A new assay for the invasive adenylate cyclase toxin of *Bordetella pertussis* based on its morphological effects on the fibronectin-stimulated spreading of BHK21 cells

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When baby hamster kidney (BHK) cells are allowed to spread on fibronectin-coated substrata in the absence of serum and the presence of agents which elevate intracellular 3′:5′-cyclic AMP (cAMP) levels they adopt an abnormal, stellated morphology. To determine whether the invasive adenylate cyclase (AC) toxin of *Bordetella pertussis* induced the same response, cell extracts were prepared from several *B. pertussis* strains. They were characterized for AC toxin production by enzymic assay and by immunoblotting with an AC-toxin-specific monoclonal antibody. Extracts of strains producing AC toxin induced elevated levels of intracellular cAMP in BHK cells and promoted a stellation response during cell spreading. Extracts prepared from strains defective in AC toxin production showed no effect. Using image analysis to quantify the morphological change, we have demonstrated that the effect of AC toxin on cell spreading is dose dependent. This technique is a rapid and sensitive assay for the invasive AC toxin.

**Keywords:** *Bordetella pertussis*, adenylate cyclase toxin, fibroblast, cell shape

**INTRODUCTION**

The adenylate cyclase (AC) of *Bordetella pertussis* is a secreted toxin which has been implicated in the pathogenesis of whooping cough (Weiss et al., 1984; Goodwin & Weiss, 1990). The toxin is a protein of 1706 amino acid residues with an apparent molecular mass of 210 kDa in SDS-PAGE. It is a bifunctional protein composed of two domains (Glaser et al., 1988a; Rogel et al., 1989; Hewlett et al., 1989): an N-terminal domain of 400 amino acid residues with AC activity which is strongly stimulated by the eukaryotic regulatory protein calmodulin (Wolff et al., 1980; Ladant et al., 1989); and the remainder of the molecule, which confers haemolytic activity to the toxin (Bellalou et al., 1990). The haemolysin domain has structural and functional homology to the pore-forming (RTX) toxins produced by other Gram-negative bacteria, including the α-haemolysin of *Escherichia coli* and the leucotoxin of *Pasteurella haemolytica* (for reviews, see Welch, 1991; Coote, 1992). Both the pore-forming and enzymic activities of AC toxin are required for its effects on a range of mammalian cells, which result from a dramatic increase in intracellular 3′:5′-cyclic AMP (cAMP) (Hewlett et al., 1989; Rogel et al., 1989; Bellalou et al., 1990).

Production of AC toxin requires the expression of at least five genes which are coordinately regulated at a single (yac) locus (Glaser et al., 1988a, b). A 210 kDa protein which has enzymic activity but lacks pore-forming and cell-invasive activities is encoded by the ycaA gene (Glaser et al., 1988a; Brownlie et al., 1988; Rogel et al., 1989). The mature toxin is formed by a post-translational modification of CyaA which requires the product of the yacA gene (Barry et al., 1991; Sebo et al., 1991). The remaining genes (ycaB, ycaD and ycaE) are essential for export of the toxin from the cytoplasm (Glaser et al., 1988b).

Study of the cell-invasive property of the AC toxin is hampered by the lack of a convenient assay. The most widely used assay for AC toxin relies on direct measurement of intracellular cAMP following incubation of eukaryotic cells with the toxin (Confer & Eaton, 1982;
Hanski & Farrel, 1985; Gentile et al., 1988). Alternatively, the intracellular AC enzymic activity can be measured following the removal of non-penetrating AC by treatment of the cells with trypsin (Hanski & Farrel, 1985; Bellalou et al., 1990; Rogel et al., 1991; Sebo et al., 1991). These procedures are expensive, time consuming and technically demanding. Other assays for AC toxin have been based on its pore-forming or haemolytic activity. Erythrocytes are incubated with the toxin, and the pore-forming activity is quantified by measuring haemoglobin release (Bellalou et al., 1990; Rogel et al., 1991). Assay procedures have also exploited the biological effects of AC toxin on different cellular functions such as the morphology of Chinese hamster ovary (CHO) cells (Hewlett & Gordon, 1988) or the respiratory burst of polymorphonuclear leukocytes (Friedman et al., 1987; Leusch et al., 1990). These assays are limited by their relative insensitivity or by a requirement for prolonged incubation.

