Bacillus cereus Nhe is a pore-forming toxin with structural and functional properties similar to the ClyA (HlyE, SheA) family of haemolysins, able to induce osmotic lysis in epithelia

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The mechanism by which *Bacillus cereus* causes diarrhoea is unknown. Three putative enterotoxins have been proposed, haemolysin BL (Hbl), cytotoxin K and non-haemolytic enterotoxin (Nhe). Both Hbl and Nhe are three-component cytotoxins and maximal cytotoxicity of Nhe against epithelia is dependent on all three components. However, little is known of the mechanism of cytotoxicity. Markers of plasma membrane disruption, namely propidium iodide uptake, loss of cellular ATP and release of lactate dehydrogenase (LDH), were observed in epithelia exposed to Nhe from culture supernatants of *B. cereus*, but not in those exposed to supernatants from a mutant strain lacking NheB and NheC. Consistent with an exogenous cause of membrane damage, purified Nhe components combined to form large conductance pores in planar lipid bilayers. The inhibition of LDH release by osmotic protectants and the increase in cell size caused by Nhe indicate that epithelia lyse following osmotic swelling. Nhe and Hbl show sequence homology, and Hbl component B has remarkable structural similarities to cytolysin A (ClyA), with both structures possessing an α -helix bundle and a unique subdomain containing a hydrophobic β -hairpin. Correspondingly, we show that Nhe has haemolytic activity against erythrocytes from a variety of species. We propose that the common structural and functional properties indicate that the Hbl/Nhe and ClyA families of toxins constitute a superfamily of pore-forming cytotoxins.

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INTRODUCTION

Bacillus cereus is a worldwide cause of food-associated illness in man. Although *B. cereus* was shown to cause enteritis in humans over 50 years ago (Hauge, 1955), the cellular mechanism of diarrhoea and identity of the responsible enterotoxin(s) remain unknown. Three putative enterotoxins of *B. cereus* have been proposed: haemolysin BL (Hbl), non-haemolytic enterotoxin (Nhe) and cytotoxin K (CytK), all of which are cytotoxic to epithelia *in vitro*. CytK is a haemolytic pore-forming toxin with homology to the β -barrel pore-forming toxins, including staphylococcal α -haemolysin (Hardy *et al.*, 2001a; Lund *et al.*, 2000). Hbl and Nhe are tripartite toxins, in which all three components are necessary for maximal cytotoxic activity (Beecher & Macmillan, 1991; Lindbäck *et al.*, 2004). Both are encoded by three genes co-transcribed as operons in which *hblCDA* encodes Hbl components L₂, L₁ and B, and *nheABC* encodes NheA,

NheB and NheC. Sequence homology is apparent both between the three components in each complex and between the proteins of Nhe and Hbl (Granum *et al.*, 1999; Ryan *et al.*, 1997; Table 1). With no sequence homology to other toxins, Nhe and Hbl have remained unclassified as toxins.

Hbl has been shown to possess a variety of biological effects, including haemolytic, dermonecrotic and vascular permeability activities and, significantly, possesses enterotoxigenic ability, as indicated by fluid accumulation in rabbit ligated ileal loops (reviewed by Schoeni & Wong, 2005). Nhe was identified in *B. cereus* following a large food-poisoning outbreak in Norway (Lund & Granum, 1996). The recovered isolate, NVH 0075/95, lacked both *hbl* and *cytK* (Ehling-Schulz *et al.*, 2005) but was still cytotoxic, thus permitting the discovery of Nhe. The initial study indicated that the toxin was non-haemolytic.

Although the toxin profile may differ between strains, it appears that Nhe is a dominant cytotoxic component of *B. cereus*. In a study of over 100 *B. cereus* strains, cytotoxicity correlated well with the amount of Nhe produced but

Abbreviations: EC buffer, extracellular bathing solution; LDH, lactate dehydrogenase; PI, propidium iodide.

Table 1. Identity table giving the values for percentage identity between ClyA from *E. coli* K-12 and the mature proteins of the Hbl and Nhe complexes from *B. cereus* ATCC 14579, obtained using Smith–Waterman global alignments and the Blossum 50 similarity matrix

	Hbl L ₂	Hbl L ₁	Hbl B	NheA	NheB	NheC
ClyA	18 %	20 %	20 %	18 %	19 %	20 %
Hbl L ₂		18 %	23 %	23 %	21 %	19 %
Hbl L ₁			25 %	18 %	40 %	32 %
Hbl B				20 %	27 %	25 %
NheA					22 %	22 %
NheB						44 %

poorly with the concentration of Hbl (Moravek *et al.*, 2006). The same group (Dietrich *et al.*, 2005) found that mAb 1E11 raised against NheB prevented cytotoxicity in 20 out of 20 strains of *B. cereus* isolated from food. In a study of *Bacillus thuringiensis* strain 407 Cry[−] (indistinguishable from *B. cereus*), culture supernatants from *hbl* and *cytK* deletion mutants showed no impairment in cytotoxicity towards HeLa and Caco-2 cells (Ramarao & Lereclus, 2006), implying that Nhe was the major virulence factor.

