



# *Clostridium perfringens* beta-toxin forms multimeric transmembrane pores in human endothelial cells

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Beta-toxin is one of the lethal toxins of *Clostridium perfringens*. It shares sequence homology with the pore-forming alpha-toxin of *Staphylococcus aureus* and structural homology has been indicated by mutagenesis studies. Human endothelial cells are sensitive to the toxic effect of alpha-toxin and in order to investigate the function of beta-toxin we have looked at the effect of the protein on human umbilical vein endothelial cells. We show that like alpha-toxin beta-toxin induces release of arachidonic acid in a dose dependent manner. In addition we show that both toxins cause leakage of inositol from the cells, consistent with the formation of transmembrane pores. The effect of toxin mutants on endothelial cells correlates with the lethal dose of each mutant in mice. Furthermore, we demonstrate the formation of heat stable toxin multimers in the cell membrane. Multimer formation was not observed on other cell types tested. We conclude that beta-toxin is a cell specific pore-forming toxin, structurally and functionally related to alpha-toxin of *Staphylococcus aureus*.

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**Key words:** beta-toxin, *Clostridium perfringens*, pore-forming toxin, human endothelial cells.

## Introduction

Vascular endothelial cells are target cells for the toxic effect of many bacterial pore-forming exotoxins including *Escherichia coli* haemolysin and *Staphylococcus aureus* alpha-toxin, proteins

considered to be prototypes for an increasing number of pore-forming toxins identified [1, 2]. Haemolytic activity is a feature shared by many of the pore-forming toxins. It has become increasingly evident however that their importance in pathogenesis stems mainly from their effect on nucleated cells [3–5]. The structure of the alpha-toxin pore has been solved, revealing a heptameric membrane spanning protein [6].

Beta-toxin is a major virulence factor of *Clostridium perfringens*. This 34 kDa protein shares

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17–29% sequence similarity with *S. aureus* alpha-toxin and related toxins [7]. Site-directed mutagenesis studies indicate that this similarity extends to the protein structure [8]. The biological function of this lethal toxin has been largely unclear, although it is known to be an important factor in the pathogenesis of necrotic enteritis in humans and animals. Unlike the staphylococcal alpha-toxin beta-toxin is not haemolytic and studies using other cell types have failed to reveal a useful experimental system.

In this study we use human umbilical vein endothelial cells (HUVEC) as an experimental system to address the question whether the structural similarity of beta-toxin with alpha-toxin of *S. aureus* is reflected in the biological activity of the toxin.