We have therefore investigated the use of alternative assay systems, and have developed a new assay based on the effect of cAMP elevation on the morphology of fibroblast cell spreading. The adhesion and spreading of animal cells on growth surfaces is mediated by an interaction between membrane receptors (integrins) and attachment proteins which form part of the extracellular matrix (Grinnell, 1978). The best characterized of the attachment proteins is fibronectin, a glycoprotein containing the cellular recognition sequence Arg-Gly-Asp (RGD) (Ruoslathi & Pierschbacher, 1986). Adhesion of fibroblasts to fibronectin-coated substrata is a passive process which proceeds rapidly (< 10 min) in the absence of serum. This is followed by an active, energy-dependent process which leads to the transition from a predominantly round morphology to a flattened cell shape with the characteristic bipolar appearance of fibroblasts. Spreading is initiated by the extension of the plasma membrane into long filipodia which form new adhesive contacts with the substratum. The resulting tension on the membrane stretches the cell into a new shape which is further modified by re-organization of the cytoskeleton (Grinnell, 1978).

Agents which elevate intracellular cAMP levels have a marked effect on the spreading of fibroblasts (Edwards et al., 1993). Baby hamster kidney (BHK) cells allowed to spread on fibronectin-coated substrata in the presence of dibutyryl cAMP and in the absence of serum have an arborized or stellated shape. The cells are characterized by long, branched processes and resemble nerve cells rather more closely than fibroblasts.

In this study, we have investigated the effect of AC toxin on this shape change using cell extracts from AC-producing strains and from AC-defective mutants of B.pertussis. The sensitivity of the response was determined with an AC preparation which had been partially purified by calmodulin-agarose affinity chromatography. We show that cell spreading may be used as a rapid, sensitive and specific assay for the AC toxin.

**METHODS**

**Bacterial strains.** Strains BP348 (Weiss et al., 1984) and BPDE386 (Barry et al., 1991) are insertion mutants derived from the Tohama strain of B. pertussis. Both are deficient in the production of the AC toxin. BP348 carries a Tn5 insertion in the cyaC gene (Glaser et al., 1988a) and expresses no detectable enzymic activity or toxic activity. BPDE386 contains an oligonucleotide insertion which inactivates the cyaC gene product but does not affect expression of cyaA. This strain produces a full-length proteo toxin which has AC-enzymic activity but is non-invasive for S49-lymphoma cells (Barry et al., 1991). In B. pertussis strain BP348(pBRM1), the insertion mutation of BP348 is complemented by pRM1, a recombinant plasmid containing the entire cya locus (Brownlie et al., 1988). Characterization of this strain revealed elevated expression of enzymic and toxic activities (Rogel et al., 1989). Strain 18-323 is the reference strain of B. pertussis which is used for mouse intracerebral challenge and vaccine potency tests.

**Growth conditions.** B. pertussis strains were grown on Bordet-Gengou (BG) medium (Gibco-BRL) containing 20% (v/v) defibrinated horse blood for 72 h at 37 °C in a humid atmosphere. Bacteria harvested from a single lawn plate were subcultured in 1 litre of defined liquid medium (Stainer & Scholte, 1972) in 2 litre dimpled flasks. Strain BP348(pBRM1) was grown in the presence of 10 μg tetracycline ml⁻¹ and 20 μg kanamycin ml⁻¹, and strain BP348 was grown in the presence of 20 μg kanamycin ml⁻¹. The flasks were incubated in an orbital incubator at 150 r.p.m. for 48 h at 37 °C. Cells were harvested by centrifugation at 9000 g for 30 min at 4 °C.

**Production of urea extracts.** Cell pellets (1 g wet wt) were resuspended in 8 ml 10 mM Tricine/HCl, 0.5 mM EDTA, 0.5 mM EGTA, pH 8.0 (buffer A), containing 4 M urea and 1 mM PMSF. Suspensions were stirred for 1 h at 4 °C before centrifugation at 15000 g for 30 min at 4 °C. The supernate was cleared by centrifugation at 160000 g for 1 h, and the resulting urea extracts were stored at −20 °C.