Previously, we have shown that maximal cytotoxic activity of Nhe against Vero cell epithelia is dependent on all three Nhe components (Lindbäck *et al.*, 2004). Cytotoxicity was quantified as inhibition of protein synthesis based on the impairment of leucine uptake (Lindbäck & Granum, 2006). With rapidly cytotoxic proteins this assay may be more a measure of uptake of radiolabelled leucine across the epithelial plasma membrane than of protein synthesis *per se*. Thus, following microscopic observations of increasing size of blebs in cells in response to *B. cereus* culture supernatants, we chose to investigate the nature of the plasma membrane damage using more direct markers. Using the *B. cereus* strain NVH 0075/95 and an isogenic *nheBC* mutant expressing only NheA, we show that Nhe acts as a pore-forming toxin to induce cell lysis. Additionally, we show structural and functional correlates between Nhe, Hbl and the pore-forming haemolysin cytolysin A (ClyA) from Gram-negative enteric bacteria.

METHODS

Cells, buffers and antibodies. Monolayers of Vero monkey kidney epithelia and human intestinal Caco-2 cells were grown under standard tissue culture conditions using minimal essential medium (MEM) and RPMI 1640 respectively, with 5 % (v/v) fetal calf serum. MEM was supplemented with penicillin (100 U ml^{−1}) and streptomycin (0.1 mg ml^{−1}), and RPMI 1640 with gentamicin (0.05 mg ml^{−1}) and glutamine (2.4 mM). Cells were subcultured from 25 ml flasks when confluent using cell scrapers and trituration, except for flow cytometric experiments, in which EDTA was used to detach cells from the flasks. All experiments on cultured cells were carried out in extracellular bathing solution (referred to as EC buffer) containing NaCl (135 mM), HEPES (15 mM), MgCl₂ (1 mM), CaCl₂ (1 mM) and glucose (10 mM), adjusted to pH 7.0–7.1 with Tris. The non-neutralizing mAbs 1A8, raised against NheA (Dietrich *et al.*,

2005), and 1C2, raised against NheB (Dietrich *et al.*, 1999), were used for immunoblotting. mAb 1E11, raised against NheB, is able to neutralize the cytotoxic activity of Nhe, as described by Dietrich *et al.* (2005).

Creation of the *nheBC* mutant. A truncation mutant in *nheB* of *B. cereus* NVH 0075/95, isolated from an outbreak of food poisoning in Norway (Lund & Granum, 1996), was created fortuitously whilst deleting *nheC*. Thus, the method described here is that devised to delete *nheC* alone. Subregions of the *nheB* and *nheC* genes were amplified by PCR using primer pairs GCGAAGCAATGGTTAGATGTA and CACAACCTGAACAGGAGCTTC, and GCGATTGATCAAAAGGATAG and AAAAATAAACAGCAGAACTAGTCCAC, respectively. PCR was carried out for 30 cycles using an annealing temperature of 50 °C. PCR products of *nheB* and *nheC* were cloned into pCR T7/NT-TOPO and pCR 2.1-TOPO (Invitrogen), respectively, and transformed into *Escherichia coli* One Shot TOP10 (Invitrogen). *nheC* was excised from pCR2.1-TOPO using *EcoRI*, and ligated into the *EcoRI* site of pMAD (Arnaud *et al.*, 2004). *nheB* was excised from pCRT7/NT-TOPO using *BamHI* and *HindIII*, and ligated into the *BamHI* site of pMAD together with the spectinomycin resistance cassette from pDG1726 (Guérout-Fleury *et al.*, 1995) digested with *BamHI* and *HindIII*, producing pMADΔ*nheC*. DNA sequencing confirmed that the plasmid did not contain any mutations in *nheB* introduced during PCR. This plasmid was electroporated into *B. cereus* NVH 0075/95 (Masson *et al.*, 1989), and transformants were subjected to allelic exchange as described elsewhere (Arnaud *et al.*, 2004). DNA sequencing of the obtained double-crossover mutant confirmed the deletion of *nheC*, while a single-base deletion of the 967th base (guanosine) was introduced into *nheB*. This deletion is likely to result in a truncated form of NheB due to a frameshift, as the last 80 amino acids were absent.

Crude toxin preparation (culture supernatants). *B. cereus* NVH 0075/95 and the isogenic *nheBC* mutant were grown in a modified casamino acids/glucose/yeast extract (CGY) broth as defined by Lund & Granum (1997), but with the 1 % glucose replaced by 1 % sucrose (Ouhib *et al.*, 2006). A 1 % inoculum of an overnight culture was incubated in 50 ml CGY (in a 250 ml flask) at 32 °C shaken at 100 r.p.m. for 5 h (early stationary phase). The supernatant was centrifuged and filtered through a 0.2 µm pore-size membrane filter and stored in aliquots at −80 °C.

Purification of Nhe components. NheA and NheB were purified from 5 h culture supernatants of *B. cereus* NVH0075/95 as described previously (Lindbäck *et al.*, 2004), with the following modifications: 1 l CGY was used to culture the organism rather than 3 l, and the proteins were precipitated with 50 % (w/v) ammonium sulphate. NheB was analysed by DEAE chromatography at pH 8. NheC was purified as a recombinant hexahistidine-tagged protein expressed in *E. coli* (Lindbäck *et al.*, 2004). Protein concentrations were estimated using the absorbance method of Whitaker & Granum (1980), in conjunction with visual assessment of titrations of the purified Nhe components from band densities on SDS–PAGE gels.