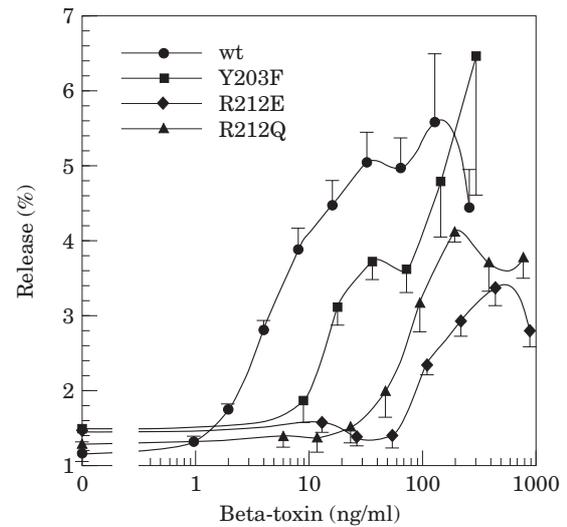
## Results

### Induction of arachidonic acid release by wild type and mutant beta-toxin

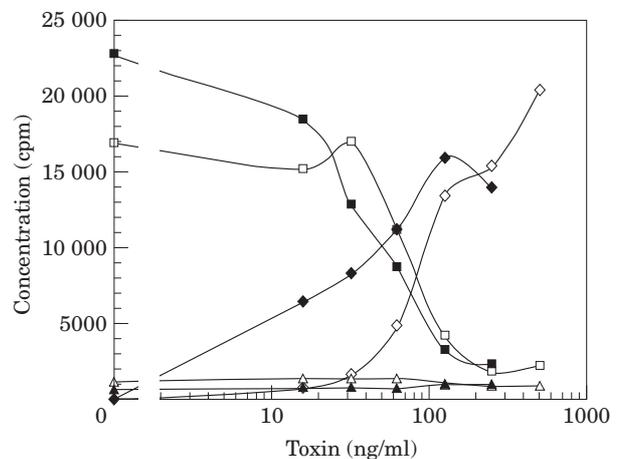
Confluent monolayers of HUVEC were labelled with tritiated arachidonic acid. Cells were incubated with increasing amounts of beta-toxin and the release of arachidonic acid into the culture supernatant was determined. A dose dependent effect of the wild-type toxin is shown in Fig. 1, where increased release is seen with as little as 2 ng/ml of the toxin. The effects of three mutations of two different amino acids on arachidonic acid release were also investigated. The LD<sub>50</sub> in mice of mutant proteins Y203F, R212Q and R212E is some 2.5, 6 and 12-fold that of the wild type protein respectively [8]. This is reflected in the effect on endothelial cells, the higher the LD<sub>50</sub> dose of a protein, the higher the threshold value for arachidonic acid release. In addition, the maximum effect is considerably lower for the mutant toxins than for the wild type protein (Fig. 1).

### Effect of alpha- and beta-toxins on cellular levels of inositol and inositol phosphates

Physiological activation of arachidonic acid release is usually caused by activation of phospholipase C (PLC) causing generation of inositol-triphosphate which in turn causes release of

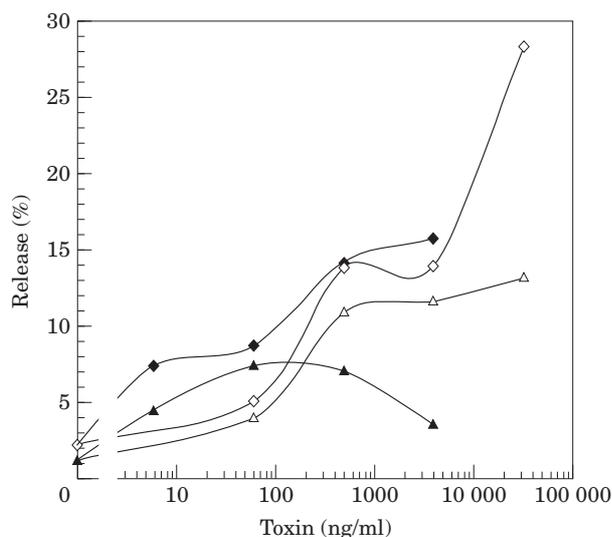


**Figure 1.** Effect of wild-type and mutant beta-toxin on arachidonic acid release from HUVEC. Cells were incubated with serial two-fold dilutions of beta-toxin for 1 h at 37°C. Mean and standard error of four independent experiments is shown. Release is expressed as the increase in counts in the culture supernatant as a percentage of total counts/plate. Zero value (0 ng/ml) represents the value obtained by incubation with beta-toxin negative *B. subtilis* culture supernatant.



**Figure 2.** Effect of beta-toxin (filled symbols) and staphylococcal alpha-toxin (open symbols) on concentrations of inositol (squares) and inositol phosphates (triangles) in HUVEC. Leakage of inositols into the medium is indicated by diamonds. Data represents mean values from one of three experiments performed in duplicate.

Ca<sup>++</sup> from internal stores. To determine if beta-toxin causes arachidonic acid release by this mechanism we studied the effect of toxin on inositol phosphate levels. As shown in Fig. 2

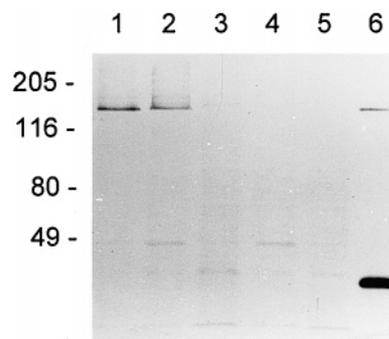


**Figure 3.** Effect of calcium on arachidonic acid release from HUVEC. Cells were incubated with the indicated amounts of wild type beta-toxin (filled symbols) or staphylococcal alpha-toxin (open symbols) in the presence (diamonds) or absence (triangles) of extracellular calcium. The results from one representative experiment are shown and are expressed as in Fig. 1.

