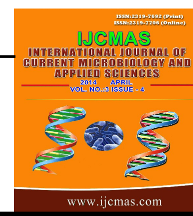

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Original Research Article

Flow cytometry assay of adenylate cyclase toxin (CyaA) preparations of *B.pertussis* on phagocytosis

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Keywords

Bordetella pertussis; childhood respiratory disease; invasive toxin

A B S T R A C T

Bordetella pertussis is the etiological agent of whooping cough, a highly contagious childhood respiratory disease, characterized by bronchopneumonia and paroxysmal coughing interrupted by inspiratory whoops. Two purified forms of CyaA with different enzymic and invasive properties were produced. These were: the native enzymatically-active, invasive toxin (CyaA), an invasive derivative lacking AC enzymic activity (CyaA*). Different concentrations of CyaA and CyaA* were used to investigate dose-dependent effects of the toxins on phagocytosis in U937 human monoblastic cells, J774.2 mouse macrophage-like cells and fresh human granulocyte cells (whole blood used). Important effects were seen with 0.2 mg protein/ml of CyaA. In instance, there was almost complete (80%) inhibition of phagocytosis by J774.2 cells and 70% inhibition of phagocotosis by human granulocyte cells, but CyaA* did not have a significant effect on either. The results of this study showed that both enzymatic and invasive functions are required for the cytotoxic effects of adenylate cyclase toxin.

Introduction

Adenylate cyclase toxin (CyaA) is one of the major virulence factors produced by *Bordetella pertussis*, the whooping cough agent. Among the variety of toxins produced by *B.pertussis*, the adenylate cyclase (CyaA) is a crucial factor in the virulence strategy of the bacteria during the early phases of respiratory tract colonization (Mohammed El-Azami-El-Idrissi et al., 2003; Smith et al., 2001). The toxin allows the pathogen to escape host immune surveillance, mainly by intoxicating neutrophils and macrophages causing phagocyte impotence and inducing macrophage apoptosis (*Confer, and Eaton,* 1982; *Gueirard,* et al., 1998). The role of CyaA in the pathogenesis of *B.pertussis* was clearly demonstrated in the mouse respiratory model. Indeed, genetically modified *B. pertussis* strains deficient in the expression of CyaA were impaired in their ability to induce pulmonary lesions and to cause lethal infection (Khelef, et al., 1994; Weiss, *and*

Goodwin, 1989). Inaddition, CyaA was shown to induce protective immunity against *B. pertussis* lung colonization in a mouse model (Betsou, et al., 1993; Betsou et al., 1995). CyaA is a polypeptide chain of 1,706 aa residues belonging to the RTX (repeat in toxin) family of bacterial toxins. CyaA is synthesized as an inactive protoxin that is converted to an active toxin by posttranslational fatty acylation. The N-terminal part of the protein contains the catalytic domain, whereas the C-terminal part mediates its binding to the target cell membrane and delivery of the catalytic moiety into the cytosol. After membrane translocation, the catalytic domain is activated by Ca^{2+}/calmodulin, thereby acquiring the ability to effectively convert cellular ATP into cAMP (Ladant and Ullmann . 1999; Hewlett, et al., 2006). CyaA can also form cation-selective pores in cell membranes independently of translocation, thereby perturbing ion homeostasis (Benz et al., 1994). CyaA triggers the sustained elevation of intracellular Ca^{2+} through cAMP-dependent, L-type Ca^{2+} channels.

The suppressive activities of CyaA on immune cells have been largely ascribed to its capacity to increase intracellular cAMP (Paccani, et al., 2008; Martín, et al., 2010), which acts as a potent immunosuppressant by interfering with the signaling pathways initiated by immunoreceptors (Taskén, and Stokka . 2006). Upon entry into the cell, the N-terminal AC enzymic moiety is activated by host calmodulin to produce supraphysiological levels of cyclic AMP (cAMP). In immune effector cells, this impairs their phagocytic and bactericidal capabilities and induces apoptosis, features that are assumed to assist survival of the bacterium in the initial stages of respiratory tract colonisation (Gueirard et al., 1998). At high concentrations, CyaA forms pores or channels which makes the toxin cytolytic (El Azami El Idrissi et al., 2002). Anti-CyaA antibodies have been shown to enhance phagocytosis of *B. pertussis* through, neutralisation of CyaA which normally inhibits phagocytosis by neutrophil polymorphonuclear leukocytes (Mobberley-Schuman et al., 2003).

