Clostridium perfringens iota toxin: characterization of the cell-associated iota b complex

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Clostridium perfringens type E iota toxin consists of two unlinked proteins designated as iota a (Ia; molecular mass ≈ 47 kDa), an ADP-ribosyltransferase and iota b (Ib; molecular mass ≈ 81 kDa) which binds to the cell surface and facilitates Ia entry into the cytosol. By Western-blot analysis, Ib incubated with Vero cells at 37 °C generated a cell-associated, SDS-insoluble oligomer of Ib (molecular mass > 220 kDa) within 15 s, which was still evident 110 min after washing cells. Ib oligomerization was temperature, but not pH, dependent and was facilitated by a cell-surface protein(s). Within 5 min at 37 °C, cell-bound Ib generated Na^+/K^+ permeable channels that were blocked by Ia. However, Ib-induced channels or oligomers were not formed at 4 °C. Two monoclonal antibodies raised against Ib that recognize unique, neutralizing epitopes within residues 632–655 either inhibited Ib binding to cells and/or oligomerization, unlike a non-neutralizing monoclonal antibody that binds within Ib residues 28–66. The Ib protoxin (molecular mass ≈ 98 kDa), which does not facilitate iota cytotoxicity but binds to Vero cells, did not oligomerize or form ion-permeable channels on cells, and neither trypsin nor chymotrypsin treatment of cell-bound Ib protoxin induced large complex formation. The link between Ib oligomers and iota toxicity was also apparent with a resistant cell line (MRC-5), which bound to Ib with no evidence of oligomerization. Overall, these studies revealed that the biological activity of iota toxin is dependent on a long-lived, cell-associated Ib complex that rapidly forms ion-permeable channels in cell membranes. These results further reveal the similarities of *C. perfringens* iota toxin with other bacterial binary toxins produced by *Bacillus anthracis* and *C. botulinum*.

Key words: channel, cytotoxicity, monoclonal antibody, oligomer, protoxin.

INTRODUCTION

Clostridium perfringens iota toxin represents one of four 'major' lethal and dermonecrotic toxins produced by this ubiquitious anaerobic bacterium, and is composed of two immunologically distinct proteins implicated in animal enterotoxemias [1]. Iota toxin belongs to a fascinating family of unlinked binary proteins synthesized by various Gram-positive, spore-forming bacilli that include *C. spiroforme* iota-like [2], *C. botulinum* C2 [3] and *Bacillus anthracis* anthrax [4] toxins, as well as *B. cereus* vegetative insecticidal proteins [5]. Poisoning begins when the non-enzymic component of the toxin binds to a cell. The receptor-binding components of C2 (C2II), anthrax [*B. anthracis* protective antigen (PA)] and iota (iota b, Ib) toxins are produced by the bacterium as inactive monomers that shed a 20 kDa N-terminal peptide after proteolysis by serine-type proteases, thus generating homoheptamers in solution and/or activated monomers that target specific proteins or carbohydrates on the cell surface and subsequently form large cell-associated complexes [6–9]. Cell-bound complexes of C2II, PA or Ib then act as a docking platform on the cell surface, which facilitates entry of a unique enzymic protein(s) into the cytosol via endosomal trafficking [6,7,10], like iota a (Ia) of iota toxin which ADP-ribosylates monomeric actin and subsequently prevents formation of cytoskeletal filaments [11]. Functional mapping of C2II, PA and Ib reveals that the C-terminus (domain 4) of each protein intimately binds to different, cell-surface protein/glycoprotein receptors [12–18] and the N-terminus (domain 1) subsequently docks with an enzymic component(s) [16,19]. Although C2II, PA and Ib share functional domains and amino acid sequence similarity primarily within the central region (domains 2 and 3) of these proteins [19–22], the only evidence for biological complementation between heterologous binding and enzymic components amongst this toxin family is with *C. perfringens* iota and *C. spiroforme* iota-like toxins [23].

Numerous studies [4,24,25] have characterized PA oligomerization and subsequent transport of various proteins into the cytosol of targeted cells, thus making anthrax toxin a prototypical vehicle within this family of binary bacterial toxins. To date, relatively less is known about C2 and iota toxins with respect to oligomerization and transport capabilities [7,9,26,27]. The present study further explores various aspects of the binding and complex formation of Ib and the Ib protoxin (Ibp) on cells in relation to the biological activity of iota toxin.

EXPERIMENTAL

Toxin, antisera and monoclonal antibodies (mAbs)

Purified components of *C. perfringens* type E iota toxins (Ia, Ib and Ibp) as well as rabbit anti-Ib serum and Ib mAbs 4F6, 1D11, 10A6, 4H7 and 1E12 were generated as described previously [2,16,28]. Goat *C. spiroforme* and *C. perfringens* type C antisera were purchased from TechLab (Blacksburg, VA, U.S.A.).

Cells

African green monkey kidney (Vero) and human lung (MRC-5) cells were cultured (37 °C, 5% CO_2, 97% relative humidity) in

Abbreviations used: HBSS, Hanks balanced salt solution; Ia, iota a; Ib, iota b; Ibp, Ib protoxin; mAb, monoclonal antibody; MEM, minimum essential medium; PA, *Bacillus anthracis* protective antigen.

