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Immunological and functional comparison between *Clostridium perfringens* iota toxin, *C. spiroforme* toxin, and anthrax toxins

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Abstract

Clostridium perfringens iota and *C. spiroforme* toxins consist of two separate proteins. One is the binding component and the other the enzymatic component. The two toxins secreted by *Bacillus anthracis* are composed of binary combinations of three proteins: protective antigen, lethal factor, and edema factor. As shown by Western blotting and ELISA, the binding component of anthrax toxin shares common epitopes with that of iota toxin and *C. spiroforme* toxin which are closely related immunologically. However, no functional complementation was observed between iota toxin and anthrax toxin components. The binding components can form toxins active on macrophages only in combination with their respective enzymatic components. Agents which prevent acidification of endosomes do not have the same effects on anthrax toxin activity as they do on iota and *C. spiroforme* toxins. Therefore, the mechanisms of entry into the cells are presumably different. Since the binding components of anthrax toxins and iota toxin share a conserved putative translocation domain, these binding components could have a common mode of insertion into the cell membranes.

Keywords: *Clostridium perfringens* iota toxin; *Clostridium spiroforme* toxin; Anthrax toxin; *Bacillus anthracis*

1. Introduction

Clostridial binary toxins, which encompass *Clostridium perfringens* iota toxin, *C. spiroforme* toxin, *C. botulinum* C2 toxin and *C. difficile* ADP-ribosyltransferase (CDT), are organized according to the A-B model. One domain (A) possesses enzymatic activity, whereas the other (B) domain is involved in binding and translocation of the toxin into the cell. The functional domains of these clostridial toxins are localized on separate protein components [1]. Iota and *C. spiroforme* toxins share a common structure consisting of A components (Ia (47.5 kDa) and Sa (45 kDa)), and B components (Ib (94 kDa) and Sb (92 kDa)) [2]. The binding components must undergo limited proteolysis to be functionally active (mature proteins 70–80 kDa). The enzymatic components ADP-ribosylate cellular monomeric actin causing a disruption of the cytoskeleton [3].

The two anthrax toxins are composed of three different proteins: edema factor (EF; 89 kDa), lethal factor (LF; 83 kDa), and protective antigen (PA; 85 kDa) (for a review, see reference [4]). PA is the com-

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mon receptor binding domain of the toxins and interacts with the two effector domains, EF and LF, to mediate their entry into target cells. In contrast to Ib, the undigested PA binds to a cell surface receptor, where it is cleaved by a cell surface protease such that it becomes able to bind the enzymatic component [5]. The combination of PA and EF forms the edema toxin. EF is a calmodulin-dependent adenylate cyclase which induces an increase in intracellular levels of cyclic AMP in eukaryotic cells [4,6]. The lethal toxin is composed of PA and LF. It has been proposed that LF is a Zn^{2+}-binding metalloprotease. However, the intracellular target of LF and the molecular mechanisms leading to death after intravenous injection of lethal toxin remain unknown [4].

Considering conservative amino acid exchanges, Ib shares 33.9% identity and 54.4% similarity with PA. The maximum homology between both proteins has been found in the central region which is supposed to be the translocation domain permitting the passage of the enzymatic component across the cell membrane [2]. These data suggest that Ib and PA could share a similar mode of action.

2. Materials and methods

2.1. Toxins, antitoxins, PAGE and immunoblotting

Toxin components (Ib, Sb, PA and LF) were purified as previously described [7,8]. Specific rabbit antibodies were raised with purified proteins as previously described [7]. SDS-PAGE and immunoblotting were carried out according to [2].

2.2. ELISA

Each well of 96-well polystyrene plates (Nunc) was coated with 100 ng of the appropriate antigen in PBS, and ELISA was performed as previously described [8].

2.3. Cytotoxicity assay

Cells were cultivated in Dulbecco modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum. Vero (African green monkey kidney) cells were plated into a 96-well Falcon tissue culture plate (Becton Dickinson Labware, Oxnard, CA) and grown for 24 h to form monolayers. Serial twofold dilutions of samples in a final volume of 100 μl were added to the monolayers. The cells were incubated for 18 h and the actin cytoskeleton was visualized by immunofluorescence with fluorescein isothiocyanate (FITC-phalloidin (1 μg/ml); Sigma Chemical Co., L'Isle d'Abeau, France). The biological activity of anthrax lethal toxin was tested on monolayers of J774 cells [9], and cytotoxicity quantitated using a colorimetric MTT assay [10].