SDS–PAGE and immunoblotting. PAGE and Western immunoblotting were carried out as described previously (Lindbäck *et al.*, 2004), using mAbs 1A8 (Dietrich *et al.*, 2005) and 1C2 (Dietrich *et al.*, 1999). Biotin-conjugated goat anti-mouse antibody was used as a secondary antibody (1:1000). A complex of streptavidin and biotinylated alkaline phosphatase was used at a dilution of 1:3000, prior to developing with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

Propidium iodide (PI) uptake by Caco-2 and Vero epithelia. For microscopy studies, Vero and Caco-2 cells were grown on coverslips in 6-well microplates and tested after 3 days, having reached 80–90 %

confluence. Cells were washed with 1 ml EC buffer solution and incubated with dilutions of culture supernatants. Cells were incubated in 5 % CO₂ for up to 60 min. At various time points the medium was replaced with fresh medium containing 5 µg PI ml⁻¹ (7.5 µM) and the coverslips were removed for examination by epifluorescence microscopy at the end of the experiment. A halogen light source was set to 570/620 nm excitation/emission wavelengths. Time-course experiments of PI fluorescence in epithelia were performed using a Wallac Victor³ fluorimeter (Perkin Elmer). Caco-2 cells were cultured in 24-well microplates and tested 10–14 days after seeding, while Vero cells were tested 2–4 days after seeding. After equilibrating cells in EC buffer for 10–15 min, microplates were transferred to the heated chamber of the Victor³ (held at 37 °C), the bathing solution (buffer) was replaced with one containing PI (5 µg ml⁻¹), and dilutions of culture supernatant were added. Microplates were 'bottom read' to record fluorescence in cells attached to the base of the well using excitation/emission wavelengths of 575/595 nm. Wells were read at 1 min intervals for 1 s duration. Saponin (0.0025 %, w/v, in EC buffer) was used as a positive control to monitor fluorescence. Results are shown without subtraction of background fluorescence; hence baseline values increased with increasing volumes of culture supernatant. Note also that since divalent cations quench fluorescence (Gibbons *et al.*, 2001), absolute values are not of any significance. This was particularly noticeable in calcium- and magnesium-free buffer, which gave higher basal readings than the standard EC buffer. The rate of PI uptake was expressed as the mean change in fluorescence per minute measured over a 10–15 min period, starting with the lowest reading obtained before fluorescence increased.

Epithelial ATP content and lactate dehydrogenase (LDH) release. Cell culture medium was replaced with EC buffer and allowed to equilibrate for at least 10 min before exposure to culture supernatant in 1 ml final volumes. For measurement of LDH release following exposure of Vero and Caco-2 monolayers to the culture supernatant, duplicate samples of EC buffer were removed at various intervals and briefly centrifuged before analysis on an ADVIA 1650 autoanalyser (Bayer). Total cell monolayer ATP was measured by replacing the entire cell bathing solution after exposure to toxin at various time points with 1 ml buffer containing 1 % (v/v) Triton X-100. After 10 min incubation, duplicate 100 µl samples of the lysed cell suspensions were diluted 1:100 before mixing with 100 µl luciferase reaction buffer (BacTiter-Glo Microbial Cell Viability Assay, Promega) for 5 min and measurement in a Glomax 20/20 luminometer (Promega). Results were expressed in the original arbitrary lux units. Total monolayer LDH was measured at the end of the experiment after lysis with 1 ml 1.0 % Triton X-100 in EC buffer.

For osmotic protection of LDH release, glucose (30 mM, hydrodynamic radius 0.36 nm), PEG 400 (30 mM, 0.68 nm), PEG 1000 (28 mM, 0.94 nm) and PEG 8000 (10 mM, 3.78 nm) were dissolved in EC buffer. Osmotic protectants were added in reducing molarities so as to offset the increase in osmolarity in the final bathing solutions. Cells were preincubated for 10–15 min in the prewarmed solutions containing PEG before addition of 20 µl ml⁻¹ culture supernatants. Hydrodynamic radii were taken from Scherrer & Gerhardt (1971) and Planchot *et al.* (2000).

Planar lipid bilayer experiments. Investigation of the ability of purified Nhe to form single channel-like pores in synthetic phosphatidylethanolamine–phosphatidylserine (PE–PS) lipid bilayers was carried out as described previously (Hardy *et al.*, 2001b). Purified Nhe components in 0.1 M NaCl phosphate buffer in a ratio of 1:1:0.3 NheA:NheB:NheC, using approximately 40 ng NheA, were premixed before addition to the earthed bathing solution. Recordings were filtered at 500 Hz and acquired to computer disc using a Digidata 1200 AD converter at 5 kHz, and analysed off-line.

Single-channel conductances were estimated individually using Win EDR (Strathclyde Electrophysiology Software).

Flow cytometric measurement of cell size and PI uptake. Vero cells were detached from culture flasks by standard protocols with trypsin/EDTA inactivated by exposure to 10 % fetal calf serum in the growth medium. Prior to exposure to the toxin the cells were equilibrated at 37 °C in EC buffer with 5 µg PI ml⁻¹ for 10 min. Vero cell suspensions were assayed for forward light scatter and PI uptake using a FACSCalibur flow cytometer (Becton Dickinson Biosciences) with a 488 nm wavelength argon laser with linear amplification of the forward- and side-scatter light signals and logarithmic amplification of the fluorescence signals of each cell. Data were collected from 10 000 cells per time point. Cells were held at room temperature for the duration of the experiment (15 min). Analysis was carried out using CellQuest Pro software (Becton Dickinson Biosciences). Following normalization of cell size at time zero, statistical comparison between the effect of 20 µl ml⁻¹ culture supernatants of NVH 0075/95 and the *nheBC* mutant on cell size (mean forward scatter value) was carried out on the area under the curve for the two samples using the Mann–Whitney test.

