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Bordetella pertussis Adenylate Cyclase Toxin: Intoxication of Host Cells by Bacterial Invasion

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Bordetella pertussis produces extracytoplasmic adenylate cyclase toxin (AC toxin) which penetrates target cells and, upon activation by host calmodulin, generates high levels of intracellular cyclic AMP (cAMP). As a result, bactericidal functions of immune effector cells are impaired. Since a considerable amount of AC toxin is associated with the bacterium, it was proposed that the toxin may be delivered by direct interaction of the organism with the target cells (E. L. Hewlett, M. C. Gray, and R. D. Pearson, Clin. Res. 35:477A, 1987). Incubation of CHO cells with intact _B. pertussis_ led to formation of intracellular cAMP at levels comparable to those produced in CHO cells by equivalent activities of isolated AC toxin. cAMP accumulation induced by the whole bacteria appeared after a lag of 40 to 60 min and reached high levels within 2 to 3 h, whereas adherence of the bacteria proceeded rapidly and reached a maximal level within 80 min. Sera of pertussis patients completely blocked cAMP accumulation induced by the whole bacteria without having a major effect on either bacterial adherence or cAMP production by the AC toxin. Cytochalasins B and D, inhibitors of bacterial invasion, abrogated the cAMP response to the whole bacteria but not the response to the AC toxin. These agents did not affect bacterial adherence. Transmission electron micrographs revealed that _B. pertussis_, within the time course of cAMP induction, invaded CHO cells. We suggest that cAMP induction by _B. pertussis_ is caused by the entry of the whole bacteria into CHO cells rather than by delivery of AC toxin during bacterial adherence. This route of cell intoxication may be relevant to the pathogenesis of whooping cough.

Bordetella pertussis is generally believed to be a noninvasive respiratory tract pathogen that causes whooping cough in humans through the action of several virulence factors (7, 28). These include, among others, an adenylate cyclase (AC) that exhibits unusual properties. Its location in the bacterium is mainly extracytoplasmic, and the enzyme is accessible to exogenous substrate or trypsin (17, 19). Several *B. pertussis* strains release considerable amounts of the enzyme into the culture medium (19). The activity of the bacterial enzyme is greatly stimulated by the eucaryotic Ca^{2+}-binding protein calmodulin (CaM), which is absent in *B. pertussis* (30). Recently, *B. pertussis* AC has been purified (15, 18, 22, 23) and cloned (1, 9), and its gene sequence was determined (9). It encodes a polypeptide of 1,706 amino acids that is secreted by the bacterium by a rather unusual mechanism, which probably does not involve the formation of a periplasmic intermediate (10). *B. pertussis* AC is a toxin that may act by suppressing host defenses. It penetrates into eucaryotic cells, interacts with CaM, and generates high levels of cyclic AMP (cAMP) (2, 14). These levels impair immune effector cell chemotaxis, phagocytosis, superoxide generation, and microbial killing (7, 28). Recently, it was established that *B. pertussis* AC is an essential virulence factor; a *B. pertussis* insertion mutant deficient in AC was shown to be of much reduced virulence in an animal model of pertussis infection (29), and introduction of a cloned AC gene into this mutant restored full virulence (1).

Hewlett et al. have recently proposed that *B. pertussis* AC toxin can be delivered into target cells via direct contact between the organisms and the cell (16). Here we demonstrate that this cAMP accumulation is caused by invasion of target cells by whole *B. pertussis* organisms. As a result, the AC associated with the bacterium gains access to the cell cytoplasm and produces cAMP. This route of cell intoxication has a comparable efficiency to that of the isolated AC toxin and may be relevant to the disease, since it is blocked by sera of pertussis patients.

MATERIALS AND METHODS

Materials. [α-^{32}P]ATP (80 Ci/mmol) and [^{35}S]-L-methionine (1,157 Ci/mmol) were purchased from Radiochemical Center (Amersham, United Kingdom) and from NEM (Dreieich, Federal Republic of Germany), respectively. ATP, cAMP, CaM, cytochalasins B and D, and heptakis(2,6-di-*O*-methyl)-β-cyclodextrin were obtained from Sigma. All other chemicals were of the highest purity available.