An immune response to this toxin might therefore be useful in preventing colonisation of the host by *B. pertussis*. Immunisation with CyaA, purified from *B. pertussis* or in recombinant form from *Escherichia coli*, protected mice against intranasal challenge with virulent *B. pertussis* (Betsou et al., 1993; Hormozi et al., 1999). In addition, coadministration of CyaA or CyaA*, a derivative lacking AC enzymic activity, with an ACV was shown to enhance the protective effects of an ACV in mice (Cheung et al., 2006).

CyaA requires calcium to acquire a translocation-specific conformation that allows the delivery of the catalytic domain into the cell cytosol (Rogel, A., and Hanski, E. (1992; Rose, et al., 1995) CyaA can penetrate at least to some extent a wide range of cell types, including the mammalian erythrocytes lacking membrane trafficking (Basar, et al., 2001; Bellalou, et al., 1990). However, CyaA toxicity effects such as the abrogation of phagocytic capacity and the induction of apoptosis were mainly elucidated on immune cells, namely neutrophils and macrophages (Khelef, N., and Guiso, N. (1995; Khelef, et al., 1993). In this study, we investigated the role of different concentrations of CyaA and CyaA* on phagocytosis in U937 human monoblastic cells, J774.2 mouse macrophage-like cells and fresh human granulocyte cells (whole blood used).

Materials and Methods

Preparation of Recombinant CyaA From *E. coli*

Twenty mL of an overnight culture of *E. coli* BL21/DE3 containing the relevant plasmids were diluted into 500 mL of LB containing appropriate antibiotics, incubated at 37C with shaking at 200 rpm until an OD600nm of between 0.4-0.45 was obtained (~3 h). Isopropyl-1-thio--D-galactoside (IPTG) was added to a final concentration of 1 mM and shaking continued at 37C for 3 h. Finally, cells were harvested at 10,000 xg for 25 min and the supernatant discarded. Cells pellets could be stored at -20C if necessary.

Expression and Purification of CyaA

E. coli BL21/DE3 (F − *ompT* rB− mB−) was used as the host strain for production of CyaA. The source of plasmids used in this study (pGW44 and pGW54) was described previously (Khosravani et al., 2007; Westrop et al., 1996; Paccani, et al., 2008; Martín, et al., 2010). Co-expression of pGW44 with pGW54 generates fully active acylated, invasive CyaA, pGW44-188, pGW54 generates Non-active AC/ invasive (CyaA*). The recombinant proteins were purified as described previously with the following modifications; the CyaA inclusion bodies were washed twice with 1% (w/v) *N*-octyl -d glucopyranoside (Sigma, Sweden in 20mM histidine buffer (pH 6.0), twice with 2M urea in 20mM histidine buffer (pH 6.0) and once with pyrogen-free water before solubilisation in 8M urea, 20mM histidine buffer (pH 6.0). The solubilised crude CyaA was purified by Q-Sepharose, Germany Amersham) and phenyl-Sepharose chromatography (Figures 1 and 2).

Oxidative Burst Procedure

The phagotest Kit (OPREGEN Pharma; BD Biosciences, Oxford, UK) allows the quantitative determination of leukocyte phagocytosis. It contains fluorescently (FITC- fluorescein isothiocyanate) labelled, opsonized bacteria (*E.coli*-FITC) and measures the overall percentage of macrophages and granulocytes showing phagocytosis in general (ingestion of one or more bacteria per cell) and the individual cellular phagocytic activity (number of bacteria per cell).