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minimum essential medium (MEM), containing Earle's salts, non-essential amino acids and 10% heat-inactivated foetal bovine serum. Cells were detached from T-150 Costar culture flasks (Corning Inc., Corning, NY, U.S.A.) in Hanks balanced salt solution (HBSS) lacking calcium and magnesium, but containing 50 mM EDTA. Cells (> 95% viable as determined by Trypan Blue exclusion) were washed with HBSS containing 0.2% BSA (hereafter described as HBSS + BSA) and used for flow-cytometry [18] or oligomerization experiments as described below.

Flow cytometry

Inhibition of Ib binding to Vero cells (10^6/tube) was examined by preincubating Ib (10 μg/ml) with mAb (50 μg/ml) in HBSS + BSA for 30 min at 25 °C before adding to cells at 37 °C for 10 min. After washing with ice-cold HBSS + BSA, cells were incubated for 1 h on ice with rabbit anti-Ib sera (diluted 1:400 in HBSS + BSA), washed and then incubated for 1 h on ice with anti-rabbit FITC conjugate (Organon Teknika, West Chester, PA, U.S.A.). Cells were finally washed and analysed via a FACSort cytometer (Becton Dickinson, Mountain View, CA, U.S.A.). The recorded signal indicates peak fluorescence from a histogram plot using 10000 events (cells) and is representative of three experiments.

MAbs 4F6, 1D11 and 1E12 were also individually tested for detection of Ib (10 μg/ml) previously bound to the Vero surface for 10 min at 4 or 37 °C. After ice-cold HBSS + BSA washes, each mAb (20 μg/ml) was added to the cells for 1 h on ice. Cells were washed, incubated with anti-mouse FITC conjugate and analysed as described above.

Oligomerization of Ib and Ibp on cells

Ib and Ibp oligomerization was performed with 6×10^6 Vero (or MRC-5) cells per reaction in HBSS + BSA employing various incubation periods and temperatures. After incubation with Ib or Ibp (10 μg/ml), cells were washed three times (2 ml each) in ice-cold HBSS + BSA and then lysed in 200 μl sample buffer containing SDS and 20 μl of DNase (10000 units/ml; Roche Diagnostics, Indianapolis, IN, U.S.A.) for 15 min on ice. The cell lysates, as well as Ib and Ibp protein controls, were not heated and did not contain reducing agent. Rainbow high-molecular-mass markers (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) were heated in sample buffer with reducing agent before electrophoresis (30 μl/sample) on NuPAGE (4–12% polyacrylamide gels) in Mops–SDS running buffer (Invitrogen, Carlsbad, CA, U.S.A.). Proteins were electrophoretically transferred on to nitrocellulose that was subsequently blocked overnight (4 °C) in PBS containing 5% (w/v) non-fat dried milk. Blots were probed with rabbit anti-Ib sera (diluted in PBS containing 3% milk and 0.1% Tween 20) for 1 h at 25 °C, washed in PBS/0.1% Tween 20 (PBST) and incubated for 1 h at 25 °C with goat anti-rabbit IgG conjugated to horseradish peroxidase (Sigma). After PBST washes, immunoreactive bands were detected on film by using the ECL® Western-blot system (Amersham).

Additional Western-blot experiments were performed with cell-bound Ibp to determine if Ib oligomers formed after trypsin or chymotrypsin treatment. Ibp (15 μg/ml) was bound to Vero cells for 10 min at 37 °C and after cold HBSS washes, cells were incubated with 2.5 mg/ml trypsin (Sigma) or chymotrypsin (Boehringer Mannheim, Mannheim, Germany) in HBSS for 30 min at 37 °C. Cells were extensively washed with cold HBSS containing Complete protease inhibitors (Roche) as described previously [18] and then processed for Western-blot analysis. To ensure that each protease cleaved/activated Ibp into Ib, Ibp (1 μg/ml) was incubated with either trypsin or chymotrypsin (2.5 mg/ml) in HBSS without cells for 30 min at 37 °C.

Effects of pH on Ib binding and oligomerization with Vero cells

The effects of pH on binding of monomeric Ib (10 μg/ml) and subsequent oligomerization were tested with Vero cells. Monomeric Ib was kept on ice in HBSS + BSA at pH 4, 7 or 9 for 1 h before incubating (10 min at 37 °C) with cells previously washed in the appropriate medium. Following incubation with Ib, cells were washed in cold HBSS at the appropriate pH and processed for Western-blot analysis.

Inhibition of Ib binding/oligomerization with Vero cells by proteases, heat or antibodies

Vero cells (6×10^6) were preincubated with 100 μl of Pronase (0.02 or 2 mg/ml in HBSS) or papain (2 mg/ml) for 30 min at 25 °C and then washed five times (2 ml/wash) in HBSS + 1% BSA containing protease inhibitors. These cells were then incubated with Ib (10 μg/ml) in HBSS containing 1% BSA plus protease inhibitors for 10 min at 37 °C and binding/oligomerization assessed by Western-blot analysis. As an additional control for oligomerization, Ib was heated at 60 °C for 15 min in HBSS containing 1% BSA, cooled to 37 °C and then incubated with Vero cells for 10 min at 37 °C. The cells were washed immediately in ice-cold HBSS and processed for Western-blot analysis.