Bacterial and cell cultures. *B. pertussis* (Tohama phase I) was passaged twice on Bordet-Gengou agar plates supplemented with 15% (vol/vol) defibrinated sheep blood. The plates were incubated at 36°C for 48 to 72 h prior to inoculation into liquid medium. A modified Stanier-Scholte medium was used for liquid cultures. This medium was composed of the basic Stanier-Scholte medium (25), which was supplemented with 1 mg of bovine serum albumin per ml, 0.1 mg of heptakis(2,6-di-*O*-dimethyl)β-cyclodextrin per ml, 2.5 ng of L-methionine per ml, and 10 μM 2-mercaptoethanol. The methionine was kept as a stock of 2.5 mg/ml in a solution containing 10 mM 2-mercaptoethanol. The bacteria were inoculated into the modified Stanier-Scholte medium at an A_{580} of $\approx$0.05 and grown for 18 to 48 h until an A_{580} of 0.5 to 0.8 was reached. Under these conditions, the AC activity associated with whole bacteria constituted 10 to 20% of the activity secreted by the bacteria into the culture medium. For labeling of *B. pertussis* by [^{35}S]methionine, the bacterial cultures were grown under similar conditions, except that 0.8 mCi of [^{35}S]-L-methionine (42 mCi/ml, 1,157 Ci/mmol) was added to 50-ml cultures. About 12 to 22% of

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TABLE 1. Effects of cytochalasins and of sera on bacterial adherence and on the activities of isolated and bacterium-associated *B. pertussis* AC toxin[a]

Treatment[b] and concn or dilution	Bacterium-associated toxin[c]			Isolated toxin[c]	
	AC activity	Penetration (cAMP accumulation)	Bacterial adherence	AC activity	Penetration (cAMP accumulation)
Cytochalasin B (5 μg/ml)		29	97		105
Cytochalasin D (0.5 μg/ml)		2	99		102
Serum of patient 1					
1:150	101	1	80	109	102
1:500	104	27	92	98	95
Serum of patient 2					
1:150	98	1	79	105	98
1:500	99	34	90	109	110
Serum of unvaccinated infant					
1:50	105	109	98	110	96
Neutralizing serum (guinea pig)					
1:100	6	1	90	1	2

[a] Penetration and CaM-dependent AC activities of the isolated and of the bacterium-bound AC toxins and bacterial adherence were assayed as explained in Materials and Methods. Penetration activities of the isolated and of the bacterium-bound AC toxins were 620 and 310 pmol of cAMP formed per 10^6 CHO cells in 120 min incubation, respectively. The CaM-dependent AC activities of these preparations applied to the various assays were 32 and 33 pmol/min, respectively. The adherence of *B. pertussis* to the CHO cell was 5.4×10^6 cpm of ^{35}S-labeled bacteria attached to 10^6 CHO cells.

[b] Isolated and bacterium-associated AC toxins were preincubated with sera, and CHO cells were treated with cytochalasins as described in Materials and Methods.

[c] All values are expressed as percentages of the control.

the radioactive methionine was incorporated into the bacteria.

Chinese hamster ovary (CHO) cells (ATCC CCL61) were maintained in culture flasks in Dulbecco modified Eagle medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (without antibiotics), under an atmosphere of 8% CO_2. At 24 h before use, the cells were trypsinized, suspended in DMEM containing 10% fetal bovine serum, and plated into 24-well culture plates at a density producing 80 to 90% confluent monolayers.