**Calmodulin-agarose affinity chromatography.** The urea extracts (60 ml containing 180–300 mg protein) were diluted with an equal volume of buffer A to give a final urea concentration of 2 M. CaCl₂ was added to a concentration of 2.5 mM before mixing with 10 ml packed calmodulin-agarose (Sigma), as described by Hewlett et al. (1991). The slurry was rotated gently at 4 °C for 2 h. After loading on a column (EconoColumn, 10 x 2.5 cm), the agarose was washed with 3 bed volumes (30 ml) of 10 mM Tricine/HCl, 1 mM CaCl₂, 0.5 M NaCl, pH 8.0 (buffer B), followed by 30 ml buffer A. AC was eluted with 30 ml buffer A containing 8 M urea. Purified extracts were stored at −70 °C for up to 6 months.

**Tissue culture and preparation of cells.** BHK21 clone 13 cells were grown to 60% confluence in Glasgow-modified MEM buffered with 22 mM HEPES, pH 7.4, and supplemented with 2% (v/v) calf serum. Assays were performed in Hanks' HEPES (HH) buffer containing 140 mM NaCl, 5.4 mM KCl, 1.27 mM MgCl₂, 1.3 mM CaCl₂, 5.6 mM D-glucose and 10 mM HEPES, pH 7.4. Hanks' Saline (HS) was the same medium without divalent cations. Monolayers were washed in HS, and cells were removed from culture surfaces by the low-trypsin/EDTA method (Edwards et al., 1975). Cells were aspirated in growth medium and washed twice by centrifugation and resuspension in HH. This procedure produces a single-cell suspension without affecting the rate of attachment and spreading on fibronectin-coated surfaces.

**Cell-spreading assay.** Bovine plasma fibronectin was purified from calf serum by affinity chromatography on gelatin-
Sepharose (Engvall & Ruoslahti, 1977). Cell-spreading assays were performed in 24-well plastic tissue-culture plates. Wells were coated with 0.5 mg fibronectin (25 μg ml⁻¹) in HH for 15 min. The coating solution was replaced with 0.5 ml HH containing 0.5 mg haemoglobin ml⁻¹, and the plates were incubated for a further 15 min before rinsing with HH. Haemoglobin was used to block free adsorption sites. Urea extracts were dialysed twice against 100 ml of 100 mM phosphate-buffered saline, pH 7.2. Cells were stained with Kenacid Blue (0.1% in water/methanol/acetic acid, 50:50:7, by vol.), rinsed twice in water and mounted in Gurr's Clearmount.

**Image analysis.** Images were obtained with the 40 x objective on a Leitz Diavert microscope equipped with a Hamamatsu Vidicon C1000 camera and were digitized to 512 x 512 x 8 bits. The digitized images were analyzed with a program written in Acorn Risc Machine Assembly Language for an Acorn Archimedes digitizer (Watford). Analysis was carried out with a program written in Acorn Risc Machine Assembly Language for an Acorn Archimedes 310 microcomputer (Edwards et al., 1993). This program measures cell perimeter and area and calculates the parameter dispersion as described by Dann & Brown (1986). Dispersion is a measure of how much the cell shape differs from a circle. Cell-spreading assays were performed in duplicate wells. Mean perimeter and mean area values were determined by analysis of > 25 cells per well. A convenient parameter of stellation was calculated by dividing the mean cell area by the mean cell perimeter. Stellation was accompanied by a decrease in the ratio of area to perimeter.

**Determination of intracellular cAMP.** Cells (0.4-0.5 x 10⁵) were incubated with dialysed B. pertussis urea extracts in 0.5 ml HH buffer containing 0.1 mM 3-isobutyl-1-methylxanthine (IBMX) for 1 h at 37 °C. IBMX inhibits cAMP phosphodiesterase and enhances the yield of cAMP from AC-treated cells (Hanski & Farrel, 1985). The cells were chilled on ice for 10 min, washed three times by centrifugation in HH buffer and resuspended in 0.5 ml 50 mM Tris/HCl, pH 7.4, containing 4 mM EDTA. Cells were lysed by boiling for 5 min, and cell debris was removed by centrifugation for 15 min in a microfuge. The cAMP content of the supernate was determined by a modification of the protein-binding assay (Gilman, 1970) as described by Leusch et al. (1990). All invasive AC activities were determined using duplicate cell suspensions, and the cAMP content of each suspension was determined in duplicate. The results are expressed as nmol cAMP per 10⁵ cells (mg bacterial protein)⁻¹ and were calculated from the mean of the four cAMP determinations (Hanski & Farrel, 1985; Brownlie et al., 1988).  