Inhibition of Ib binding was further investigated by preincubating various mAbs (100 μg/ml) or 1:5 diluted antisera towards *C. spiroforme* or *C. perfringens* type C with 10 μg/ml Ib for 30 min at 25 °C before adding to Vero cells for 10 min at 37 °C. Cells were then processed for Western-blot analysis.

Detection of Ib-induced, ion-permeable channels on Vero cells

Vero cells were grown in 6-well plates, washed twice with MEM and then incubated with Ib (10^{-7} M) diluted in MEM (1 ml/well) at 37 °C in a CO_2 incubator. Cells were washed with 125 mM triethanolamine at the indicated time periods and lysed with 1% (v/v) Triton X-100. Fluorescent probes were added to a final concentration of 1 mM CD222 (K^+ assay) and 10 mM sodium green (Molecular Probes, Eugene, OR, U.S.A.) with 50 μl of lysed cells. Fluorescence was determined on a Fluoroskan II spectrofluorimeter (Labsystems, Helsinki, Finland) using excitation/emission wavelengths of 380/475 and 485/538 nm for CD222 and sodium green respectively. The 0% baseline was established with Vero cells washed with triethanolamine and lysed with 1% Triton X-100, whereas the 100% reading represented cells incubated at 37 °C for 5 min with MEM containing 0.1% Triton X-100, washed with triethanolamine and then lysed with 1% Triton X-100. Data are expressed as percentage fluorescence quenched and represent the means ± S.D. of five assays.

RESULTS

Effects of time, temperature and pH on Ib binding and oligomerization with cells

Western-blot analysis of time-course studies revealed that the proteolytically activated form of the iota toxin component responsible for cell binding, Ib (molecular mass ≈ 81 kDa; [21]), rapidly formed a large (molecular mass > 220 kDa) complex on Vero cells within 15 s at 37 °C and was still detectable on washed

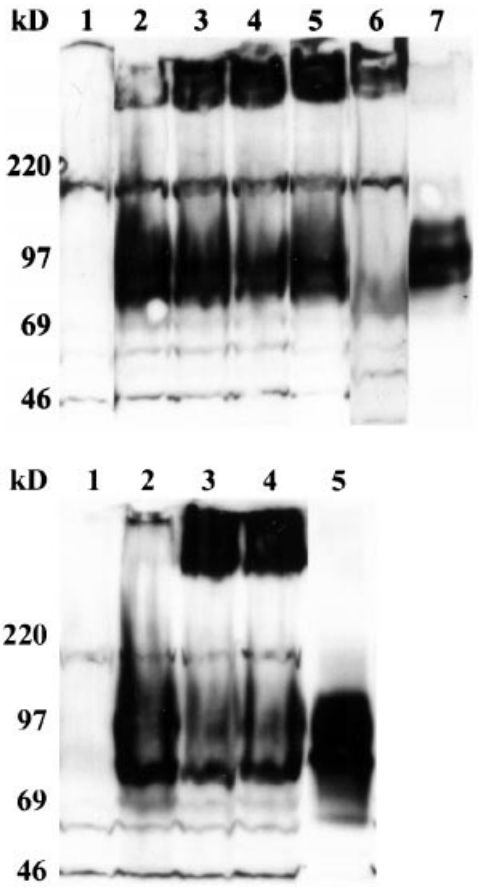

Figure 1 Western-blot analysis of Ib (10 μg/ml) oligomerization on Vero cells over time at 37 °C and at different temperatures

Positions of the molecular-mass markers (in kDa; kD) are located on the left-hand side. Upper panel: cells only (lane 1); cells + Ib, 15 s (lane 2); cells + Ib, 1 min (lane 3); cells + Ib, 3 min (lane 4); cells + Ib, 10 min (lane 5); cells incubated with Ib for 10 min, washed and incubated for an additional 110 min (lane 6); 2 ng Ib (lane 7). Lower panel: cells only (lane 1); cells + Ib, 4 °C/10 min (lane 2); cells + Ib, 25 °C/10 min (lane 3); cells + Ib, 37 °C/10 min (lane 4); 2 ng Ib (lane 5).

cells 110 min later (Figure 1A). The Ib complex for these and all other blots is located at the top of the gel, whereas monomeric Ib is evident between the 69 and 97 kDa markers. Although a time course was not performed at 25 °C, the Ib complex was also readily apparent at this temperature within 10 min (Figure 1B). However, at 4 °C, there was only monomeric but not oligomeric Ib associated with Vero cells, suggesting that formation of the cell-associated Ib complex is an active process requiring membrane fluidity and/or low temperature, perhaps preventing the proper conformation of Ib needed for oligomerization. These binding results are corroborated by previous flow-cytometry experiments [18], but this technique does not distinguish between monomeric and multimeric forms of Ib on the cell surface. In addition to the time and temperature effects on Ib interactions with Vero cells, Western blots revealed that pH (4, 7 or 9) had no effect on Ib monomer and oligomer formation on these cells (results not shown).