The investigation of phagocytosis can be performed either by flow cytometry or by fluorescence microscopy. Because of the quantitative analysis, very accurate work is important, especially when day to day comparisons are required. The detailed instructions result from specific experience and precise validation assays.
The ingestion activity of peripheral human granulocyte cells in whole blood, J774.2 and U937 cell lines was evaluated in the presence and absence of recombinant CyaA protein using the flow-cytometry based Phagotest kit according to the manufacturer's instructions for conjugated *E. coli*. Briefly, 100 µl of whole blood or a volume of 0.4 x 10^6 of J774.2 mouse macrophages or human monoblastic U937 cells were added at the bottom of a 5 ml Falcon tube. Samples were incubated (120 minutes, 37ºC, 5% CO_2) with either CyaA or CyaA* diluted in 8 M urea, 20mM histidine (0.05, 0.1 or 0.2 µg/ml) or PBS, then incubated for another 20 minutes at 37ºC in 5% CO_2 with opsonized *E. coli*-FITC cells (6 cells per leukocyte). A sample with PBS and no Bioparticle and another sample with PBS (120 min) and opsonized FITC-labeled *E. coli* cells (20 min) remained on ice for the whole period of experiment, these acted as negative controls.

At the end of the incubation time all samples were placed on ice in order to stop phagocytosis. A volume of 100 µl of ice-cold quenching solution was added and mixed gently. Then 2ml of washing solution was added to each tube after which the tubes were centrifuged at 4°C for 5 min at 250 x g. The cell samples were incubated in washing solution containing 1% paraformaldehyde for 10 min, washed again and centrifuged as described. Finally, 200 µl of DNA staining solution were added to each tube, mixed and incubated for 10 min on ice. Cells were analysed by flow cytometry (FACS Calibur, BD Biosciences) and 5000 granulocyte events acquired to obtain the percentage and number of cells ingesting bioparticles, as well as their mean fluorescence intensity (MFI) using Cellquest Pro software (BD Biosciences). The nucleated cells were discriminated by a setting in the red fluorescence channel (DNA staining FL2-H,), detecting those events which have the DNA content of a human diploid cell (to exclude bacterial aggregates). The nucleated events were then discriminated; into lymphocytes, monocytes and polymorphonuclear cells by combined measurements of the forward angle light scatter (FSC) and side angle light scatter (SSC). The granulocyte cluster was then gated in the analysis program in the scatter diagram (FSC vs SSC), and its green fluorescence histogram was analysed). The SSC vs FL-1 diagram also was also set to make sure that the appropriate cell population data was collected.

Results and Discussion

Using flow cytometry, the phagocytic ingestion of *E. coli* by human peripheral blood cells from healthy donors, J774.2 mouse macrophages and U937 human monoblastic cells was determined after exposure to different concentrations of CyaA and CyaA*. This technique was developed for the evaluation of phagocytosis activity in human peripheral blood (neutrophils and monocytes) and other cells. Ingestion activity was measured as the mean fluorescence intensity (MFI) produced after 20 minutes of incubation with FITC-conjugated *E. coli*. All tubes were read by flow cytometry at a wavelength of 488 nm after quenching and DNA staining.

Exposure of granulocytes or J774.2 cells to different concentration of CyaA and CyaA* (0.05, 0.1, 0.2 µg/ml final concentrations) resulted in significantly lower mean neutrophil ingestion in the presence of CyaA compared to CyaA* at the same concentrations, and the unexposed controls cells (Table 1 and Figure 1). The results also showed that phagocytosis was significantly impaired by increases in CyaA concentrations for granulocytes and J774.2 cells compared to cells treated with PBS or CyaA*. With 0.2 µg protein/ml of CyaA there was almost complete (92%) inhibition of phagocytosis by J774.2 cells and 63% inhibition of phagocyotosis by human granulocyte cells. CyaA* had no obvious affect on phagocytosis except at the highest concentration of toxin used (0.2 µg/ml) where 10 % inhibition with granulocytes was seen but this was not significant. The flow cytometry histograms that represents the amount of ingested *E. coli,* also indicated the dose-dependent inhibitory effect of CyaA on J774.2 and granulocytes (Fig 1, images 2 and 4), while there was lack of inhibitory effect of CyaA* on J774.2 and granulocytes (Fig 2, images 1 and 3). In similar experiments, undifferentiated and differentiated U937 human monocytes were treated with CyaA

only at 0.1μg/ml. As Table 3.7.1 shows, only differentiated cells were affected by CyaA, showing 42% inhibition of phagocytosis. Overall the results of this assay would imply that inhibition of phagocytosis by CyaA was due mainly to the AC enzymic activity of the protein.