cAMP and adherence assays. Bacterial cultures were centrifuged at 10,000 × *g* for 20 min, and the resulting culture supernatants were removed and passed through a 0.2-μm-pore-size filter before further use. The bacterial pellet was resuspended in DMEM, washed three times by centrifugation as described above, passed three times through a 21-gauge needle, and then resuspended in DMEM at an A_{580} of 1.0, which corresponded to 2.5×10^9 CFU/ml. A similar ratio between optical density at 580 nm and CFU per milliliter was reported by Steinman et al. (26). The toxic form of *B. pertussis* AC was extracted from bacterial pellet of the recombinant strain BP348pRMB1 (23) by 4 M urea. This form was separated from nontoxic forms of *B. pertussis* AC by gel filtration chromatography, as previously described (14, 22). To assay for cAMP accumulation induced by *B. pertussis* AC and by whole bacteria, CHO cells were washed twice with DMEM and then incubated with one of the following: bacterial culture medium, the toxic form of *B. pertussis* AC, or bacterial suspension. The incubation was performed for the designated times in a final volume of 1 ml of DMEM containing 0.3 mM isobutylmethylxanthine to inhibit cAMP phosphodiesterase. The cells were washed three times with 2 ml of DMEM and extracted with 0.5 ml of boiling 50 mM sodium acetate, pH 4.2. The extracts were spun for 10 min in an Eppendorf centrifuge, and the cAMP content of the cell-free supernatant was determined as previously described (14). To assay for bacterial adherence, CHO cells were incubated with ^{35}S-labeled bacteria and then washed as described above. The washed cells were detached and dissolved by the addition of 1 ml of phosphate-buffered saline containing 1% (wt/vol) sodium dodecyl sulfate. The DNA was pelleted by spinning of the corresponding samples in an Eppendorf centrifuge for 30 min. Then samples of 50 to 100 μl were removed and counted.

AC assay. AC activity was determined in a total volume of 50 μl at 36°C for 15 min. The assay mixture contained 50 mM Tris hydrochloride (pH 7.5), 60 μM $CaCl_2$, 1 mM [α-^{32}P]ATP (specific activity, 40 cpm/pmol), 10 mM $MgCl_2$, and 10 μM CaM. The [^{32}P]cAMP formed was isolated according to the procedure of Salomon et al. (24).

Effects of antibodies and of cytochalasins. Sera of two patients with pertussis and of an unvaccinated infant were used. Diagnosis of pertussis in suspected cases was based on high titers of immunoglobulin A against whole-cell lysates of *B. pertussis* and of immunoglobulin G against pertussis toxin (PT) and the presence of pertussis toxin-neutralizing antibodies (5a). A serum sample of a 4-month-old unvaccinated infant, which did not contain anti-pertussis toxin and anti-AC antibodies (5a), was used as a control. Serum neutralizing both enzymatic and toxic activities of AC toxin was produced in a guinea pig, as previously described (22). The effects of the various sera on bacterial adherence and on the AC and penetration activities of the isolated and bacterium-associated AC toxin were determined after preincubation of the sera with the bacterial suspension and the toxic form of AC. Bacterial suspensions (1 ml in DMEM) were preincubated for 2 h at 4°C, with the sera at dilutions indicated in Table 1. Then, three samples of 10 μl were withdrawn for determination of AC activity. The rest of the bacterial suspension was used for cAMP induction, which was determined as described above. For determination of bacterial adherence, suspensions of ^{35}S-labeled bacteria were used. Preincubation with the various sera and quantification of adherence were performed as described above. The isolated toxic form of the enzyme (6 μmol of cAMP/min/mg) was preincubated at activity of 30 nmol/min with the indicated dilution of the various sera in a final volume of 0.2 ml of DMEM. Then, three samples of 10 μl were withdrawn, diluted 50-fold, and assayed for AC activity. The rest of the sample was used for production of intracellular cAMP in CHO cells, which was quantified as described above.

CHO monolayers were cultured with cytochalasins B and

D at final concentrations of 5 and 0.5 μg/ml, respectively, for 18 h prior to the experiment. Control cells were cultured in the presence of dimethyl sulfoxide at a final concentration in the cell cultures of 1% (wt/vol), which was the final dimethyl sulfoxide concentration in the cytochalasin solutions. The viability of cytochalasin-treated monolayers was similar to that of control cells, as judged by trypan blue exclusion and responsiveness to the AC toxin (Table 1).