Generation of cell-associated Ib complexes was a specific phenomenon as determined by various experiments. Cell-associated oligomerization was not due to binding of high-molecular-mass components from the cell lysate, as Ib added after lysis

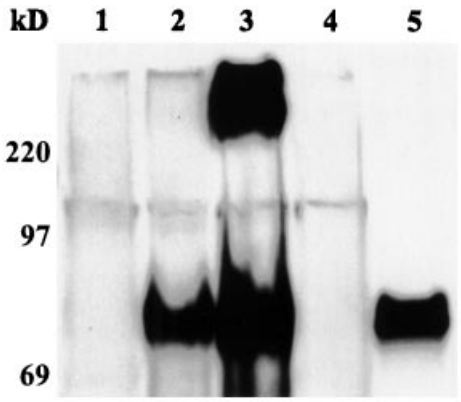

Figure 2 Ib does not form oligomers on iota toxin-resistant MRC-5 cells (37 °C/10 min)

Western-blot analysis showing molecular-mass markers (in kDa; kD) on the left-hand side. Samples included MRC-5 cells only (lane 1), MRC-5 cells + Ib (lane 2), Vero cells + Ib (lane 3), Vero cells only (lane 4), 2 ng Ib (lane 5).

yielded only monomeric Ib on blots (results not shown). Preheating Ib at 60 °C for 15 min does not prevent binding to Vero cells, but it inhibits Ia docking [18], iota toxicity [29] and Ib oligomerization on Western blots (results not shown). Incubation of Ib with an iota toxin-resistant cell line (MRC-5) [18] subsequently revealed monomers but not SDS-insoluble oligomers of Ib (Figure 2). Finally, preincubation of Ib with specific polyclonal antibodies or mAbs that prevent Ib binding, as determined by flow cytometry, and neutralized iota cytotoxicity also prevented Ib oligomerization on Vero cells ([18]; described below). Overall, these results revealed that Ib oligomerization was an active process that occurred on the membranes of cells susceptible to iota toxin. With our experimental conditions it also appeared that Ib, unlike PA [4,8,10] or C2II [6], did not readily generate preformed oligomers in solution, which subsequently bound to cells.

Cell-associated oligomerization of Ib is mediated by a protein receptor

Previous flow-cytometry experiments have shown that the Vero receptor for Ib is a Pronase-sensitive protein [18]. Western-blot experiments were performed to determine if binding, and subsequent oligomerization, of Ib was linked to a surface receptor susceptible to Pronase. Relative to Ib-treated cells not preincubated with Pronase, levels of monomeric and oligomeric Ib were clearly decreased by Pronase (2 mg/ml) pretreatment (Figure 3). Inhibition of Ib binding and oligomerization by Pronase was probably not due to proteolysis of Ib by residual Pronase. Ib found in culture fluid after 10 min at 37 °C incubation with Pronase-pretreated Vero cells still yielded oligomers on cells not treated with Pronase (results not shown). Additionally, pretreatment of Vero cells with papain (2 mg/ml), which readily digests Ib in solution, similarly to Pronase, does not inhibit Ib binding by flow-cytometry [18] or Western-blot analysis (results not shown).

Effects of iota toxin antibodies on Ib binding and oligomerization

Specificity of Ib binding and oligomerization was demonstrated further with various antibodies. Preincubation of Ib with *C. spiroforme* antiserum, which neutralizes iota toxicity [29], effec-

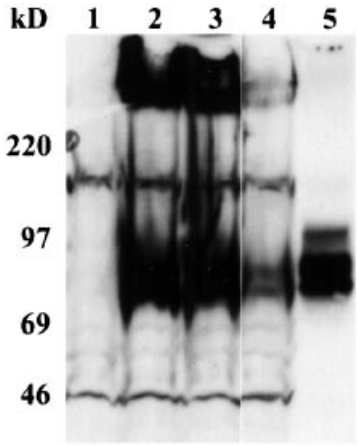

Figure 3 Pronase pretreatment of Vero cells inhibits Ib (10 μg/ml) binding and oligomerization at 37 °C for 10 min

Western-blot analysis showing molecular-mass markers (in kDa; kD) on the left-hand side. Samples included cells only (lane 1), cells + Ib (lane 2), cells + 0.02 mg/ml Pronase + Ib (lane 3), cells + 2 mg/ml Pronase + Ib (lane 4), 2 ng Ib (lane 5).