The statistical analysis of data by t test was as follows: CyaA (0.0 5μg/ml) vs PBS, $P < 0.044$,, ; CyaA (0.1, μg/ml) *vs* PBS, $P < 0.004$, CyaA (0.2 μg/ml) *vs* PBS, $p < 0.005$, CyaA* (0.2 μg/ml) *vs* PBS, $p < 0.19$, CyaA* (0.2 μg/ml) *vs* CyaA, $p < 0.001$ Phagocytosis provides a specialised mechanism for regulated ingestion and intracellular destruction of microbial pathogens as well as of apoptotic host cells and debris. In general, professional phagocytes, including neutrophils (which migrate from the blood to a site of infection) and macrophages (which constitutively reside in tissues and are less motile than neutrophils), accomplish most phagocytosis. By confining the mechanisms of microbial killing and digestion to distinct intracellular compartments (lysozomes) of these cells, damage to host cells and tissues is minimised during the process of killing offending microbes. In addition to disposing of microbial pathogens, phagocytosis (especially by macrophages and dendritic cells) initiates the process of antigen processing and presentation for development of adaptive immune responses (Ramachandra et al., 1999).

In order to better understand the importance of this defence mechanism, a flow cytometry assay was developed to study oxidative burst of neutrophils stimulated by bacteria (Khosravani et al., 2013). A flow cytometry investigation was made in order to choose appropriate target cells, opsonisation conditions, and fluorochromes. These authors used FITC-*E. coli* for phagocytosis experiments and the extracellular fluorescence was quenched by trypan blue to differentiate it from that due to intracellular bacteria.

Host defence against *B. pertussis* relies to some extent upon phagocytosis and killing of the bacterial cells. The adenylate cyclase toxin appears to be an important virulence factor against phagocytosis, as evidenced by the efficient phagocytosis of mutants that fail to express this toxin (Weingart, and Weiss, 2000). These authors also showed that CyaA, added separately, blocks phagocytosis even when bacteria are not expressing the toxin.

In this study, different concentrations of CyaA and CyaA* were used to evaluate their modulatory effects on phagocytosis. The results of the ingestion activity experiment suggested that CyaA inhibits the phagocytosis of *E. coli* by J774.2 macrophage-like cells and by human granulocytes as it caused a decrease in the mean fluorescent intensity (MFI) measured by flow cytometry.

Although the concentration of CyaA used in the assay caused appreciable cell killing of J774.2 cells as determined by the MTT assay, it should be emphasised that the effect of CyaA on phagocytosis was measured only in viable cells, by the flow cytometric method here. In contrast, no significant decrease of phagocytosis activity was noted in response to treatment of J774.2 macrophage-like cells or human granulocytes with various concentrations of CyaA*. The poor AC enzymic activity of CyaA* is presumably responsible for the lack of effect on phagocytosis activity in these cells, when compared with CyaA.

Table.1 Suppression of phagocytosis activity of granulocytes, J774.2 and U937 cells by CyaA preparations

Toxin conc (µg/ml)	% Inhibition of J774.2 cells (mouse macrophages)	% Inhibition of granulocytes (Human peripheral blood)	% Inhibition of U937 cells (human monocytes) (without PMA)	% Inhibition of U937 cells (human monocytes) (with PMA)
CyaA (0.05)	26	28	0	18
CyaA (0.1)	79	58	0	42
CyaA (0.2)	92	63	0	59
CyaA* (0.05)	0	0		
CyaA* (0.1)	0	0		
CyaA* (0.2)	0	10		
PBS	0	0		

