Contact-haemolysin production by entero-invasive *Escherichia coli* and shigellae

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Summary. Entero-invasive *Escherichia coli* (EIEC) and shigellae were tested for contact-haemolysin (CH) with red blood cells (RBCs) of guinea-pig, rabbit, rat, mouse, monkey, man, sheep and chicken; all bacteria showed the best lysis with guinea-pig RBCs. The best culture medium for CH activity of shigellae was tryptic soy broth, and for EIEC it was casamino acid-yeast extract broth with 1 mM CaCl₂. CH production by all species was best at the slightly alkaline pH which is optimal for growth; it was also dependent on the presence of a large (140-Mda) plasmid. Pre-treatment of bacteria with homologous antisera inhibited CH activity. Various treatments of bacterial cells and RBCs suggested that CH may be a protein molecule, and that a chitotriose-like moiety may serve as CH receptor. RBCs that were incubated with bacteria at 4°C, or with heat-killed bacteria at 37°C, were not lysed; also, isolated cell-surface components (lipopolysaccharide and outer-membrane protein) did not lyse RBCs. This suggests that metabolically active cells are required for CH activity. Production of CH by both EIEC and shigellae is consistent with a common mechanism for the virulence of these organisms.

Introduction

Disease caused by shigellae involves a complex process of invasion and multiplication in the mucosa of the human colon.¹ The process of invasion requires entry of the bacterium into the enterocytes, and lysis of the membranous vacuole which surrounds it after invasion, to allow free multiplication of the pathogen within the cytoplasm. This leads to spread of the organisms to the lamina propria, triggering a severe inflammatory response in the gut mucosa with abscesses and ulceration.^{2,3} All these features are regulated by a large non-self-conjugative plasmid of 120–140 Mda called the virulence plasmid. Entero-invasive *Escherichia coli* (EIEC) produces dysentery like that caused by shigellae; it also possesses a 140-Mda plasmid which is associated with virulence.^{4,5}

Sansonetti *et al.*⁶ described a virulence-plasmid-mediated, thermoregulated contact-haemolysin (CH) in *Shigella*, which was active against sheep red blood cells (SRBC). Close contact was required between virulent bacteria and SRBC which was achieved by centrifugation. The production of CH correlated with its capacity to destroy the membranous vacuole surrounding the bacteria after invasion of HeLa cells, and with efficient intracellular multiplication. CH was also demonstrated in *S. dysenteriae* and *S. sonnei* strains in another study.⁷

The large virulence-plasmids of EIEC and all species of *Shigella* have extensive homologous sequences and are functionally exchangeable.⁸ As the pathogenesis of EIEC and *Shigella* spp. is similar, this study was initiated to investigate whether CH was also produced by *S. boydii* and EIEC, other species of *Shigella* being included for comparison. Factors influencing the production and activity of CH were investigated by the use of virulent and avirulent pairs. Preliminary experiments were also performed to study the chemical nature of CH.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains are shown in table I. Dr D. N. Taylor, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, kindly provided 26 strains of EIEC from patients with invasive diarrhoea in Thailand. Five similar isolates were from central Australia (2), Bangladesh (2) and Hungary (1).⁹ The avirulent EIEC strain 2/33 is an isogenic mutant strain of virulent EIEC strain 2, isolated in Hungary;⁹ these were kindly supplied by Dr P. Echeverria (AFRIMS, Bangkok). *E. coli* strain 36 000 was obtained from the Centers for Disease Control, Atlanta, GA, USA. All *Shigella* strains were isolated from patients at the Clinical Research Centre, International Centre for Diarrhoeal Disease Research, Bangladesh. The CH

Table I. CH activity and HeLa-cell invasiveness of shigellae and EIEC

Organism			Number of strains	CH activity (OD ₄₉₀)	Number* of bacteria/HeLa cell
<i>S. dysenteriae</i> type 1	Virulent		5	2.19	53
	Avirulent		5	0.89	0
	Rough		4	0.12	0
<i>S. sonnei</i>	Virulent		5	1.65	54
	Avirulent		5	0.12	0
	Rough		4	0.15	0
<i>S. boydii</i>	Virulent		5	1.16	11
	Avirulent		5	0.34	0
<i>S. flexneri</i>	1a	Virulent	2	1.06	25
	1b	Virulent	2	0.72	5
	2a	Virulent	5	2.43	70
	2b	Virulent	2	0.71	3
	3a	Virulent	2	1.10	15
	3b	Virulent	2	1.14	15
	4a	Virulent	1	1.10	12
	5	Virulent	2	2.00	51
	Y	Virulent	2	2.27	65
	6	Virulent	2	1.44	20
	2a	Avirulent	5	0.51	0
EIEC	O28	Virulent	24	2.72	152
	O29	Virulent	2	1.35	15
	O143	Virulent	1	0.87	3
	O144	Virulent	1	0.82	6
	O164	Virulent	1	0.90	6
	O124	Virulent	1	0.56	4
	O136	Virulent	1	1.01	27
	O143	Avirulent	1	0.50	0
<i>E. coli</i>	36000	Avirulent	1	0.10	0