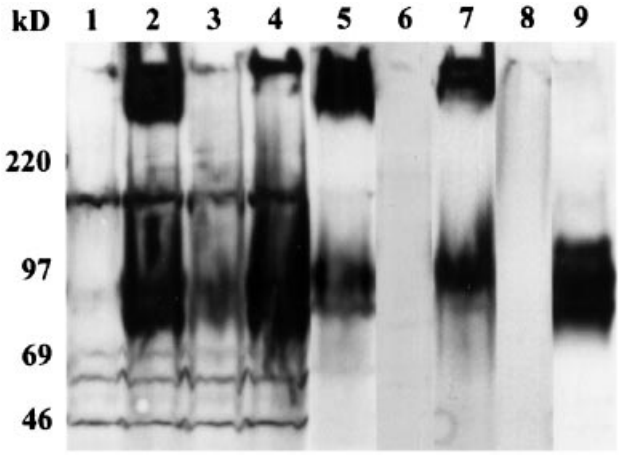

Figure 4 Toxin-neutralizing antibodies towards Ib inhibit binding and/or oligomerization on Vero cells

Western-blot analysis showing molecular-mass markers (in kDa; kD) on the left-hand side. Samples included cells only (lane 1), cells + Ib (10 μg/ml, lane 2), cells + Ib + mAb 4F6 (100 μg/ml, lane 3), cells + Ib + mAb 1D11 (100 μg/ml, lane 4), cells + Ib + mAb 1E12 (100 μg/ml, lane 5), cells + mAb 1E12 (lane 6), cells + Ib + 1:5 dilution of *C. perfringens* type C antiserum (lane 7), cells + Ib + *C. spiroforme* antiserum (lane 8), 2 ng Ib (lane 9).

tively prevented Ib binding and subsequent oligomerization on Vero cells in Western blots (Figure 4). However, *C. perfringens* type C antiserum lacking antibodies against iota toxin had no effect on Ib binding/oligomerization.

Various Ib mAbs (4F6 and 1D11) that bind within C-terminal residues 632–655 and neutralize iota cytotoxicity [16], as well as non-neutralizing mAbs 10A6, 4H7 and 1E12 that recognize an epitope between N-terminal residues 28–66, were also tested for effects on Ib binding and oligomerization. Preincubation of Ib with any of these mAbs revealed that only 4F6 effectively inhibited the Ib-specific fluorescence on Vero cells, as determined by flow cytometry (Table 1) and subsequent oligomerization (Figure 4). Since the epitope recognized by mAb 4F6 is important

Table 1 MAb inhibition of Ib binding to Vero cells by flow cytometry

Vero cells were incubated with an Ib (10 μg/ml) cocktail consisting of normal mouse sera (NMS; 1:30 dilution) or mAb (50 μg/ml) in HBSS + BSA. Ib was detected on the surface with rabbit anti-Ib sera and an FITC conjugate. An additional negative control consisted of medium-treated cells subsequently incubated with anti-Ib sera and FITC conjugate. The recorded signals indicate peak fluorescence from 10000 cells and are representative of three experiments.

Treatment	Fluorescence signal
Media	4.3
Ib + NMS	11.3
Ib + mAb 10A6	10.8
Ib + mAb 4H7	10.4
Ib + mAb 1D11	13.2
Ib + mAb 4F6	4.8

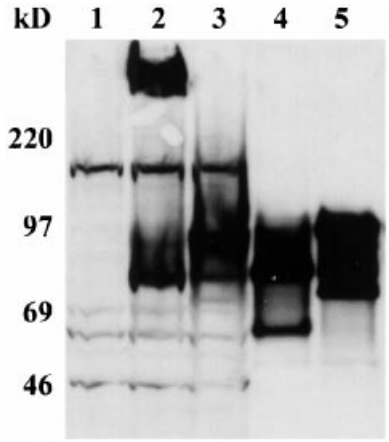

Figure 5 Ibp binds to, but does not form a large complex on, Vero cells at 37 °C after 10 min

Western-blot analysis showing molecular-mass markers (in kDa; kD) on the left-hand side. Samples included cells only (lane 1), cells + Ib (10 μg/ml, lane 2), cells + Ibp (10 μg/ml, lane 3), Ib only (2 ng, lane 4), Ibp only (2 ng, lane 5).

for Ib binding to a protein receptor [16,18], this suggests receptor-mediated oligomerization of Ib on toxin-sensitive cells. However, mAb 1D11, which recognizes a unique epitope within residues 632–655 also neutralizes iota cytotoxicity [16], but did not inhibit Ib binding (Table 1). Western-blot analysis revealed that mAb 1D11 significantly reduced formation of a detergent–stable Ib complex relative to those from the Ib + cells control (Figure 4), thus demonstrating further the importance of Ib oligomerization for the biological activity of iota toxin. These results with mAb 1D11 also suggested that residues 632–655 are important for Ib monomer interactions that generate oligomers. In contrast with the C-terminal binding antibodies, mAb 1E12, which has no effect on iota cytotoxicity [16] did not alter Ib binding or oligomerization on Vero cells (Figure 4).

To learn more about the cell-associated monomer and oligomer of Ib, we incubated Ib with Vero cells at 4 or 37 °C for 10 min, followed by various mAbs. Flow cytometry revealed that none of these antibodies recognized Ib bound to the Vero surface (results not shown), suggesting that the N-terminus (residues 28–66; domain 1) and C-terminus (residues 632–655; domain 4) of Ib in a cell-associated monomer or oligomer were not antibody-accessible.