TEM. CHO cells, grown on 40-mm-diameter plates to 90% confluency, were incubated with the bacterial suspension, washed four times with 2 ml of DMEM, and fixed in situ for 24 h at 4°C with 2.5% (wt/vol) glutaraldehyde which was dissolved in 0.1 M cacodylate buffer at pH 7.4. Cells were flat embedded in Epon and processed by standard procedures. Sections less than 40 nm thick were examined with a model 410 Phillips transmission electron microscope.

Data analysis. The data presented are from representative experiments repeated two to four times. Within the same experiments, the determinations of cAMP, AC, and adherence differed by less than 8%; all results shown are means of duplicate or triplicate determinations.

RESULTS

Elevation of cAMP in CHO cells by whole *B. pertussis* bacteria. We chose to use CHO cells as targets for the study of cell intoxication by whole *B. pertussis* bacteria for the following reasons: CHO cells can be used as a useful model for bacterial invasion (6), *B. pertussis* efficiently adheres to CHO cells (21), and CHO cells accumulate high levels of cAMP in response to the isolated *B. pertussis* AC toxin (12). Exposure of CHO cells to whole *B. pertussis* bacteria caused accumulation of high levels of cAMP. A comparison of the kinetics of cAMP accumulation induced by *B. pertussis* culture medium containing AC toxin and that induced by intact *B. pertussis* organisms, by using equivalent amounts of enzymatic AC activity, is shown in Fig. 1. As had been described before by us (5) and by others (12), the cAMP accumulation induced by AC toxin was rapid and occurred without any noticeable lag time. The maximal levels obtained were dependent on the toxin concentration used. In contrast, the cAMP accumulation induced by intact bacteria had a lag time of 40 to 60 min, and the maximal levels of cAMP were independent of bacterial AC activity (Fig. 1). The cAMP induced by intact bacteria was not generated by the free toxin which could have been released into the medium during incubation of the bacteria with CHO cells, since such medium elevated cAMP only slightly (data not shown). The bacteria used in this study were grown under conditions which allowed secretion of AC in a highly toxic form. Comparable results to those shown in Fig. 1 were obtained when the isolated AC toxin, at similar levels of AC activity, was used.

The relationship between adherence and cAMP accumulation. To further explore the requirements for cAMP induction by whole *B. pertussis*, we measured bacterial adherence to CHO cells. Adherence of *B. pertussis* preceded cAMP accumulation (Fig. 2). This provides further evidence that cAMP accumulation induced by intact bacteria requires direct contact between the organism and the target cells. The adherence of the bacteria to CHO cells occurred without any noticeable lag time and reached after 80 min a constant level of 100 to 300 organisms attached per CHO cell; similar values were reported by Relman et al. (21).

TEM examination of CHO cell monolayers infected with *B. pertussis*. It has recently been reported that *Bordetella* organisms can efficiently invade HeLa and respiratory epithelial cells in primary cultures (3, 4). To examine whether CHO cell invasion by *B. pertussis* occurs during the time course of cAMP accumulation, we performed TEM of CHO cells exposed to *B. pertussis*. After 10 min of exposure, only a small number of bacteria adhered to the cells (Fig. 3A). The number of adherent bacteria rose dramatically after 120 min of exposure, in accordance with the direct measurement obtained by using ^{35}S-labeled bacteria (Fig. 2). Moreover, after 120 min of cell exposure, many bacteria appeared