there is no increase in inositol phosphates after toxin treatment whereas histamine at 11  $\mu$ M, an agonist causing arachidonic acid release in HUVEC, caused an eight-fold increase (not shown). Fig. 2 also shows a dose dependent decrease in the inositol levels of toxin treated cells. This represents leakage of inositol from the cells as demonstrated by the increase in extracellular inositol. The same effect is obtained when these cells are treated with staphylococcal alpha-toxin as shown in Fig. 2. Both toxins cause inositol to leak from the cells in a dose dependent manner.

### Effect of Ca<sup>++</sup> on arachidonic acid release

Arachidonic acid release is tightly regulated by a diverse family of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) enzymes with variable dependency on Ca<sup>++</sup> for activity [9]. We therefore studied the effect of extracellular Ca<sup>++</sup> on the release of arachidonic acid after beta-toxin treatment. As shown in Fig. 3 arachidonic acid release caused by low to moderate doses of beta-toxin occurs in the absence of extracellular Ca<sup>++</sup>. However, increasing the toxin concentration to high levels causes a great increase in arachidonic acid release in the



**Figure 4.** Beta-toxin bound to HUVEC detected by immunoblotting. Cell monolayers on 35 mm plates were incubated with 200 ng toxin preparations in a total volume of 1 ml at 37°C. After incubation for 30 min cells were washed and harvested. Lysed cells were boiled and subjected to SDS-PAGE under reducing conditions and analysed by Western blotting. Lanes 1–5 show lysates of cells incubated with: (1) wild type beta-toxin, (2) Y203F, (3) R212E, (4) R212Q and (5) beta-toxin negative *B. subtilis* culture supernatant. Lane 6: wild type beta-toxin preparation (30  $\mu$ g/ml). Numbers to the left of the figure indicate molecular mass standards (in kilodaltons).

presence of Ca<sup>++</sup> whereas in the absence of Ca<sup>++</sup> the release is reduced to basal levels. Fig. 3 also shows that arachidonic acid release by staphylococcal alpha toxin is only affected by Ca<sup>++</sup> at a high toxin dose. In contrast to the toxin effect the arachidonic acid release caused by treatment with the Ca<sup>++</sup> ionophore A23187 is almost totally prevented by the absence of Ca<sup>++</sup> in the medium (not shown).

### Beta-toxin forms stable oligomers in endothelial cell membranes

In order to confirm multimeric beta-toxin pore formation HUVEC were incubated with wild-type and mutant beta-toxin, and solubilized cell membranes were subjected to SDS-PAGE analysis and immunoblotting (Fig. 4). A band of high molecular weight protein is detected with the wild type protein and mutant Y203F (lanes 1 and 2). This band is absent in the negative control (lane 5). No monomeric protein is observed, indicating that most of the membrane bound protein is in a multimeric form and that the oligomerized protein is heat stable. No difference was seen between unboiled and boiled samples (not shown). In lanes 3 and 4 a faint high molecular weight band of mutant proteins R212E and R212Q can be observed, suggesting

that binding or multimerization of these proteins is less efficient than the wild type protein. No monomeric size protein is present. This is consistent with this residue, arginine 212, being important for protein binding to the cell membrane.

Some oligomer formation can occur in solution as seen in lane 6 which shows a toxin preparation with a beta-toxin concentration of 30 µg/ml. This step is time and concentration dependent and not essential for toxicity since more dilute toxin preparations with no apparent oligomer formation display the same relative activity. Boiling in 2% SDS or electrophoresis in 8 M urea had no apparent effect on the beta-toxin protein complex.

The cell specificity of beta-toxin binding was tested by applying a high toxin concentration to different cell types and analysing by immunoblotting. No beta-toxin binding was detected on sheep fibroblasts and choroid plexus cells, treated with a concentration of 0.7 µg/ml and sheep peripheral blood mononuclear cells and red blood cells treated with up to 5 µg/ml of beta-toxin for 30 min at 37°C (not shown).