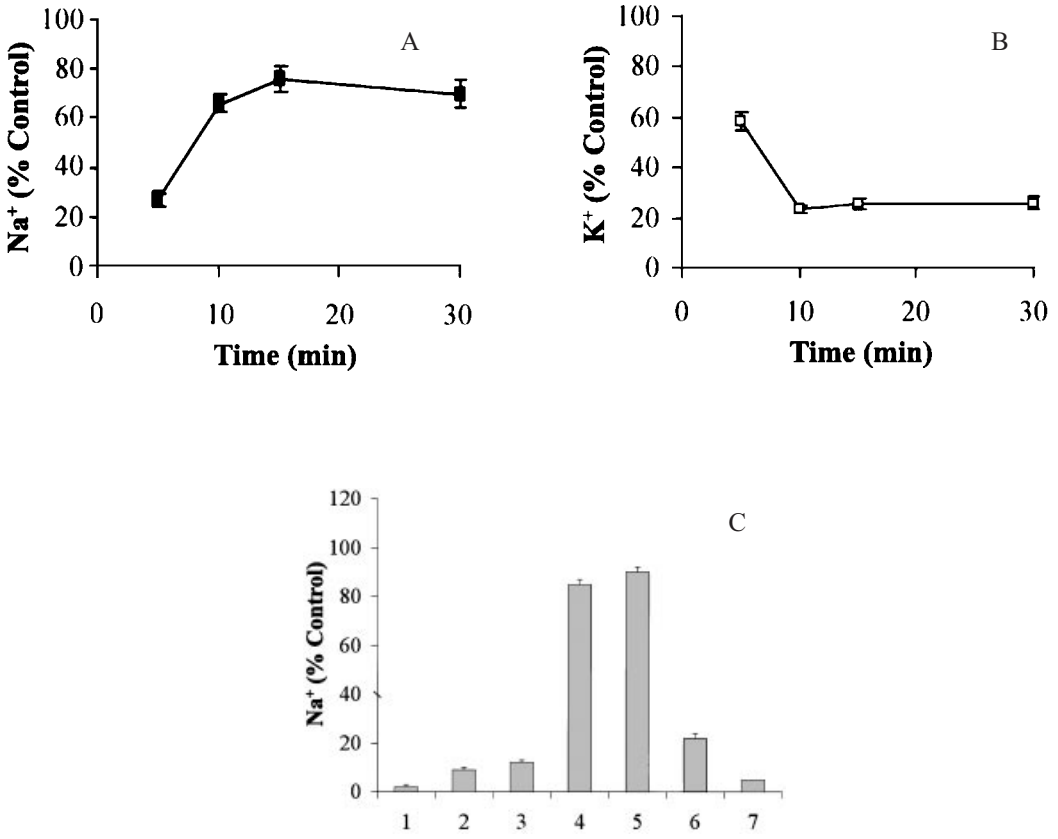

Figure 6 Ib generates ion-permeable channels in Vero cell membranes

(**A**) Na^+ influx with Vero cells incubated at 37 °C with Ib (10^{-7} M) over time; (**B**) K^+ efflux from Vero cells incubated at 37 °C with Ib (10^{-7} M) over time; (**C**) effects of varying Ia concentrations with Ib (10^{-7} M) on Na^+ influx of Vero cells incubated at 37 °C for 15 min. Ib + 10^{-7} M Ia (column 1), Ib + 0.5×10^{-7} M Ia (column 2), Ib + 0.1×10^{-7} M Ia (column 3), Ib + 0.5×10^{-8} M Ia (column 4), 10^{-7} M Ib only (column 5), 10^{-7} M Ibp only (column 6), 10^{-7} M Ib at 4 °C (column 7).

Ibp binds to Vero cells but does not form a complex

It has been known for many years that iota toxin is produced as a protoxin requiring proteolytic activation [30]. It was recently shown [28] that Ibp (molecular mass $\approx$ 98 kDa; [21]), which contains a 20 kDa N-terminal peptide removed by various serine-type proteases, such as trypsin or chymotrypsin, binds to Vero cells but does not effectively interact with Ia and facilitate iota cytotoxicity [18]. In contrast with Ib, incubation of Ibp at 37 °C for 10 min with Vero cells yielded monomeric but not oligomeric forms of Ibp in Western blots (Figure 5). These results support further the existing paradigm that bacterial binary toxins like iota form an oligomeric complex on cells composed of protease-activated cell-binding protein.

A previous study [4] showed that the PA protoxin (PA83) from anthrax toxin binds to a target cell as a monomer, and furin, a surface-associated serine-type protease, generates an activated PA63 molecule that subsequently forms homoheptameric complexes on cells. Ibp does not contain a furin-cleavage site (RKKR) and unlike PA83, the proteolytic activation of Ibp once bound to the cell surface has not been described. Therefore additional Western-blot studies were performed with cell surface-associated Ibp (15 μg/ml) and subsequent treatment with 2.5 mg/ml chymotrypsin or trypsin (37 °C for 30 min). These experiments revealed no proteolytic conversion of surface-bound Ibp into Ib and subsequent oligomers on the cell; however, protease readily generated Ib from Ibp in solution under the same conditions (results not shown), thus suggesting that once Ibp binds to a cell, it is not readily activated by these common gastrointestinal proteases.