Haemolysis assays. Blood cells from different species were washed three to six times in PBS. Purified Nhe proteins were used at a ratio of 6:6:1 NheA:NheB:NheC, using approximately 100 ng NheA ml⁻¹. Volumes of culture supernatants were incubated with 2 % (v/v) bovine blood, and freshly combined Nhe components were incubated with blood samples from different species diluted to concentrations obtaining the same OD₆₃₀ as 1.5 % human blood. After incubation at 37 °C for 60 min on a roller incubator, samples were centrifuged and haemolysis was determined from the A₅₄₀ of the supernatants. The percentage of haemolysis was calculated by comparing the A₅₄₀ of the samples with positive (100 % lysis by 1 % Triton X-100) and negative controls. All experiments were performed at least twice.

RESULTS

Characterization of the *nheBC* double mutant

During attempts to create an isogenic *nheC* deletion mutant in *B. cereus* strain NVH 0075/95 we were only able to obtain mutants which contained an additional frameshift mutation in *nheB*, resulting in an *nheBC* double mutant. Immunoblotting analysis of supernatants from 5 h cultures failed to detect NheB in the Δ *nheBC* strain, in contrast to the wild-type strain NVH 0075/95 (Fig. 1). Growth curves were indistinguishable between the two strains, each yielding 10⁸ c.f.u. ml⁻¹ after 5 h incubation at 32 °C.

Microscopic cytotoxicity of Nhe on Caco-2 and Vero cells

We examined the effect of Nhe on Caco-2 cell monolayers by microscopy. Cells often developed blebs at the plasma membrane, typically a single bleb that increased in size with time and eventually burst. Fluorescent dyes such as PI are excluded from cells with intact plasma membranes but will enter cells if pores of sufficient size are created in the plasma membrane. Upon intercalation with nucleic acid these dyes undergo Stokes shift and can be detected by their fluorescence. Microscopic examination of both Vero

Fig. 1. Nhe content and haemolytic activity in *B. cereus* culture supernatants. Western immunoblot of culture supernatants from *B. cereus* NVH 0075/95 (75/95) and the *nheBC* mutant (Δ BC) probed with antisera against NheA and NheB. The size marker (Std.) used was SeeBlue Plus2 Pre-stained Standard (Invitrogen). Both proteins yield two bands as a result of minor degradation, as observed previously (Lund & Granum, 1996, 1997).

and Caco-2 cells exposed to NVH 0075/95 supernatant demonstrated uptake of PI, detectable down to $6 \mu\text{g ml}^{-1}$. Photomicrographic images of Vero cells exposed to NVH 0075/95 supernatant, co-illuminated with both epifluorescent and phase-contrast illumination, showed development of clear blebs (i.e. free from organelles), which subsequently burst, leaving a granular appearance of what is likely to be the cytoplasm surrounding a red fluorescent nucleus (Fig. 2a). In contrast, cells exposed to up to $50 \mu\text{g ml}^{-1}$ supernatant from the *nheBC* mutant for up to 60 min excluded PI and appeared morphologically indistinguishable from cells exposed to culture medium alone (Fig. 2b).

Nhe induces PI uptake in Caco-2 cell monolayers

To quantify the rate of PI uptake in real-time we developed a fluorometric assay using epithelial monolayers grown in 24-well microplates. Fig. 3(a) shows the increase in PI fluorescence from Caco-2 cells over 20 min following exposure to NVH 0075/95 culture supernatant, and the rate of propidium fluorescence increasing in a dose-dependent manner (Fig. 3b). In contrast, Caco-2 cells exposed to up to $50 \mu\text{g ml}^{-1}$ culture supernatant from the *nheBC* mutant exhibited no increase in fluorescence (Fig. 3a). PI uptake in Caco-2 cells exposed to $20 \mu\text{g ml}^{-1}$ NVH 0075/95 culture supernatant was not dependent on extracellular divalent cations, since cells bathed in extracellular buffer with no added calcium or magnesium also exhibited prompt increases in PI fluorescence (data not shown). The increase in PI fluorescence was abolished in cells exposed to toxin that had been held at 60°C for 20 min (data not shown) or preincubated with the neutralizing mAb against NheB, 1E11 (Fig. 3a).

Fig. 2. Cytotoxicity of *B. cereus* NVH 0075/95 culture supernatant to Vero cells. (a) Photomicrograph taken 30 min after addition of $12.5 \mu\text{g ml}^{-1}$ NVH 0075/95 culture supernatant with dual phase-contrast and epifluorescent illumination in a bathing solution containing $5 \mu\text{g PI ml}^{-1}$. Note the numerous bright nuclei and clear swollen extrusions. (b) Vero cells exposed to $12.5 \mu\text{g ml}^{-1}$ culture supernatant from the *nheBC* mutant for 60 min. Bars, 0.1 mm.

PI uptake induced by Nhe is not blocked by glycine or induced by extracellular purinergic (ATP) activation in Caco-2 epithelia

PI uptake has been demonstrated in cells exposed to maitotoxin (Schilling *et al.*, 1999), cells that have been metabolically poisoned and in macrophages undergoing pyroptosis due to salmonellae (Fink & Cookson, 2006). In all three situations, PI uptake has been shown to be inhibited by 5 mM glycine but not by L-leucine. To determine whether the plasma membrane permeability to PI induced by Nhe was related to the activation of these undefined endogenous pores we exposed Caco-2 epithelia to NVH 0075/95 culture supernatants ($20 \mu\text{g ml}^{-1}$) in the presence of 5 mM glycine and leucine. PI uptake was unaffected by either amino acid (data not shown).