3. Results and discussion

The antigenic relatedness of Ib, Sb and PA was investigated by ELISA (Table 1). Ib and Sb gave similar ELISA titers with the different antibodies tested and are therefore immunologically closely related.

Anti-PA antibodies did not significantly recognize the clostridial binding proteins in ELISA, and PA reacted weakly with anti-Ib and anti-Sb antibodies (Table 1). PA is therefore only distantly related to Ib and Sb.

Antigenic cross-reactivity between PA and Sb was investigated by Western blotting. Rabbit polyclonal antibodies raised against Sb cross-reacted in immunoblots with PA and, reciprocally, polyclonal anti-PA antibodies recognized Sb (Fig. 1). However, more PA than Sb (approximately 6000 times more) was required for a similar immunological reaction with anti-Sb antibodies; reciprocally, a higher amount of Sb than PA (approximately 250 times more) was required with anti-PA antibodies (Fig. 1). Similar results were obtained between Ib and

Table 1
Comparative analysis of PA, Ib and Sb with their specific antibodies by ELISA

Antibody	Toxin component		
	PA	Ib	Sb
Anti-PA	400 000	< 100	< 100
Anti-Ib	1 200	1 600 000	410 000
Anti-SB	600	102 000	102 000

The results are expressed as the reciprocal of the highest antiserum dilutions giving an OD of 0.5.

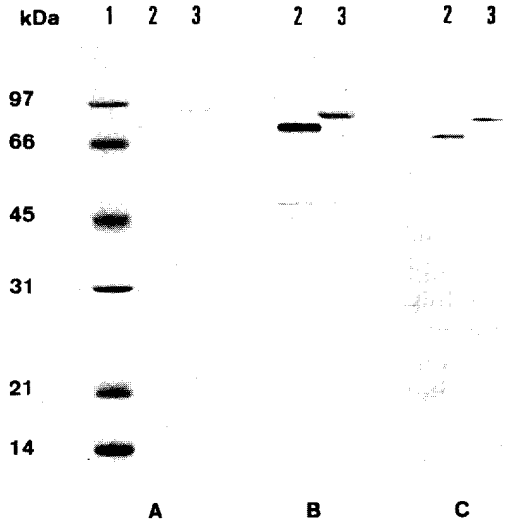

Fig. 1. Analysis of Sb and PA by Western blotting. A: SDS-PAGE (10% acrylamide) of Sb (0.34 μg) (lane 2) and PA (0.5 μg) (lane 3). Western blot with specific anti-Sb antibodies (B): Sb (1.7 ng) (lane 2) and PA (10 μg) (lane 3), and with specific anti-PA antibodies (C): Sb (510 ng) (lane 2) and PA (2 ng) (lane 3).

PA with anti-PA and anti-Ib antibodies (data not shown).

The ELISA and immunoblotting gave slightly different results. Immunoblotting involved analysis of denatured proteins whereas ELISA was done in non-denaturing conditions permitting the recognition of linear and conformational epitopes. The difference in reactivity between PA and Sb or Ib by both methods presumably reflects the diversity of the common epitopes of these toxin components. The presence of partial immunological relatedness between PA, Sb, and Ib indicates that these toxin components, although immunologically different, share common epitopes.

Macrophages J774, which are sensitive to the lethal anthrax toxin [9], were used to test if PA is able to induce the internalization of Ia. Excess Ib (1 μg/ml) alone did not perturb the actin cytoskeleton, and in the presence of Ia (7 ng/ml), a complete disorganization of the actin cytoskeleton was observed indicating that macrophages J774 are also sensitive to iota toxin. In contrast, excess PA (1 μg/ml) with various concentrations of Ia (7, 17.5, 35, and 140 ng/ml) did not induce any morphological changes, indicating that PA is unable to internalize Ia into macrophages J774. Identical results have been obtained using CHO and Vero cells (data not shown).

The association of LF (100 ng/ml) with various concentrations of PA (10–1000 ng/ml) caused macrophage lysis and death after 4 h incubation as assayed by the MTT test. The same concentration of LF, even with large amounts of Ib (up to 800 ng/ml), did not reduce macrophage viability (data not shown). The lack of response of LF in association with Ib indicates that Ib cannot internalize LF into macrophages J774.

Thus, PA is not able to mediate internalization of Ia, nor Ib that of LF. This could be due to different binding sites for their respective enzymatic components in PA and Ib molecules. The N-terminal parts of EF and LF contain regions of similarity and are probably involved in PA binding [4,11]. There is no significant similarity between Ia and EF or LF, suggesting that PA and Ib binding sites are different and that these B components can interact only with their respective A components.