## Discussion

The role of beta-toxin in the pathogenesis of *C. perfringens* has hitherto been unclear. Analysis of the beta-toxin gene and the predicted structure of the protein product indicated a relationship with a group of *S. aureus* pore-forming toxins [7, 8]. In the current study we have looked at this relationship by comparing the effect of beta-toxin and staphylococcal alpha-toxin on HUVEC, and asked whether beta-toxin forms multimeric transmembrane pores in these cells.

Sensitivity of endothelial cells to beta-toxin is illustrated by dose responsive release of arachidonic acid. This is analogous to the effect of staphylococcal alpha-toxin on endothelial cells [1, 10].

In HUVEC [11], as in other cell types [12], arachidonic acid release by physiological agonists is commonly mediated by activation of the phosphoinositide messenger system. Ca<sup>++</sup> ionophores bypass this mechanism by causing influx of Ca<sup>++</sup>. Neither of these mechanisms seems to mediate the arachidonic acid release caused by toxin treatment, as there is no increase in the level of inositol phosphates and the release of arachidonic acid is Ca<sup>++</sup> independent. This

Ca<sup>++</sup> independence at low to moderate toxin doses suggests the involvement of the Ca<sup>++</sup> independent phospholipase A<sub>2</sub> (iPLA<sub>2</sub>). The physiological function of this enzyme is thought to involve phospholipid remodelling rather than agonist induced activation [13]. However, in a recent study of Fas mediated apoptosis it was concluded that iPLA<sub>2</sub> was responsible for the accompanying arachidonic acid release [14]. In this study the inhibitor of iPLA<sub>2</sub>, bromoenolactone, had no effect on arachidonic acid release by either alpha- or beta-toxin in the absence of Ca<sup>++</sup> (not shown) thus making this enzyme an unlikely candidate. The finding that the Ca<sup>++</sup> independent arachidonic acid release is observed at lower toxin doses and Ca<sup>++</sup> dependent release is only apparent at higher doses for both toxins is consistent with a mechanism of action that has been reported previously for staphylococcal alpha-toxin. Jonas *et al.* [15] observed that alpha-toxin at low doses caused Ca<sup>++</sup> impermeable pores and apoptotic death in T lymphocytes. At higher doses of toxin the cell membrane became permeable to Ca<sup>++</sup> and the cells died non-apoptotically as judged by DNA laddering. The pores formed by the low doses of toxin were permeable to K<sup>+</sup> ions and recently a role for K<sup>+</sup> efflux in the activation of apoptosis has been proposed [16]. The reduction of arachidonic acid release that we observe at high doses of beta-toxin in the absence of Ca<sup>++</sup> is caused by the *B. subtilis* culture supernatant, rather than the toxin itself, as the volume of supernatant applied with the highest toxin dose prevents release otherwise seen at lower toxin doses (not shown). Finally, our observation that both toxins cause loss of inositol from the cells, and a corresponding extracellular increase, indicates that inositol has leaked from the cells, presumably through pores created by the toxins. This loss of inositol occurs at low toxin doses at which arachidonic acid is released in the absence of Ca<sup>++</sup>. This is consistent with the formation of Ca<sup>++</sup> impermeable pores at low toxin concentrations as suggested by Jonas *et al.* [15] for alpha-toxin.

The reduced activity observed with mutant proteins R212E and R212Q compared to the wild-type toxin can be explained by reduced membrane binding. Similarly, the corresponding residue in staphylococcal alpha-toxin, arginine 200, is thought to be involved in ligand binding and mutant proteins are defective in haemolysis [6, 17].

The stability of beta-toxin oligomers once

formed is remarkable. Oligomers formed in concentrated solutions or on cell membranes appear completely resistant to boiling in detergent under reducing conditions. Complex formation in concentrated solutions has been observed previously, confounding purification of the toxin. Furthermore when beta-toxin was expressed in *E. coli* it was recovered in oligomeric form from the periplasmic space [7].