Purinergic activation of certain cell types is also able to cause PI uptake (Pelegrin & Surprenant, 2006). However,

Fig. 3. PI uptake in Caco-2 epithelia is dependent on all three Nhe proteins. (a) Time-course of propidium fluorescence in Caco-2 epithelia exposed to 20 $\mu\text{l ml}^{-1}$ culture supernatant from NVH 0075/95 (75/95; $\circ$). The fluorescence increase (arbitrary units; a.u.) is abolished in cells bathed in buffer solution containing 10 $\mu\text{g mAb 1E11 ml}^{-1}$ against NheB (75/95 + mAb; ∇). PI fluorescence did not increase when 20 $\mu\text{l ml}^{-1}$ culture supernatant from the *nheBC* mutant ($\Delta\text{BC-}$; $\square$) was added. Results are representative of six to eight wells for both 75/95 and $\Delta\text{BC-}$, and of three wells for 75/95 + mAb. (b) Concentration-dependent increase in fluorescence following exposure to NVH 0075/95 culture supernatants. Fluorescence is expressed as change in fluorescence measured (mean $\pm$ SE, $n=6-8$ wells for each dataset).

PI uptake in Caco-2 cells was not detected following exposure of the cells to 0.3 M ATP (data not shown). Thus, PI fluorescence in Caco-2 cells exposed to Nhe in *B. cereus* culture supernatants is not due to the undefined endogenous death pore activated by pyroptosis or purinergic activation by ATP.

Purified Nhe forms pores in planar lipid bilayers

The above data are consistent with increases in plasma membrane permeability induced by Nhe rather than activation of the endogenous channels linked with the inflammasome and cell death. To support this distinction, we added purified Nhe components to planar synthetic lipid bilayer membranes, which by definition will lack endogenous channels or receptors. Fig. 4(a) (upper trace) shows the prompt step-like increases in membrane conductance observed following addition of the three purified Nhe components. Typically, multiple levels of current were observed in which openings and closings of the channels could be seen before the current exceeded the headstage or the bilayer was disrupted. The time taken for channels to insert in the bilayer was 1.5 ± 0.2 min (mean $\pm$ SE, $n=14$). The specificity of the pore formation being due to Nhe rather than co-purifying contaminants was confirmed by the prevention of the appearance of channels in the bilayer by addition of the neutralizing antibody against NheB (1E11) to the bathing solution prior to the addition of Nhe, at least within a period up to 15–17 min (Fig. 4a, lower trace). In contrast, addition of

non-specific IgG (affinity-purified goat anti-rabbit antibody) did not significantly increase the time taken for channels to appear (2.3 ± 0.6 min, mean $\pm$ SE, $n=7$, $P>0.05$, unpaired t test). The range of channel conductances in 0.1 M NaCl is shown in Fig. 4(b). Channels of 200–700 pS dominated (mean 400 ± 58 pS in 0.1 M NaCl), but both lower and higher conductances were observed. The pores were able to conduct both cations and anions; however, replacement of sodium with a larger cation (N-methyl-D-glucamine) reduced the mean conductance to 235 ± 26 pS in 0.1 M sodium gluconate, whereas replacement of chloride ions with the larger gluconate ion had less effect, yielding channels with mean conductance of 400 ± 33 pS (0.1 M sodium gluconate). Thus, cations appear to permeate preferentially with respect to anions in the large conductance channels formed by Nhe in planar lipid bilayers.

Nhe causes ATP and LDH release from Caco-2 and Vero cell monolayers

The rapidity of action and the size of the putative pores in cell membranes were studied by measuring both cellular ATP and released LDH in Caco-2 monolayers following exposure to culture supernatants. Fig. 5(a) shows the prompt fall in intracellular ATP, in which over 90% of ATP was lost after 5 min, in Caco-2 monolayers exposed to 20 $\mu\text{l ml}^{-1}$ NVH 0075/95 supernatant. In contrast, supernatant from the *nheBC* mutant had no significant effect on ATP levels in the cell monolayers. An indication

Fig. 4. Nhe forms pores in planar lipid bilayers. (a) Representative recording of current flow across lipid bilayer induced by Nhe and the effect of a mAb against NheB. Upper trace, channel-like step increases in current occurring approximately 1 min after addition of purified Nhe proteins to the bathing solution (taken from one of 14 bilayers); lower trace, addition of 10 μg mAb 1E11 ml^{-1} to the bathing solution prior to the addition of Nhe prevented any channel insertions (one of three bilayers). Bilayers were bathed in 0.1 M NaCl, 5 mM HEPES, pH 7.0, and held at +20 mV. Traces were filtered off-line at 100 Hz. (b) Frequency histogram of observed Nhe-induced channel conductance in bilayers bathed in 0.1 M NaCl with 5 mM HEPES, pH 7.0. The results are from a total of 171 single-channel events. The channel sizes were grouped into 100 pS divisions.

of the extent of membrane permeability was obtained by measuring the release of the high-molecular-mass LDH (140 kDa) from Caco-2 monolayers into the bathing solution. Fig. 5(b) shows that 88% of total cell monolayer LDH was released at 60 min after exposure to NVH 0075/95 supernatant, whereas $\Delta nheBC$ supernatant again failed to induce any loss of LDH. Similar release of LDH was observed in Vero cell monolayers exposed to supernatant from NVH 0075/95 but not in those exposed to supernatant from the *nheBC* mutant. LDH release indicates either complete breakdown of the membrane or the formation of large pores. In the latter case, LDH release should be delayed or prevented by osmotic protectants. To test this we used PEG of varying molecular size as an osmotic protectant of LDH release in Vero cells. Fig. 5(c) shows the delay in LDH release induced by the presence of the largest diameter osmotic protectant, PEG 8000 (approximate diameter 7–8 nm). The time taken to reach 50% release of LDH increased from 13 min (control) to

28 min in the presence of PEG 8000. LDH release in the presence of additional glucose (0.9 nm diameter), PEG 400 (1.2–1.4 nm) and PEG 1000 (1.8–2.2 nm) was indistinguishable from that of cells without added osmotic protectant.