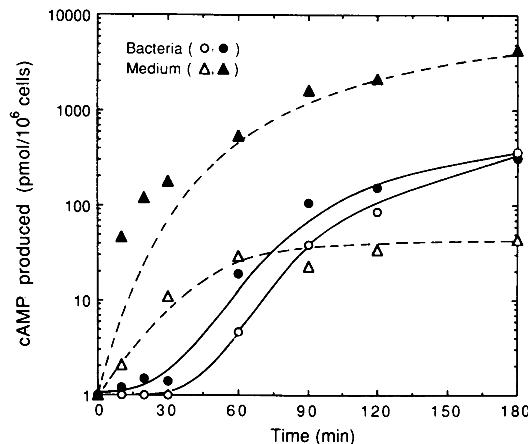

FIG. 1. Time courses of cAMP accumulation induced by intact *B. pertussis* and bacterial medium in CHO cells. *B. pertussis* (Tohama phase I) was grown in modified Stanier-Scholte medium as described in Materials and Methods. Two bacterial suspensions (A_{580} = 1) containing CaM-stimulated AC activities of 32 nmol/min (●) and 1.6 nmol/min (○) were added to CHO cell monolayers (1 ml of bacterial suspension per 10^6 CHO cells). At the indicated times, the amount of intracellular cAMP formed was determined as described in Materials and Methods. Samples of bacterial culture medium containing AC activity of 32 nmol/min (▲) and 1.4 nmol/min (△) were added to the CHO cell monolayers, and the cAMP content was determined.

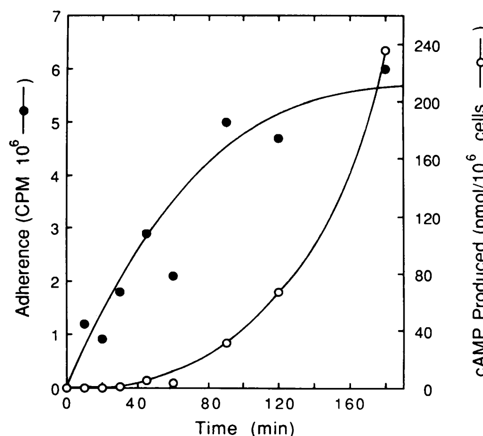

FIG. 2. Time courses of bacterial adherence and of cAMP accumulation induced by intact bacteria. Suspensions of ^{35}S-labeled *B. pertussis* (A_{580} = 1, 2.5 × 10^9 CFU/ml) containing 5 × 10^7 cpm/ml and AC activity of 8 nmol/min were incubated with CHO cell monolayers (1 ml of suspension per 10^6 CHO cells). At the times indicated, bacterial adherence and cAMP accumulation were determined as described in Materials and Methods. The number of bacteria adhering to CHO cells can be calculated at each time point by multiplying the cpm value by the factor of 5 × 10^{-5}.