**Haemolysis assay.** Defibrinated sheep erythrocytes were washed by centrifugation and resuspension in HH buffer. Dialysed urea extracts were assayed for haemolytic activity by serial twofold dilution in HH. The dilutions (final volume 0.5 ml) were mixed with an equal volume of a 1% (v/v) sheep erythrocyte suspension and incubated for 180 min at 37 °C. The suspensions were cleared by centrifugation at 1500 g for 5 min at 25 °C. The percentage lysis was determined by measuring the A₅₄₀ of the resulting supernates (100% lysis was determined by measuring the A₅₄₀ after addition of water and saponin to 1%, v/v).

**AC enzymic assay.** AC enzymic activity was determined at 30 °C in 100 μl 25 mM Tris/HCl, 10 mM MgCl₂, pH 7.5, containing 1 μM bovine-brain calmodulin (Sigma) and 1 mM ATP with 10³ c.p.m. of [α-³²P]ATP (Amersham). Reactions were terminated after incubation for 10 min by the addition of 100 μl of a solution containing 1% (w/v) SDS, 20 mM ATP and 625 mM cAMP and immediate transfer to an ice-water bath. The cAMP formed was isolated by the double-column chromatography method of Salomon et al. (1974).

**SDS-PAGE and immunoblotting.** SDS-PAGE was performed according to the method of Laemmli (1970) on 7.5% thick SDS-PAGE gels. For immunoblotting, proteins were separated on 7.5% SDS-PAGE gels, transferred to Hybond C membrane (Amersham) and blotted, as described by Towbin et al. (1979), with the anti-AC monoclonal antibody 9D4 (Hewlett et al., 1991).

**Protein assay.** Protein concentrations were determined by the method of Bradford (1976).

### RESULTS

**AC activity in B. pertussis urea extracts.** Urea extracts were prepared from late-exponential-phase cultures of four B. pertussis strains. These were characterized for AC production by AC enzymic and toxicity assays (Table 1) and by immunoblotting with AC-specific monoclonal antibody 9D4 (obtained from E. Hewlett, Department of Medicine, University of Virginia School of Medicine, Charlottesville, USA). Strain BP348(pRMB1) produced > 10-fold higher amounts of AC enzyme than either strain 18-323 or BPDE386, which is in keeping with previous work (Brownlie et al., 1988). Strain BPDE386 produced comparable levels of AC to the wild-type strain 18-323, whereas BP348 extracts had no detectable activity, even at the highest level tested, and must therefore produce > 1000-fold less enzyme than 18-323. SDS-PAGE and immunoblotting with monoclonal antibody 9D4 confirmed that urea extracts of each of the AC-producing strains contained high levels of full-length 210 kDa protein (Fig. 1). The intensity of staining on the immunoblot was consistent with the enzymic activities.

Before investigating the effect of various extracts on BHK cell spreading, their invasive AC activities were determined by incubating BHK cells with dialysed extracts and measuring intracellular cAMP. Sub-confluent monolayers of BHK cells were routinely trypsinized during the preparation of cell suspensions for spreading experiments. These cells may be removed from the substrata without trypsin by EDTA chelation of divalent metal cations. However, trypsin is required to break the cell-cell contacts and generate a single-cell suspension free of aggregates. Such proteolytic degradation could also destroy cellular receptors for attachment proteins or bacterial toxins. It was therefore important to determine the invasive AC activities of the extracts with the trypsinized suspensions of BHK cells used in cell-spreading assays.

The cAMP content of the BHK cell suspensions treated with the various urea extracts is shown in Table 1. No cAMP could be detected in untreated cells or in cells treated with extracts from strains BP348 and BPDE386. Thus, on the basis of the detection limit of our protein-binding assay, these cells contained < 0.07 nmol cAMP.