Nhe increases epithelial cell size prior to cell death

Using flow cytometry, changes in cell size can be ascertained by measuring forward light scatter as a measure of cell diameter. In Vero cell suspensions the mean cell size increased upon exposure to culture supernatants of NVH 0075/95, with concomitant (and progressive) uptake of PI (Fig. 6). The changes in cell size were significantly greater than those observed in cells exposed to supernatant from the $\Delta nheBC$ strain, which did not take up PI (i.e. remained intact). Addition of mAb 1E11 to the culture supernatant prior to addition to the cells abolished both the increase in

Fig. 5. Epithelial loss of ATP and LDH induced by Nhe. (a) The time-course of total ATP content of Caco-2 epithelial monolayers was monitored after exposure to NVH 0075/95 supernatant (●) and $\Delta nheBC$ supernatant (■). Values represent mean $\pm$ SE, $n=3$, for each data point. At time zero the total cellular ATP concentration was approximately $10 \mu M$ as judged against a standard solution of ATP. The final concentration of cellular ATP at 30 min for the cells treated with NVH 0075/95 supernatant was approximately $0.2 \mu M$. (b) Extracellular LDH was measured over time following exposure to NVH 0075/95 (●) and $\Delta nheBC$ supernatants (■). Maximum release of LDH from the monolayers induced by 1% (v/v) Triton X-100 is indicated by (▲). Values are mean $\pm$ SE, $n=3$. (c) Effect of PEG compounds on LDH release in Vero cell monolayers. LDH release induced by NVH 0075/95 supernatant was monitored in the presence of glucose, PEG 400, PEG 1000 and PEG 8000 (◑). Traces for all compounds except PEG 8000 overlaid each other and were indistinguishable from results obtained with the bathing solution without added osmotic protectant. Values shown are mean $\pm$ SE, $n=3$.

Fig. 8a). NheB, NheC and Hbl L₁ showed sufficient sequence similarities to Hbl B for generation of 3D homology models using the Hbl B structure as the template. The homology models of NheB and NheC created using SWISS-MODEL (Schwede *et al.*, 2003; swissmodel.expasy.org) are shown in Fig. 8(b, c). The 3D structures are predominantly α -helical, containing four-helix bundles that wrap around each other in left-handed supercoils, associated with a shorter fifth carboxy-terminal helix. A subdomain consisting of a β -hairpin flanked by two short α -helices is located between the third and fourth helices of the main bundle folded up against the side of the larger domain. Hbl B, NheB and NheC all contain a predicted hydrophobic segment that correlates perfectly with the β -hairpin.

Nhe and Hbl show structural similarity to ClyA

A search of the PDB using the VAST structural comparison algorithm (Gibrat *et al.*, 1996) revealed Hbl B to be structurally similar to a 34 kDa pore-forming haemolysin from *E. coli* named cytolysin A (ClyA) (Fig. 8d). (Wallace *et al.*, 2000). Hbl B aligns to ClyA with a VAST structure-similarity score of 8.9 and a DaliLite (Holm & Park, 2000) Z-score of 13.8. The two proteins can be superimposed (Fig. 8e) with a root mean square deviation of $4.0 \text{ \AA}$ (0.4 nm) for 242 C α residues, while the crystal structures of Hbl B and ClyA contain 331 and 301 residues, respectively. The subdomain containing the β -hairpin appears to have a different orientation in ClyA compared to Hbl B, as if tilted out in a more extended conformation. Although an alignment showed only weak similarity between the amino acid sequences of Hbl B and ClyA (Table 1, Fig. 7), they share strong structural similarities and several conserved residues.

Haemolytic activity of Nhe

In the light of the predicted structural and haemolytic activity of Hbl and ClyA we examined the activity of Nhe

cell size and PI uptake, such that the changes in forward scatter overlaid those observed in the cells exposed to the *nheBC* mutant.

Homology models of Nhe based on the Hbl B structural template

The three Hbl components L₂, L₁ and B are homologous in sequence to the components of Nhe, which implies that all six Hbl and Nhe proteins are members of the same protein family (Fig. 7, Table 1). Recently, the crystal structure of Hbl component B at $2 \text{ \AA}$ (0.2 nm) resolution was deposited in the Protein Data Bank (PDB; www.pdb.org) (PDB ID: 2nrj;