Ib, but not Ibp, forms ion-permeable channels on Vero cells that are blocked by Ia

Experiments were performed to determine if Ib oligomerization on Vero cells resulted in ion-permeable channels, as indicated by Na^+ influx and K^+ efflux (Figure 6). A recent report by Knapp et al. [31] reveals that Ib, but not Ibp, forms ion-permeable channels in artificial lipid membranes that are blocked by Ia. Nagahama et al. [9] have recently shown that Ib causes a dose- and temperature-dependent efflux of K^+ from Vero cells, but they did not investigate Na^+ influx, whether or not Ibp forms cell-associated oligomers and ion-permeable channels or if Ia has any effects on Ib-induced channels. Relative to Vero cell controls, Na^+ (Figure 6A) and K^+ (Figure 6B) movement was clearly evident within 5 min and maximal between 10 and 15 min after incubation at 37 °C with 10^{-7} M Ib, thus corroborating Western-blot results showing that Ib oligomers rapidly formed in this same time period on Vero cells at 37 °C (Figure 1A). Additional experiments revealed that incubation of Ib with cells at 4 °C, which did not promote Ib oligomerization (Figure 1B), did not promote Na^+ influx (Figure 6C). In contrast with Ib, Ibp did not substantially alter Na^+ influx (Figure 6C) and

provided further evidence that oligomerization is necessary for the formation of Ib-induced channels.

Once it was determined that ion-permeable channels were formed by Ib, different concentrations of Ia (molecular mass ≈ 47 kDa; [21]) were co-incubated for 15 min (37 °C) with Ib and Vero cells to establish whether Ia blocked Ib-induced channels. An equal molar or 10-fold less concentration of Ia versus Ib effectively prevented Na^+ influx induced by Ib (Figure 6C).

DISCUSSION

The purpose of the present study was to investigate various parameters involved in iota poisoning, in particular Ib binding and oligomerization on toxin-sensitive (Vero) and -resistant (MRC-5) cells. *C. perfringens* iota toxin resembles many biologically diverse plant and microbial toxins that employ a classic A-B model in which the receptor-binding (B) subunit facilitates entry of an enzyme (A) into a targeted cell [1,3,4,29,32–34]. Among the bacterial binary toxins composed of unlinked A and B subunits, the best characterized is that produced by *B. anthracis* [4], thus making anthrax toxin the prototype for toxins like *C. perfringens* iota. Anthrax toxin consists of a cell-binding component (PA83) that is proteolytically activated into PA63 by serine-type proteases in solution or on the cell surface [4,8], generating a homoheptameric platform for docking with enzymic components, such as lethal and/or oedema factors [10,19,35]. To date, there is no uniform consensus regarding how many molecules of lethal or oedema factor bind to a PA heptamer. The enzyme-docking experiments with PA heptamers were performed in solution, since proteolytically activated PA as well as C2II from *C. botulinum* C2 enterotoxin readily form SDS-insoluble oligomers in solution [6,10,35]. In contrast, chymotrypsin-treated Ib weakly forms a detergent stable complex of approx. 530 kDa in solution [7]. Therefore determining the mole ratio of Ia/Ib may be more difficult than that established for anthrax toxin [10,35]. Recent gel electrophoresis studies by Nagahama et al. [9] report that the Vero cell-associated Ib complex is approx. 500 kDa, suggesting a hexamer or heptamer. However, it is possible that this Ib complex also contains cell-surface receptor and perhaps associated protein(s) that could alter electrophoretic migration and estimated size.

Our Western-blot studies of cell-associated Ib did not distinguish internal versus external oligomers; however, previous flow-cytometry studies suggest that Ib can be found on the cell surface at least 2 h after addition to cells at 37 °C [18]. The present study revealed that Ib oligomers (molecular mass > 220 kDa) rapidly formed within 15 s at 37 °C, thus suggesting a very efficient process evident for at least 110 min after cell washing. Recent studies by Nagahama et al. [9] also show that the Ib complex remains associated with Vero cells for at least 360 min at 37 °C. Overall, these results seemingly concur with earlier *in vivo* studies by Sakurai and Kobayashi [36] showing that Ib injected intravenously 2 h before Ia results in mouse lethality. Ib evidently binds rapidly to cells *in vivo*, as Ib-specific antiserum given just 5 min later does not protect against lethality induced by a subsequent injection of Ia [36].

Similar to C2II [14] and Ib [18], the surface receptor for PA is also a protein [13,15]. However, unlike the recent discovery [13] of the PA receptor as a type I membrane protein with an extracellular von Willebrand factor A domain, further identity of the Ib receptor remains elusive. Receptor binding studies show that Pronase pretreatment of Vero cells readily prevents Ib binding and subsequent oligomerization ([18]; present study). One possible critique of these results is that residual protease may remain and subsequently degrade Ib, even after extensive washes with HBSS containing protease inhibitors plus 1 % BSA. This was unlikely as shown by cell pretreatment experiments with papain, an enzyme that readily degrades Ib like Pronase but does not prevent Ib binding [18]. Furthermore, when Ib (10 μg/ml) was incubated with cells (10 min at 37 °C) pretreated with Pronase and the Ib-containing media subsequently added to untreated Vero cells (10 min at 37 °C), there was no difference in monomeric/oligomeric forms of Ib by Western blots. This also suggested that Ib is not readily degraded by any residual Pronase on cells after extensive washing.