Shape-change assay for B. pertussis AC toxin

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Table 1. AC activities in urea extracts of B. pertussis strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Protein concn (mg ml⁻¹)</th>
<th>Enzymic activity* [nmol cAMP min⁻¹ (mg protein)⁻¹]</th>
<th>Toxic activity† [nmol cAMP per 10⁷ cells (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP348(pRMB1)</td>
<td>5.7</td>
<td>4791.93 ± 321.85</td>
<td>339.72 ± 22.94</td>
</tr>
<tr>
<td>BP348</td>
<td>&lt; 0.01</td>
<td>&lt; 0.01</td>
<td>&lt; 0.7</td>
</tr>
<tr>
<td>BPDE386</td>
<td>4.4</td>
<td>436.85 ± 58.03</td>
<td>&lt; 0.7</td>
</tr>
<tr>
<td>18-323</td>
<td>4.4</td>
<td>303.25 ± 26.06</td>
<td>23.93 ± 1.29</td>
</tr>
</tbody>
</table>

* Bacteria were harvested from late-exponential-phase cultures, resuspended in 4 M urea and mixed for 1 h at 4 °C. Suspensions were cleared by centrifugation at 160000 g for 1 h. The supernates were assayed for AC enzymic activity. Results represent means (±SEM) of at least six determinations.
† Toxic activities refer to the intracellular cAMP levels that result from exposure of BHK cells to dialysed B. pertussis extracts for 1 h at 37 °C. Data represent means (±SEM) of four determinations.

The BPDE386 extract had an invasive AC activity at least 30-fold lower than the 18-323 extract, despite having a similar specific enzymic activity. The levels of intracellular cAMP detected were consistent with those obtained previously with other cell types (Brownlie et al., 1988; Rogel et al., 1989; Barry et al., 1991), and demonstrate that trypsin-treated BHK cells are sensitive to AC toxin.

The effect of B. pertussis extracts on fibronectin-induced spreading of BHK cells

Cell suspensions (2 x 10⁴ cells ml⁻¹) were spread on the fibronectin-coated plates in the presence or absence of dialysed urea extracts (200 µg protein ml⁻¹ final concentration). Cell suspension (0.5 ml) was added to each well and the cells were fixed and stained after incubation for 90 min at 37 °C. Fig. 2(a) shows the bipolar fibroblast morphology obtained when BHK cells were spread in HH buffer only. Cells exposed to 1 mM dibutyryl cAMP and 0.1 mM IBMX (Fig. 2b) spread into the aberrant stellate shapes described previously (Edwards et al., 1993). Extracts from strains BP348(pRMB1) (Fig. 2d) and 18-323 (not shown) induced an identical response. In contrast, cells treated with extracts from strains BPDE386 (Fig. 2c) and BP348 (not shown) spread into typical fibroblast shapes, even in the presence of high concentrations of bacterial protein.

Fig. 1. Immunoblot analysis of B. pertussis strains. Urea extracts of B. pertussis strains were separated by SDS-PAGE, transferred to a nitrocellulose membrane and blotted with anti-AC monoclonal antibody 9D4. Equal volumes of extract containing similar protein concentrations were applied to each well. Lanes: A, BP348(pRMB1); B, BP348; C, BPDE386; D, 18-323. Molecular mass standards are indicated on the right margin.

per 10⁷ cells, and the invasive AC activities of these extracts must be < 0.70 nmol cAMP per 10⁷ cells (mg protein)⁻¹. In contrast, extracts of strains BP348(pRMB1) and 18-323 induced very high levels of intracellular cAMP which were directly related to their enzymic activities.

The stellation of BHK21 cells induced by dibutyryl cAMP was quantified using image analysis to measure spread cell area and perimeter. Mean area showed a strong positive correlation with mean perimeter (Fig. 3). The shape change can be quantified by calculating the regression coefficient or, more simply, by dividing the mean cell area by the mean perimeter. Elevation of intracellular CAMP caused a decrease in the ratio of mean area to mean perimeter (Table 2). An increase in the parameter dispersion was also detected, as previously reported (Edwards et al., 1993). The effect of B. pertussis extracts on BHK cell spreading using these parameters is shown in Fig. 4. BP348(pRMB1) and 18-323 extracts at 200 µg ml⁻¹
Shape-change assay for *B. pertussis* AC toxin

Fig. 2. Stellation of BHK cells by *B. pertussis* urea extracts. BHK cells were allowed to spread on fibronectin in serum-free HH buffer in the presence of various agents. After spreading for 90 min at 37 °C, cells were fixed and stained with Coomassie blue. Cells were photographed under brightfield optics at x10 magnification. (a) Control in HH; (b) 1 mM dibutyryl cAMP and 0.1 mM IBMX present; (c) dialysed extract of the AC-defective *B. pertussis* strain BPDE386 (200 µg protein ml⁻¹ final concentration); (d) dialysed extract of the AC over-producing strain BP348(pRMBl) (200 µg protein ml⁻¹). Bars, 200 µm.