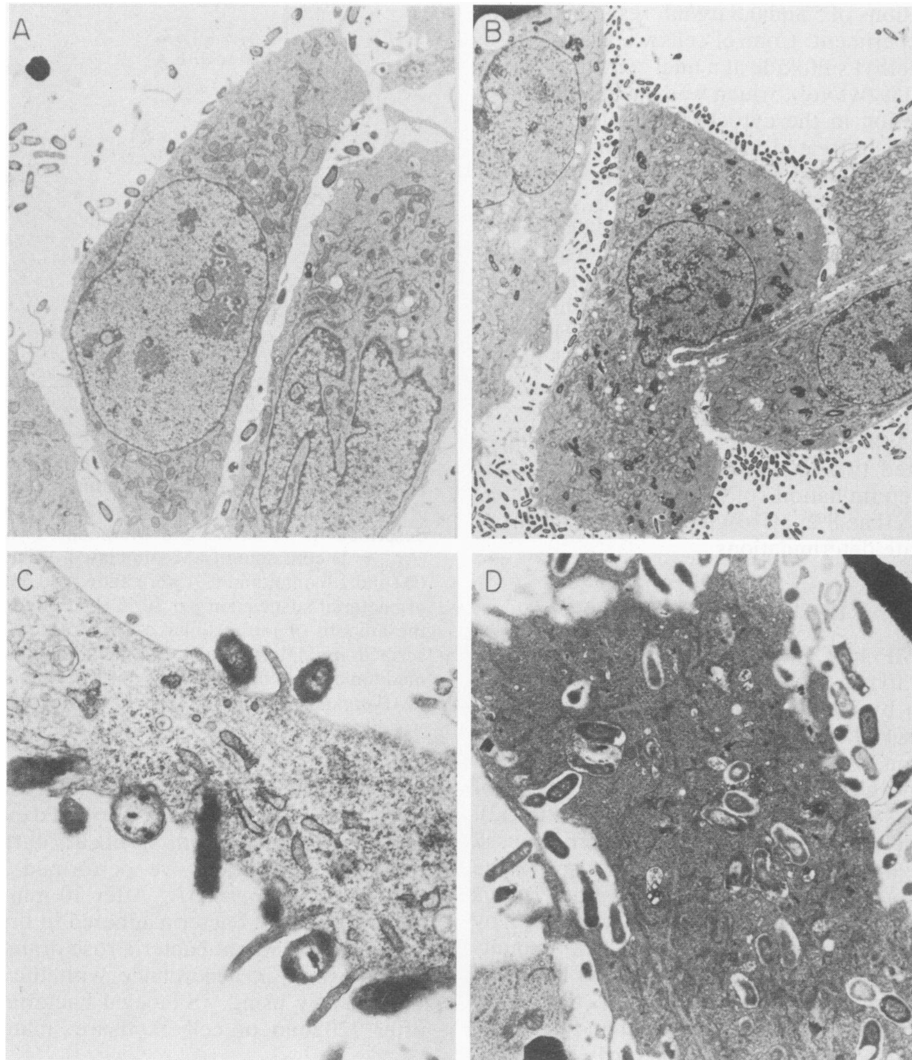

FIG. 3. Transmission electron micrographs of CHO cell monolayers infected with *B. pertussis*. Transmission electron micrographs following 10 (A) and 120 (B) min of coincubation of *B. pertussis* and CHO cell monolayers are shown. (C) *B. pertussis* during its process of entry into CHO cells at 30 min of coincubation; (D) intracellular bacteria within endocytic vacuole at 2 h of coincubation. Magnifications, ×3,000 (A); ×2,000 (B); ×14,000 (C); and ×4,000 (D).

within endocytic vacuoles (Fig. 3B). As observed by Ewanowich et al. (4), invasive *B. pertussis* organisms entered target cells through an endocytic process while being embraced by outstretched microvilli of the cell, which ultimately met and fused, reforming the continuous plasma membrane (Fig. 3C). Most of the bacteria appeared singly within the vacuoles; however, vacuoles containing two or more bacteria could be detected (Fig. 3D).

Inhibition of cAMP accumulation by cytochalasins and sera of pertussis patients. To test whether invasion of CHO cells by *B. pertussis* is responsible for cAMP accumulation in these cells, we used cytochalasins which inhibit microfilament formation, thereby blocking phagocytosis and cell movement (20). Several groups demonstrated that cytochalasins inhibit internalization of invasive bacteria (6), and recently Ewanowich et al. (4) demonstrated that these drugs also inhibit entry of *Bordetella parapertussis* into HeLa cells. Treatment of CHO cells with cytochalasins B and D did not alter the responsiveness of the cells to the isolated AC toxin; however, it effectively blocked their intoxication by the intact bacteria. In contrast, the adherence of *B. pertussis* was not affected by cytochalasin treatment (Table 1). This suggests that invasion of CHO cells by *B. pertussis* is required for cAMP induction by intact bacteria. Neutralizing polyclonal anti-*B. pertussis* AC antibodies raised in a guinea pig (23) completely blocked cAMP accumulation induced by whole bacteria. However, invasion of CHO cells by antibody-treated organisms did not appear to be significantly affected, as judged by TEM (data not shown). Therefore, the AC of the invading *B. pertussis* may be directly responsible for the cAMP production (Table 1). Sera of pertussis patients, at dilutions of 1:150 and 1:500, inhibited cAMP accumulation induced by intact bacteria but only slightly decreased bacterial adherence (Table 1). In contrast, a serum sample of an unvaccinated infant had no effect on cAMP accumulation or on bacterial adherence (Table 1). However, patient sera did not completely prevent appearance of bacteria within CHO cells, as studied by TEM (data not shown). In addition, the patient sera did not neutralize

either enzymatic or penetrative activities of the isolated AC toxin (Table 1) (5a).