Figure 1

Image 1

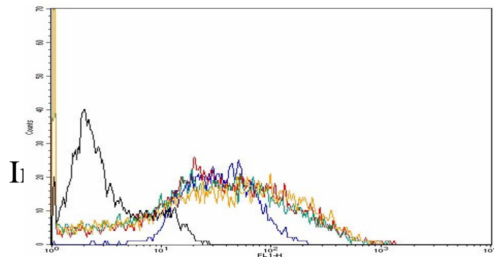
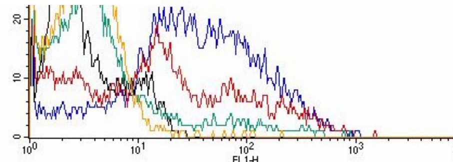


Image 2



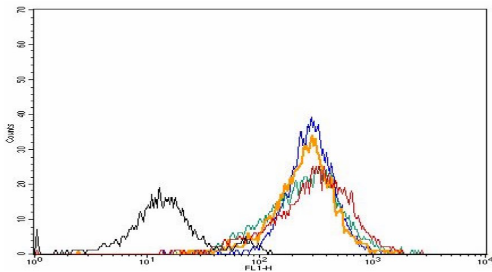


Image 3

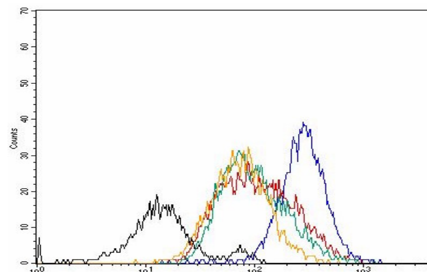


Image 4

An additional experiment was performed under the same conditions on the U937 human monoblastic cell line in the absence or in the presence of PMA. PMA is used as a stimulator to differentiate this cell-line into monocyte-like cells. The effect of PMA shown in this study was that it potentiated the phagocytosis activity of U937 cells. However, the cytotoxic effect of CyaA was greater on PMA-differentiated U937 cells than on undifferentiated cells. Thus, it can be suggested that PMA at 0.020μg at final concentration could stimulate the immature cells to differentiate, which CyaA could, then affect. Further investigation is recommended to show how differentiation of U937 cells might influence their susceptibility to CyaA, for example by increasing the number of receptors for the toxin.

It is very clear that phagocytic functions, such as migration, ingestion and release of reactive oxygen intermediates (ROI), which appear to be responsible for the killing activity, are important host defence mechanisms against *Bordetella.* Evasion of these functions may be the principal tools by which the bacteria can persist in the sites of colonization and proliferate further. One obvious strategy in defence against phagocytosis is direct attack by the bacteria upon the professional phagocytes using extracellular enzymes or toxins that kill or at least block the activity of phagocytes. Extracellular proteins that inhibit phagocytosis include the exotoxin A of *Pseudomonas aeruginosa*, which kills macrophages, and the bacterial exotoxins that are adenylate cyclases e.g. anthrax toxin EF and *B. pertussis* CyaA which decrease phagocytic activity. Thus, the reduction of ingestion and oxygen-dependent killing functions is a predominant activity *of B. pertussis* CyaA. However, the results of this study show that the full activity of CyaA is necessary for phagocytic inhibitory activity as CyaA* had little effect on phagocytosis. These results are keeping with the results reported by Galgiani *et al.* (1988). They showed that an adenylate cyclase toxin-containing extract blocked neutrophil-mediated inhibition of *N*-acetylglucosamine incorporation by arthroconidia of *Coccioides immitis* in a dose-dependent manner. The authors indicated that CyaA, rather than other virulence factors of *B. pertussis,* was responsible for the inhibitory effects on PMN. In the present study, the effects of CyaA preparations were examined and the interactions of human granulocytes and J774.2 mouse phagocyte-like cells with *E. coli in vitr*o were compared. The results suggest that CyaA impairs the functions of human granulocytes and J774.2 cells phagocytosis in a dose dependent manner and that this activity is dependent on the AC activity of the toxin**.**

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