Final protein concentration caused a similar reduction in the area:perimeter ratio to that caused by dibutyryl cAMP and IBMX, as expected from the initial visual examination. Cells treated with 200 µg ml⁻¹ of extracts from strains BP348 and BPDE386 could not be distinguished from the untreated controls. These results show that *B. pertussis* extracts containing AC toxin induce stellation of BHK cells.

**Dose dependence of AC-induced stellation**

The previous experiments using crude urea extracts were designed to determine the specificity of the shape-change response for AC. To determine the sensitivity of the cells to AC, we obtained a partially purified preparation, from a urea extract of strain BP348(pRMB1), by a single-step calmodulin affinity chromatography procedure. This preparation had a specific AC enzymic activity of 21.9 µmol cAMP min⁻¹ (mg protein)⁻¹ and a toxic activity for trypsinized BHK cell suspensions of 2.2 µmol cAMP per 10⁷ cells (mg protein)⁻¹. SDS-PAGE analysis and immunoblotting of proteins in crude and purified preparations of strain BP348(pRMB1) are shown in Fig. 5. Calmodulin-agarose chromatography resulted in a 15-fold increase in specific AC enzymic activity and > 60% recovery.

Fig. 6 shows the effect of increasing concentrations of partially purified AC on the morphology of BHK cell spreading. The stellation response, indicated by de-
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**Fig. 3.** Image analysis of spread cell shape. BHK cells were allowed to spread on fibronectin in serum-free HH buffer (○), or HH buffer containing 1 mM dibutyryl cAMP and 0.1 mM IBMX (●). Mean spread cell area and mean perimeter were determined by analysis of > 25 cells for each of two or three replicates. Results represent 15 determinations from seven experiments using separately prepared cell suspensions. Mean area shows strong positive correlation to mean perimeter for cells spread to HH (product-moment correlation coefficient (r) = 0.93; significant at P < 0.01) and dibutyryl cAMP + IBMX (r = 0.98; significant at P < 0.01). The stellation induced by cAMP elevation is indicated by a decrease in the regression coefficient of mean area on mean perimeter from 11.99 for cells spread in HH to 7.22 for cells spread in dibutyryl cAMP + IBMX.

**Fig. 4.** Effect of *B. pertussis* urea extracts on BHK cell spreading. BHK cell suspensions (2 × 10⁴ cells ml⁻¹) were prepared by trypsinization and allowed to spread on fibronectin in the presence of dialysed urea extracts (200 µg protein ml⁻¹ final concentration) of *B. pertussis* strains for 90 min at 37 °C. The mean area:mean perimeter ratio was determined as a convenient measure of cell shape, which decreases in value as the cells become stellated. Bars represent the mean (±SEM) of five determinations from two independent experiments using separately prepared BHK cell suspensions. Cells spread in (A) HH buffer, (B) HH containing 1 mM dibutyryl cAMP and 0.1 mM IBMX, or (C, D, E, F) HH containing dialysed *B. pertussis* extracts [C, BP348(pRMBl); D, BP348; E, BPDE386; F, 18-323].

A concentration of 45.33 µg protein ml⁻¹ was required for 50% haemolysis and 3.48 µg protein ml⁻¹ was required for half-maximal increase in intracellular cAMP.

A crude extract of BP348 was processed by the same affinity chromatography procedure. The resulting eluate had no detectable AC activity and had no effect on BHK cell spreading when assayed in parallel with the purified AC preparation over the same range of protein concentrations. Previous work has shown that a factor in calf serum inhibits the stellation response of BHK cells to dibutyryl cAMP (Edwards et al., 1993). The effect of purified AC on cell spreading in the presence or absence of 2% foetal calf serum (FCS) was determined. FCS (2%)

**Table 2. Stellation of BHK21 cells**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean area</th>
<th>Mean perimeter</th>
<th>Mean dispersion</th>
<th>Area:perimeter</th>
</tr>
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<tbody>
<tr>
<td>HH*</td>
<td>2802.5 ± 231.2</td>
<td>308.6 ± 17.8</td>
<td>0.40 ± 0.03</td>
<td>8.94 ± 0.35</td>
</tr>
<tr>
<td>cAMP†</td>
<td>2156.3 ± 193.2</td>
<td>382.1 ± 23.0</td>
<td>0.88 ± 0.04</td>
<td>5.54 ± 0.27</td>
</tr>
</tbody>
</table>

*BHK21 cell suspensions were allowed to spread for 90 min on fibronectin-coated plates in HH buffer.

