* studies, and *vice versa*. On the one hand, measurements of *in vivo* ACT levels may be reduced by *ex vivo* handling of samples, the presence of mucous and inflammatory contents in specimens, or the inability for saline washes to completely remove bacteria and toxin from the airway lining. The dense hyaline and necrotic debris in the lumen of the pathological sample shown in Fig. 7 does, in fact, suggest that accessibility of ACT in the airway to saline washes may be limited. On the other hand, *in vitro* measurements may be elevated in comparison to *in vivo* conditions due to the use of a different bacterial strain (BP338 versus D420, respectively) or due to culture in growth media compared with the airway environment. An important advantage of this study is the use of several different bacterial isolates: the baboon model was developed with the D420 clinical isolate (33, 34), *in vitro* studies were performed with the Tohama I-derived laboratory strain, and human samples are all potentially unique specimens. The consistency from infections with these different strains under various conditions is notable and reassuring.

B. pertussis secretes multiple virulence factors, most importantly pertussis toxin (PTX) and filamentous hemagglutinin (FHA). FHA interacts with ACT in solution, on the bacterial surface, or indirectly at the target cell (61–64). PTX catalyzes ADP ribosylation of $G_{i\alpha}$, eliminating inhibitory input to cellular adenylyl cyclase, but only in the setting of ongoing inhibition does this result in an increase in cAMP. This point is illustrated by the fact that PTX alone does not increase cAMP in J774 cells. In addition, treatment of cells with PTX for 2 h prior to or in combination with ACT did not affect ACT-induced cAMP levels or cytotoxicity in J774 cells (data not shown). Similarly, ACT-induced cAMP and cytotoxicity in J774 cells were not affected by addition of FHA (data not shown). Furthermore, the adenylate cyclase enzyme assay is specific for measurement of AC enzyme and has been used to quantify ACT activity on the surfaces of whole bacteria (49). For these reasons, we believe that measurements of ACT activity and concentrations *in vitro* or *in vivo* are not altered by the other virulence factors. In fact, Fig. 4 shows that purified recombinant ACT increased cAMP in J774 cells equivalently to *B. pertussis* when *B. pertussis* was incubated with cells for the amount of time required to secrete the same concentration of ACT. This finding also supports previously published data showing that recombinant ACT produced in *E. coli* retains catalytic and invasive activity equivalent to that of ACT produced by *B. pertussis* (39).

These *in vitro* studies not only contribute to our understanding of how much ACT may be present *in vivo* but also highlight several points that are important for future studies of *B. pertussis*. First, because newly secreted ACT is responsible for intoxication of target cells (49), the effects of secreted ACT on cells in culture will depend on how long a target cell is exposed to bacteria. Mouallem et al. have also reported a delay in ACT-induced cAMP generation in Chinese hamster ovary cells exposed to live *B. pertussis* (65). Second, the concentration of ACT at the bacterium-cell interface is higher than that in the medium, and effects of secreted ACT may be missed if bacteria are not closely apposed to target cells during incubation. For example, phagocytosis of *B. pertussis* by neutrophils is very inefficient, with <1% of bacteria in suspension phagocytized by neutrophils in 1 h, but phagocytosis is increased to $\sim10\%$ by centrifugation (63, 66). Studies of neutrophil phagocytosis using centrifugation to bring bacteria into close apposition with the target cell have shown that secreted ACT antagonizes phagocytosis under some conditions, an effect of ACT that would have been missed without concentration of ACT at the bacterium-cell interface by centrifugation.

More broadly, knowledge of bacterial protein toxin concentrations at target tissues is limited, and the techniques presented here may be applicable to other toxins, although there are specific problems associated with individual toxins. Diarrheagenic toxins have well-characterized target tissues and easily accessible human specimens. For instance, the concentration of both cholera toxin and *Clostridium difficile* toxin in stool may be >100 ng/ml during

disease caused by their bacteria (67, 68). However, there is still difficulty in estimating the amount of toxin at the site of infection. The volume of diarrhea and presence of mucins, in the case of cholera toxin (69), may reduce the measured concentration of toxin in liquid stool. Some diarrheagenic bacteria are also attached closely to epithelial cells, and the local concentration at the bacterium-cell interface may be different than the concentration measured in the lumen. Unlike most infections with *Vibrio cholerae* and *Clostridium difficile*, infection with *Bacillus anthracis* is characterized by toxemia. Anthrax lethal factor has been measured in the blood from human cases of cutaneous anthrax, at concentrations up to 1.26 ng/ml (70), and in the blood and pleural fluid of humans and monkeys with inhalational anthrax, at concentrations of >500 ng/ml (71, 72). Measurement of anthrax toxin levels in target organ tissues has not been reported and requires knowledge of the relevant target tissue and a tissue-specific method for toxin quantification.

In summary, estimation of the concentration of a secreted toxin at the site of bacterial infection has been difficult. The combination of an animal model, human specimens, and *in vitro* data used for these studies illustrates how this may be accomplished. The determination of ACT concentration and the understanding of the kinetics of secretion provide reference points for *in vitro* studies. In addition, we have presented the first immunohistochemical imaging of ACT produced by *B. pertussis* during human infection. These human data further validate the baboon model of pertussis as an invaluable tool for studying pertussis pathogenesis and vaccine efficacy.

ACKNOWLEDGMENTS

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