Fig. 6. Vero cell suspensions swell and die upon exposure to NVH 0075/95 culture supernatants. (a) Flow cytometric analysis of Vero cells showing increase in cell size as determined by mean forward scatter (MFS) value after exposure to $20 \mu\text{l ml}^{-1}$ NVH 0075/95 supernatant ($\bullet$), which was abolished by preincubation of the toxin with $10 \mu\text{g ml}^{-1}$ mAb 1E11 ml^{-1} for 15 min on ice ($\circ$). The response to NVH 0075/95 culture supernatants was significantly greater than that seen with $20 \mu\text{l ml}^{-1}$ supernatant from the *nheBC* mutant (∇ , $P < 0.05$). Cells exposed to EC buffer alone are shown as (∇). (b) Increase in PI uptake in Vero cells, expressed as mean fluorescence intensity (MFI) of cells per time point. Data were measured simultaneously with forward scatter shown in (a). Only cells exposed to $20 \mu\text{l ml}^{-1}$ NVH 0075/95 culture supernatants demonstrated PI uptake alongside cell swelling. The symbols are the same as in (a). Each data point represents 10^4 cells and the figures are representative of two to four experiments for each condition.

towards erythrocytes. Incubation of purified Nhe proteins with 1.5 % human erythrocytes at 37°C for 1 h resulted in dose-dependent haemolysis, as determined by haemoglobin release measured at 540 nm (Fig. 9a). This activity was inhibited when the Nhe components were premixed with the 1E11 antibody before testing (data not shown). The haemolytic activity of Nhe varied between species, as shown in Fig. 9(b).

DISCUSSION

The use of an isogenic mutant demonstrates that Nhe has both cytolytic and haemolytic activity, rapidly inducing disruption of epithelial plasma membranes as assayed by four markers of membrane integrity, namely PI uptake, ATP and LDH release from epithelia, and haemoglobin release from erythrocytes. Single channel formation in lipid bilayers and inhibition of lysis of epithelial cells by osmotic protectants strongly indicate that Nhe disrupts epithelial plasma membranes through the insertion of transmembrane pores. This results in colloid osmotic lysis, as indicated by observations of increases in cell size following exposure to Nhe, as measured by forward light scatter.

The use of an *nheBC* mutant in *B. cereus* is dependent on the fact that the strain used, NVH 0075/95, lacks genes encoding the other two dominant cytotoxic components, Hbl and CytK. The total loss of cytotoxicity in the *nheBC* mutant reveals the contribution of Nhe to cytotoxicity in culture supernatants. The *nheBC* mutant producing just the NheA component was employed as we have been unable to create a mutant with deletion of the entire *nhe*

operon, as was also observed by Ramarao & Lereclus (2006).

The use of unpurified supernatants obviously needs to be judged cautiously, since *B. cereus* is known to produce a variety of additional exotoxins. However, they did not appear to have any primary ability to cause PI uptake, ATP loss or LDH release, since up to $50 \mu\text{l ml}^{-1}$ of supernatant from the $\Delta nheBC$ strain had no demonstrable effect. Furthermore, the antibody neutralization effects strongly indicate that the formation of Nhe-specific pores is the cytotoxic determinant in NVH 0075/95 culture supernatants. Thus, it remains likely that the membrane damage is dependent on the activity of the three Nhe proteins and a cytotoxic contribution from other compounds need not be evoked.

The rapid time-course is consistent with direct pore formation rather than activation of inflammasome-mediated death pores. Cells undergoing caspase-dependent cell death resulting from salmonella infection permit influx of fluorescent dyes of up to 1 kDa, including propidium, a process that can be blocked by glycine. However this phenomenon is detectable in macrophages after hours rather than minutes (Fink & Cookson, 2006). More rapid is the membrane permeability to propidium dyes induced by ATP and the marine toxin maitotoxin (Estacion *et al.*, 2003). However, the former appears to use pannexin-1 hemichannel pores triggered by ATP-gated P2X_7 receptors (Pelegrin & Surprenant, 2006). We have excluded any dependence on exogenous ATP in our system as 300 mM ATP failed to induce PI uptake. The dye permeability induced by maitotoxin can be blocked by glycine, but

Fig. 7. Multiple sequence alignment of ClyA from *E. coli* K-12 and the mature sequences of the Hbl and Nhe components from *B. cereus* ATCC 14579, created using CLUSTAL W. Hydrophobic regions are boxed and in bold type, identical residues are indicated in red type on a yellow background, while blocks of identical and similar residues are indicated by blue and green backgrounds, respectively.

similar inhibition of the Nhe pore was not observed. Thus, whilst we have not measured the caspase dependence of *B. cereus* culture supernatants, and the relationship between pannexin-1 pores and the inflammasome is far from straightforward (Pelegriin & Surprenant, 2007), we believe that the results can be explained without reference to pannexin-1 channels, pyroptosis and the undefined endogenous death pore blocked by glycine.

Given the complex geometry of endogenous eukaryotic ion channels there is no simple relationship between the electrical conductance in a planar lipid bilayer and the diameter of a transmembrane pore. Nevertheless, to help assess the size of the channels formed by Nhe (200–700 pS in 0.1 M NaCl) we note that the conductances of the other pore-forming toxins of *B. cereus*, CytK and Haemolysin II, are both 600 pS, although obtained in 10-fold higher

concentrations of ions, i.e. 1 M NaCl and KCl (Hardy *et al.*, 2001a; Miles *et al.*, 2002). These toxins form pores by generating transmembrane β -barrels (Parker & Feil, 2005), and the archetypal β -barrel pore former of this group, *Staphylococcus aureus* α -haemolysin, has a diameter of 2.8 nm at the pore entrance narrowing to 1.4 nm (Song *et al.*, 1996). Thus, based on conductance values alone we expect the pore formed by Nhe to be substantially larger than this. The release of the high-molecular-mass LDH (140 kDa) is likely to be the result of cell lysis subsequent to pore formation and osmotic swelling. If so, then the delay in LDH release caused by PEG 8000 will be due to blocking of the preceding ionic fluxes through the Nhe pore.