†BHK21 cells spread on fibronectin in HH buffer containing 1 mM dibutyryl cAMP and 0.1 mM IBMX.
Fig. 5. Purification of AC toxin. AC toxin was partially purified from urea extracts of B. pertussis strains by calmodulin-agarose chromatography. (a) Proteins in crude and purified extracts were separated by SDS-PAGE and stained with Coomassie blue. Lanes: A, urea extract of BP348(pRMBl); B, eluates from calmodulin-agarose columns loaded with urea extracts of strain BP348(pRMBl); C, strain BP348. (b) Proteins from a duplicate gel were transferred to nitrocellulose and blotted with monoclonal antibody 9D4 (lanes A, B, C: as above). Molecular mass standards are indicated on the right, and the position of the 210 kDa AC protein is indicated by an arrow.

Fig. 6. Dose response of BHK cell stellation for AC toxin. BHK cell suspensions were allowed to spread on fibronectin for 90 min at 37 °C in the presence of various concentrations of an AC toxin preparation from BP348(pRMBl) which had been purified by calmodulin-agarose affinity chromatography. The area:perimeter ratio was determined by image analysis. Results are the means (± SEM) of four determinations from two independent experiments using separately prepared cells. Cells spread on fibronectin in the presence of 1 mM dibutyryl cAMP and 0.1 mM IBMX had a mean area:perimeter ratio of 4.98±0.18 (positive control). An extract of the AC-defective strain BP348 was processed by the affinity chromatography procedure. Cells treated with 3.5 μg protein from the BP348 eluate spread with an area:perimeter ratio of 8.89±0.38 (negative control).

completely abolished the stellation response, even at concentrations of AC > 10-fold higher than that required to induce maximum decrease in the area:perimeter ratio.

DISCUSSION

The stellation of BHK21 cells by B. pertussis urea extracts was dependent on AC toxin. Extracts which induced the stellation response contained the mature, cell-invasive AC toxin, as shown by direct measurement of the cAMP content of BHK cells after exposure to the extracts under similar conditions to those employed in cell-spreading assays. Extracts from strain BPDE386 which contained AC enzymic activity but had no detectable cell-invasive AC activity did not affect cell spreading. Strain BPDE386 produces a 210 kDa AC protein which is believed to be the precursor of the mature AC toxin. The ability to induce stellation is therefore dependent on activation of the proAC toxin.

The possibility that the stellation response required other bacterial proteins to act synergistically with AC seemed unlikely because the shape change induced by the B. pertussis extracts was identical to the response induced by dibutryl cAMP. Also, partially purified AC, prepared by calmodulin-agarose affinity chromatography, induced an identical response to the crude extract. Protein from an extract of the AC-defective strain BP348, which had been prepared using the same purification procedure, had no effect on cell spreading, even when cell suspensions were exposed to > 10-fold higher protein concentrations than that required for maximum stellation by the AC toxin preparation.

The trypsin treatment used to prepare BHK cell suspensions for spreading assays did not appear to modify the susceptibility of cells to AC toxin. The half-maximal invasive AC activity for BHK cell suspensions was similar to that obtained using the myelomonocytic cell-line HL60, which proliferates as a suspension culture and was used without pre-trypsinization (data not shown). It has also been reported that treatment of CHO cells with trypsin does not affect the capacity of AC to elevate intracellular cAMP (Gordon et al., 1989).

Using image analysis to quantify the shape-change response, we have shown that AC causes a linear increase in stellation, although over a narrow range of protein concentrations. Over this range, stellation was accompanied by an increase in mean dispersion and a decrease in the mean area:perimeter ratio. Stellation was visible microscopically at the higher concentrations of AC toxin; however, image analysis revealed that extreme stellation was accompanied by a marked reduction in spread cell area with lower than expected values for mean dispersion. The mean area:perimeter ratio was therefore used as the parameter of shape in this study because it was not affected by the reduction in spread cell area, even at the highest concentrations of AC toxin.