DISCUSSION

A CaM-dependent AC activity is detected both in *B. pertussis* culture medium and in intact bacteria. The toxic form of AC, i.e., the form able to penetrate target cells and catalyze the formation of cAMP, can be extracted from *B. pertussis* bacteria. This form of the enzyme appears in the medium only under certain culture conditions. Under these conditions, proteolytic cleavage of the holotoxin (to yield nontoxic forms) is probably prevented (13). Hewlett et al. proposed that AC toxin may be delivered into cells by direct contact with whole bacteria (16). Exposure of macrophage-like cells (J744.1A) to *B. pertussis* but not to bacterial culture medium resulted in a dose-dependent cAMP accumulation (16). We used nonprofessional phagocytes as target cells and grew the bacteria under conditions in which toxic AC activity was found both in culture medium and in association with bacterial cells. This allowed us to compare intoxication of cells by the isolated AC toxin with that by intact bacteria. We found that cell intoxication by whole bacteria is as efficient as intoxication by the isolated toxin. The intoxication by intact bacteria may be caused by the entry of the whole bacterium into the target cell, rather than by delivery of the toxin during bacterial adherence. This is based on the observation that cytochalasins prevented cAMP accumulation without affecting bacterial adherence. Since some bacteria were detected within CHO cells in the presence of patient sera, we cannot rule out the possibility that these sera interfered with the intracellular toxin activity and did not completely prevent the entry of the bacteria.

The slightly delayed cAMP accumulation induced by intact bacteria may be due to the time required for the entry of the bacteria, their possible processing within the host, or both. A role for intracellular processing is suggested by the observation that bacterial suspensions with the same number but with different enzymatic AC activities evoked a similar cAMP response (Fig. 1).

The route of cell intoxication by AC toxin during whooping cough is not known. In a previous study, we have demonstrated that sera of pertussis patients containing high titers of anti-*B. pertussis* AC antibodies did not neutralize enzymatic or toxic activities of the enzyme (5a). Ewanowich et al. have recently demonstrated that *Bordetella* organisms efficiently invade ciliated human respiratory cells (4). Prevention of AC intoxication, mediated by intact bacteria, by sera of pertussis patients supports the possible relevance of this route to human disease. Although effectively preventing cAMP accumulation mediated by intact bacteria, these sera did not affect bacterial adherence. Tuomanen et al. reported that sera of convalescent pertussis patients can inhibit bacterial adherence to human tracheal cells in culture (27). However, this inhibitory effect was obtained at serum dilutions much lower than the serum dilutions used in this study (Table 1). It is noteworthy that a genetic locus, *inv*, which affects entry of *Salmonella typhimurium* into epithelial cells but not bacterial adherence has recently been identified (8).

Ewanowich et al. demonstrated that *B. pertussis* AC-deficient mutants invade HeLa cells more efficiently than wild types and that the increase in invasion was eliminated by elevation of cAMP (3). These investigators proposed that *B. pertussis* invasion may provide a mechanism for intracellular bacterial survival to maintain a reservoir for transmission of pertussis (3). Since cAMP accumulation induced in target cells by intact bacteria is as effective and almost as rapid as the intoxication induced by the isolated AC toxin, we propose that it may play a more direct role in the pathogenesis of whooping cough. Ciliostasis, which occurs at the very early stages of pertussis infection, may not be solely attributed to the action of tracheal cytotoxin (28). It is possible that because of invasion of ciliated respiratory epithelial cells by intact bacteria and intoxication by the AC toxin, the cilia cease to beat, and only later on are they damaged by the action of the tracheal cytotoxin (11). A definite elucidation of the relative roles of cell intoxication induced by AC toxin in its isolated form and the form associated with the bacterium must await the construction of mutants capable of presenting the AC to the outer membrane of the bacteria but lacking the ability to secrete it.

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