Cytolysin A (ClyA), also known as HlyE and SheA, is a 34 kDa protein that was initially identified in *E. coli* K-12

Fig. 8. Structural comparison of Hbl, Nhe and ClyA. (a) Structure of Hbl component B as determined by X-ray crystallography (PDB ID: 2nrj). (b) Homology model of NheB. (c) Homology model of NheC. The models in (b) and (c) were created on the basis of the Hbl B crystal structure. The first 29 and 34 residues of the mature sequences of NheB and NheC, respectively, and the last 26 residues of NheB were not present in the models obtained. (d) Crystal structure of *E. coli* ClyA (PDB ID: 1qoy). Protein structures in (a–d) are shown in ribbon format, with the β -hairpins in blue, and drawn using MOLMOL (Koradi *et al.*, 1996). (e) Structural alignment visualized as a 3D superimposition of Hbl B (blue) and ClyA (grey) obtained using DaliLite, viewed as a $C\alpha$ -trace.

as a haemolysin expressed during anaerobic growth (Green & Baldwin, 1997; Ludwig *et al.*, 1999; Oscarsson *et al.*, 1996). It is also present in enteropathogenic *E. coli* (Ludwig *et al.*, 2004), *Shigella flexneri* (Wallace *et al.*, 2000) and *Salmonella enterica* serovars Typhi and Paratyphi A (Oscarsson *et al.*, 2002). ClyA is haemolytic and causes LDH release in nucleated cells (delCastillo *et al.*, 1997; Oscarsson *et al.*, 1996, 1999), which has led to the suggestion that it too mediates osmotic lysis through the formation of transmembrane homo-oligomeric pores (Eifler *et al.*, 2006; Tzokov *et al.*, 2006; Wallace *et al.*, 2000). The narrowest pore diameter for ClyA has been measured in cryoelectron microscope images at 4 nm. This is significantly larger than the diameter for the β -barrel pore-forming toxins (see above) and consistent with the large conductance pores created by Nhe.

The available 3D crystal structure of Hbl component B showed tertiary structure resemblance to ClyA of *E. coli* (Wallace *et al.*, 2000) in a near-global structural alignment, despite limited sequence similarity. Although four-helix bundles like those found in ClyA and Hbl B are quite common architectural features of proteins, the subdomain containing the hydrophobic β -hairpin represents a unique fold (Wallace *et al.*, 2000). The different orientation of this subdomain in the two crystal structures (Fig. 8e) potentially represents crystallization in different conformations, reflecting alternative means of burying the hydrophobic β -hairpin from the solvent which, in ClyA, is achieved by forming homodimers. 3D crystal structures have not been determined for any of the Nhe proteins, but,

judging by sequence homology, they are likely to adopt a fold similar to that of Hbl B, as structural similarities are usually highly conserved during protein evolution.

In addition to the common key structural features there are also clear functional correlates between Hbl, Nhe and ClyA: they are all bacterial pore-forming toxins with cytolytic and haemolytic activity. The pores formed by Nhe and ClyA are both weakly cation-selective in lipid bilayer experiments, and the pore conductances, 200–700 pS in 0.1 M NaCl for Nhe and 1000 pS in 0.1 M KCl for ClyA (Ludwig *et al.*, 1999), are broadly comparable, given that potassium ions have greater ionic mobility than sodium. Comparisons between the pore sizes of ClyA and Nhe remain only very tentative, since Nhe requires three separate proteins for maximum cytotoxicity, whereas ClyA is a homooligomer, and we present no data to identify the extent to which Nhe needs to oligomerize in this process.

Structural comparison methods such as VAST and DaliLite used in the current study can detect evolutionary relationships that are not apparent when judged by sequence similarities alone (Gibrat *et al.*, 1996). However, such methods may not always distinguish between remote homologues linked by divergent evolution, and analogous proteins obtained through parallel evolution, especially when only very limited sequence similarity is present. In such cases, determination of homology and classification into superfamilies is performed on combined sequence, structural and functional information, since divergent evolution from a common ancestor retains not only similar folding but also functional features (Dietmann &

Fig. 9. Haemolytic activity of purified Nhe proteins. (a) Haemolytic dose-response curve of purified Nhe proteins incubated with 1.5% human erythrocytes. Purified Nhe proteins were used at a ratio of 6:6:1 NheA:NheB:NheC, using approximately 100 ng NheA ml⁻¹ and expressed relative to this. (b) Species differences in sensitivity of erythrocytes to Nhe. Suspensions were adjusted to an OD₆₃₀ equal to that of 1.5% human blood. The amount of Nhe used corresponds to the highest concentration used in (a). The percentage of the maximal release of haemoglobin (Hb) was calculated relative to 100% lysis of controls consisting of the same type of erythrocytes. Data represent the mean of two to five experiments for each species.

Holm, 2001). Thus, the strongest argument for homology between the ClyA and the Hbl/Nhe families of toxins stems from the association of similar unique structures with a common functional property, the formation of transmembrane pores in eukaryotic cell membranes.

In summary, through the use of a deletion mutant and purified proteins we have identified a mechanism of cytotoxicity for Nhe, namely osmotic lysis following pore formation in the plasma membrane. We propose that the structural and functional similarities among Nhe, Hbl and ClyA indicate that they constitute a superfamily of pore-forming toxins.

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