The invasive AC activities of the purified AC preparation were compared using the shape-change assay, the cAMP-elevation assay and the haemolysis assay. In terms of the
amount of protein required for half-maximal invasive AC activity, the shape-change assay is > 11-fold more sensitive than the cAMP-elevation assay and > 150-fold more sensitive than the haemolysis assay.

The cAMP-elevation assay is the standard assay for AC toxin activity. This assay is time consuming, and measurement of intracellular cAMP levels involves the use of radioisotopes (Gilman, 1970). Of the alternative assay systems currently used, the haemolysis assay is the simplest, but it requires prolonged incubation (2-4 h) and up to 10-fold higher concentrations of protein for half-maximal activity than cAMP elevation (Rogel et al., 1991). Although both functions are dependent on activation of CyaA by CyaC, the invasive AC and haemolytic activities may be dissociated by altering physical conditions such as extracellular Ca²⁺ concentration (Rogel et al., 1991) or by mutagenesis of CyaA (Bellalou et al., 1990), and probably require different domains of the CyaA polypeptide.

Following penetration of the target-cell membrane, AC becomes resistant to trypsin treatment. Invasive AC has been quantified by measuring the enzymatic activity of membranes isolated from erythrocytes after treatment with toxin and digestion with trypsin (Hanski & Farfel, 1985; Bellalou et al., 1990; Sebo et al., 1991). However, less than 1% of the applied enzymic activity is internalized (Hanski & Farfel, 1985) and this procedure is relatively insensitive. The standard method for measuring AC enzymic activity requires high-activity [³²P]ATP as a substrate and includes two sequential chromatography steps (Salomon et al., 1974). The membrane-penetration assay offers no obvious advantage over direct measurement of intracellular cAMP.

AC toxin has been shown to inhibit the chemotactic and superoxide responses of polymorphonuclear leucocytes (PMNs) to chemoattractants such as opsonized Staphylococcus aureus (Friedman et al., 1987). The superoxide response can be conveniently quantified by measuring leucocyte chemiluminescence (CL), and inhibition of CL response can be used to assay for AC (Leusch et al., 1990). However, a 64% inhibition of the CL response of human PMNs required > 200-fold more protein than the induction of high levels of intracellular cAMP in Jurkat cells under similar conditions (Leusch et al., 1990).

We have developed a new assay for the invasive AC of Bordetella pertussis based on its morphological effect on the fibronectin-induced spreading of BHK cells. Unlike some alternative assay procedures described above, the stellation of BHK cells can be related directly to elevation of intracellular cAMP. Stellation is therefore an appropriate assay for investigation of the cell-invasive process. The stellation response is not confined to BHK cells and may be observed in other fibroblast cells. We found that CHO cells spread on fibronectin in the presence of AC have a stellated morphology similar to that induced in BHK cells under the same conditions. It should be emphasized that the stellated shapes are very different to the previously described elongation phenotype (Guerrant et al., 1974) induced when CHO cells were treated with cAMP-elevating agents in culture (i.e. in the presence of serum, and after cell attachment and spreading had already taken place).

Elongation of CHO cells has been used as an assay for cholera toxin, the heat-labile toxin of Escherichia coli (Guerrant et al., 1974) and for AC toxin (Hewlett & Gordon, 1988). The cell-spreading assay is different in two important respects. First, the stellated morphology is more distinctive than the cell elongation and is therefore less subjective. Secondly, the CHO-cell-elongation assay requires incubation for up to 24 h for development of the phenotype (Guerrant et al., 1974), whereas attachment of BHK cells to the fibronectin substrata is very rapid and spreading is completed within 1 h (Hughes et al., 1979). The time course for the stellation induced during cell spreading is therefore very similar to the time course for elevation of intracellular cAMP, which occurs without a lag and reaches maximum levels after 15-60 min, depending on the cell type used (Farfel et al., 1987).

Although we have used an image analysis procedure to quantify the shape change, given the dramatic difference between the stellated shapes and the normal fibroblast shape and the narrow dose response curve, a visual scoring system could be used, with the invasive AC activity expressed as a dilution end-point titre.

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