Endothelial cells appear to be target cells for beta-toxin activity. Previous work has shown that beta-toxin is not haemolytic [8] and no other sensitive cell types have been identified. Our present data indicates that the cell specificity is determined at the level of membrane binding since no bound toxin, in monomeric or oligomeric form, was detected on resistant cells. This is different from staphylococcal alpha-toxin where resistant cells have been shown to bind toxin efficiently, but membrane insertion of the functional domain was not detected [18]. Both toxins target endothelial cells with similar affinity, possibly via high affinity binding sites. However, alpha-toxin is less cell restricted. Although a receptor for alpha-toxin has not been identified, binding at low toxin concentration is thought to be receptor mediated while non-specific membrane adsorption occurs at high toxin concentration [19]. The effect of beta-toxin on artificial membranes has not been tested but it is of interest to determine whether the protein is strictly dependent on a receptor for membrane binding.

## Materials and Methods

### Endothelial cell culture

As previously described endothelial cells were cultured from human umbilical veins by a modification of the method of Jaffe and co-workers [11]. After harvesting the cells with 0.12% collagenase digestion, they were seeded on 35 mm culture dishes in Morgan's medium 199 containing 20% foetal calf serum and antibiotics (penicillin, 100 units/ml and streptomycin, 100 µg/ml). The medium was changed every 2–3 days until the cell-culture reached confluence.

### Toxin preparation

Production of recombinant wild-type and mutant beta-toxin using a *Bacillus subtilis* expression

system has been described previously [8]. Wild type protein was expressed using strains PB14 and XB10. Three mutant toxins were used containing the mutations of tyrosine 203 to phenylalanine, arginine 212 to glutamic acid and arginine 212 to glutamine. Strains Y203F, R212E and R212Q produce the corresponding mutant proteins. Following sterile filtration crude culture supernatant was used directly for functional studies of beta-toxin. Culture supernatant from a strain carrying the corresponding vector (pHB201) without the beta-toxin insert was used as a negative control in each experiment. Beta-toxin concentration was determined by ELISA as described [8]. Toxin preparations contained beta-toxin at a concentration of 4 µg/ml (wt PB14), 30 µg/ml (wt XB10), 3.6 µg/ml (Y203F), 4.4 µg/ml (R212E) and 12 µg/ml (R212Q). Staphylococcal alpha-toxin was obtained from Sigma.

### Arachidonic acid release and inositol phosphate formation

To determine the release of arachidonic acid cells were incubated for the last 24 h of culture in medium containing 1 µCi/ml [<sup>3</sup>H]-arachidonic acid. Before experiments cells were washed twice with medium 199 without serum, but containing 0.1% fatty acid free bovine serum albumin. One ml of this medium was then added to each dish. Baseline samples were obtained 20 min later and toxin added in small volumes of concentrated solution to give the indicated final concentrations. After 60 min of incubation samples were again obtained. The concentrations of arachidonic acid and its labelled metabolites in the samples were quantified by liquid scintillation counting.

The formation of inositol phosphates was determined after incubating confluent cells for 24 h in 1 ml of medium 199 with 20% foetal calf serum and 3 µCi/ml myo-[<sup>3</sup>H]inositol. Before the experiments cells were washed with medium 199 containing 20 mM LiCl. One ml of this medium together with various doses of toxin or 11 µM histamine was then added. Stimulation was terminated after 60 min by removing the medium and adding 1 ml of ice-cold trichloroacetic acid to quench cellular reactions. After extraction the water-soluble inositol-containing compounds were separated by applying the samples on columns of anion-exchange resin

as previously described [8] and quantified by scintillation counting.

### Analysis of membrane associated beta-toxin by Western blotting

Beta-toxin was added to confluent plates of HUVEC in fresh medium and incubated for 30 min at 37°C. Medium was removed and cells washed three times with phosphate buffered saline (PBS). Cells were scraped off into PBS, centrifuged for 5 min/1000 rpm in an eppendorf tube and the pellet stored at -20°C. Thawed cells were subsequently lysed 5 min on ice in a buffer containing 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM MgCl<sub>2</sub> and 0.5% (v/v) NP40, centrifuged and analysed by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and beta-toxin detected by immunostaining using anti-beta monoclonal antibody [20], alkaline phosphatase labelled anti-mouse antibodies and visualized using Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrate.

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