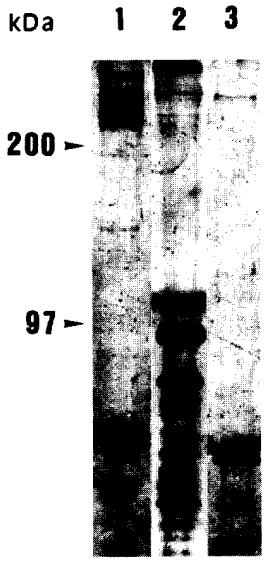

Fig. 2. Analysis of oligomer formation by PA63, proteolytically activated IB and Sb. The mature binding components were diluted at least 10-fold in 50 mM sodium acetate pH 5 (1.5 μg final amount of protein in 20 μl). The mixtures were incubated for 10 min at room temperature, mixed with 1:10 sample buffer, and loaded without boiling onto a 0.1% SDS-6% PAGE and silver stained: PA (lane 1); Ib (lane 2); and Sb (lane 3). Only PA shows significant amounts of oligomers (bands above 200 kDa).

The receptor binding domain of PA has been mapped on the C-terminal region [4]. The C-terminal parts of PA (residues 537–764) and Ib (residues 532–875) show a low level of identity (28.6%). If Ib and PA have a similar domain organization, this suggests that the two components recognize different cell surface receptors. This was confirmed by the absence of competition between PA (10-fold excess) and Ib in combination with Ia, and reciprocally between a 10-fold excess of Ib over PA in combination with LF as assessed by the cytotoxic titers of iota and anthrax toxins respectively.

Anthrax toxins enter the cell by receptor-mediated endocytosis, and translocation of the A component to the cytosol requires passage through an acidic compartment [9]. In contrast, the entry of *C. spiroforme* toxin into Hep2 cells is not blocked by ammonium chloride [7]. Preincubation of Vero cells for 1 h with the lysosomotropic amine chloroquine (1 mM), with the ionophore monensin (5 μg/ml) and with the vacuolar H^+ ATPases inhibitor bafilomycin A1 (1 μM) at active concentrations [9], did not protect the cells from rounding caused by iota and *C. spiroforme* toxins.

Brefeldin A (1.2 μg/ml), which causes disintegration of the Golgi apparatus [12], completely prevented the cytopathic effects of iota and *C. spiroforme* toxins in Vero cells, suggesting that the intracellular trafficking of iota and *C. spiroforme* toxins is different to that of anthrax toxins. Anthrax toxins are routing through an acidic compartment, and iota and *C. spiroforme* toxins are probably transported to the Golgi prior to the release of the A component to the cytosol as has been found for ricin, Shiga toxin (ST), heat-labile toxin (LT), cholera toxin (CT) and *Pseudomonas* toxin [12]. CT and LT display a K/RDEL sequence in the C-terminal part of the A component which is known to mediate the retention of eukaryotic proteins in the endoplasmic reticulum (ER). However, iota toxin and ST do not possess an ER retention sequence.

The Ib sequence contains a predicted transmembrane segment (positions 292–308) [2] which could be involved in the insertion of the B component into the cell membrane and in the translocation of the A component to the cytosol as observed with diphtheria toxin [13]. The precise mechanism of translocation of anthrax toxins and *Clostridium* binary toxins is not yet known. It has been shown that PA63 forms stable oligomers at acidic pH and the oligomers are probably associated with the channel formation in plasma membrane [14]. We did not observe that mature Sb and Ib form a significant amounts of oligomers in the acidic pH conditions which cause PA63 oligomerization (Fig. 2) or at neutral pH as analyzed by SDS-PAGE and in non-denaturing PAGE (data not shown). However, it cannot be ruled out that the binding components of *Clostridium* binary toxins could form oligomers only in cell membrane.

In conclusion, Ib and PA display significant amino acid sequence similarity. However, they are only distantly related immunologically and there is no functional complementation. PA and Ib seem to share a similar domain organization, since they are produced as precursor proteins which are proteolytically activated by removing a 20 and 14 kDa N-terminal peptide respectively. That Ib and PA can form fully active toxins only with their respective A components could be due to different A binding domains and to different cell entry mechanisms. The regions of greatest similarity are in the putative translocation domains of Ib and PA. This is consistent with the two proteins mediating a common mechanism of insertion into the cell membrane.

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