In addition to inhibiting Ib binding and oligomerization by Pronase pretreatment of cells, iota toxin-neutralizing mAbs that recognize unique C-terminal epitopes within residues 632–655 on Ib [16] revealed two mechanisms for preventing iota poisoning. MAb 4F6 prevented Ib binding to the cell surface and suggests, along with the Pronase data, that Ib oligomerization on cells is mediated by a specific protein receptor. In contrast with 4F6, mAb 1D11 did not inhibit Ib binding, but it effectively prevented cell-bound Ib from forming SDS-insoluble complexes. The C-terminal epitope recognized by 1D11 may play an intimate role in forming a cell-associated Ib complex and/or complex formation is inhibited via steric hindrance induced by the antibody. It is also feasible that an unstable, detergent-dissociated oligomer of Ib–mAb 1D11 formed on the cell surface, but was unable to translocate Ia into the cytosol.

Various bacterial toxins, including *Staphylococcus aureus* α [37], *B. anthracis* anthrax [8], *C. botulinum* C2 [6,38], as well as *C. perfringens* β [39], ϵ [40] and enterotoxin [41] generate large complexes and subsequent ion-permeable channels on cell membranes. Knapp et al. [31] report that Ib forms cation-permeable channels in artificial lipid membranes. Recently, Nagahama et al. [9] revealed that Ib forms temperature-dependent oligomers within 15 min on Vero cells, resulting in K^+ efflux. Our results showed that Ib clearly formed a large, cell-surface complex that spans the membrane within 5 min at 37 °C and facilitates Na^+/K^+ fluxes. The Ib-induced ion flow was readily blocked by varying concentrations of Ia. However, incubation of Ib with cells at 4 °C did not produce oligomers or subsequent ion-permeable channels. Overall, our results revealed a good correlation between ion permeability and Western-blot experiments showing the time course for Ib oligomerization on Vero cells. Although the mechanism for Ib coalescence on the cell is still unknown, membrane lipid rafts may play a role as they concentrate receptors for pore-forming toxins, like streptolysin O, which probably promotes oligomerization and subsequent toxicity [42]. An understanding of how other bacterial toxins form cell-associated oligomers will also enhance knowledge of Ib and iota poisoning.

Since Ib is produced by *C. perfringens* as an inactive protoxin (Ibp) that binds to Vero cells but requires proteolysis before docking with Ia [18], it was important to investigate whether Ibp produced cell surface-associated oligomers. Western-blot analysis clearly revealed that Ibp did not produce a large complex on cells after 10 min at 37 °C, further emphasizing the necessity of a cell-associated complex for ion permeability, Ia docking and subsequent iota poisoning. Additional experiments with Vero cells incubated with Ibp, washed and then treated with an excess of trypsin or chymotrypsin did not result in Ib oligomerization, suggesting that cell-bound Ibp was not readily activated by common serine-type proteases found in the gastrointestinal tract. This mechanism for iota toxin clearly differs from the cell-associated activation of *B. anthracis* PA by furin or furin-like proteases found on the cell surface [4]. To date, cell surface-associated proteases that activate the protoxin forms of either *C. perfringens* Ib or *C. botulinum* C2II have not been described.

The importance of Ib oligomerization for iota toxicity was also evident with toxin-resistant MRC-5 cells, which bound to Ib but did not yield oligomers. These results differ from those derived by flow cytometry in which Ib is not detected on MRC-5 cells [18]. This discrepancy is probably explained by differences in sensitivity between the Western-blot and flow-cytometry techniques. It is also possible that the detectable binding of Ib to MRC-5 cells is non-specific and not receptor-mediated, thus resulting in Ib monomers that do not effectively form oligomers. Anti-Ib sera may readily bind to oligomeric but not monomeric forms of Ib on the cell surface, thus cells with only monomeric Ib would generate a relatively weak Ib-specific signal by flow cytometry. Based on the cumulative Western-blot and flow-cytometry results, it is clear that if Ib binds to a cell without forming oligomers, there is no effective platform for Ia interactions and subsequent poisoning [18].

In summary, we found that the Vero cell-associated complex of Ib had a molecular mass > 220 kDa, evident within 15 s at 37 °C and lasted for at least 2 h, and generated Na^+/K^+-permeable channels at 37 °C (not 4 °C) that were blocked by Ia. The Ib oligomer was also easily detected by Western blots on cells incubated at 25 °C, but not at 4 °C, suggesting the importance of membrane fluidity and/or a proper Ib conformation necessary for monomer to oligomer conversion and channel formation. The Ibp molecule, which binds to cells but does not facilitate Ia docking and subsequent iota poisoning [18], did not form cell-associated oligomers or ion-permeable channels. MRC-5 cells, which are resistant to iota toxin, bound to Ib, as shown by Western-blot analysis, but did not form Ib oligomers. Finally, toxin-specific antiserum or C-terminal (domain 4)-binding mAbs that neutralize iota toxin prevented Ib binding and/or oligomerization on Vero cells. Overall, the present study provides important clues regarding the mechanism of action for *C. perfringens* iota toxin, particularly the Ib component, and the promising potential of this binary toxin as a biological tool.

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