* Each of these numbers is the approximate mean of results from at least three experiments. Virulent strains gave a positive Sereny result, invaded HeLa-cells and bound Congo red dye; avirulent strains did none of these. Virulent strains had a 120–140-Mda virulence plasmid; avirulent strains had either no virulence plasmid or a deleted one. Rough strains auto-agglutinated in physiological saline and did not have the virulence plasmid.

activity of bacterial components was studied with *S. flexneri* strain Z-611, *S. dysenteriae* type 1 strain Z-24623, and EIEC strain 2.

All the strains were stored at -70°C in Trypticase Soy Broth containing yeast extract 0.6% w/v (TSB; Gibco) with glycerine 15% v/v. The growth media were TSB, Casamino Acid-Yeast Extract Broth (Difco) supplemented with 1 mM calcium chloride (CYE) and minimal medium (MM) as described by Ahmed *et al.*¹⁰

Assay of CH

CH activity was measured by the method of Sansonetti *et al.*⁶ Briefly, bacteria were washed once and suspended in phosphate-buffered saline pH 7.3 (PBS) to a concentration of 2×10^{10} cfu/ml; blood was collected in Alsever's solution, washed twice, and the packed RBCs were resuspended in PBS to a concentration of 4×10^9 RBCs/ml; 50- μl volumes of bacterial suspension were mixed with equal volumes of RBCs in 96-well microtitration plates, and centrifuged at 2200 g for 10 min to achieve close contact between bacteria and RBCs. The plates were incubated at

either 37°C or 4°C for 2 h. Pellets were resuspended in cold PBS, centrifuged at 4°C for 15 min and the OD₄₉₀ of the supernates was measured with an ELISA reader (Titertek-Multiscan, Flow Laboratories).

Plasmid analysis

Plasmid DNA was isolated by the method of Birnboim and Doly¹¹ and electrophoresed in agarose 0.7% gel.¹²

Tests for bacterial virulence

Each strain was tested for virulence, both by invasion of HeLa cell monolayers⁶ and by the Sereny test.¹³ The latter was performed by inoculating 20 μl of bacterial suspension containing 3×10^8 cfu/ml into the conjunctival sac of an adult guinea-pig; strains producing keratoconjunctivitis within 72 h were considered virulent.

For the former, non-confluent monolayers of HeLa cells on 12-mm cover glass (Kimble, Toledo, OH, USA) in a vial were inoculated with bacteria, centrifuged for 10 min at 2200 g and incubated for 2 h

at 37°C. Non-invasive bacteria were washed off with PBS. Monolayers were fixed in methanol and stained with Giemsa dye, and the percentage of infected cells was determined by light-microscopy. To calculate the average number of viable bacteria in each infected HeLa cell, strains were inoculated on to monolayers of HeLa cells in a vial, centrifuged for 10 min at 2200 *g*, incubated for 15 min, washed three times with PBS, covered with 2 ml of Minimum Essential Medium (Gibco) containing gentamicin 200 µg/ml, and re-incubated for 2 h at 37°C. Monolayers were then washed, cells were trypsinised and counted, and finally cells were lysed with a solution of sodium deoxycholate 0.5% in distilled water. Dilutions of this suspension were plated to count the number of bacteria/vial. The average number of bacteria in each infected HeLa cell in a vial was calculated as follows:

$$\frac{\text{number of viable bacteria}}{\text{number of HeLa cells} \times \text{percentage of infected HeLa cells}}$$

For comparison of the invasiveness of different organisms, the chosen multiplicity of infection was 100 bacteria/HeLa cell. Each strain was tested at least three times; results are presented as the mean from three experiments.

Treatment of erythrocytes

Erythrocytes (RBCs) were treated at 37°C for 1 h with various reagents (Sigma): N-acetylneuraminic acid 1% w/v (NANA), lysozyme 250 µg/ml, neuraminidase 100–1000 U/ml, and lectins—wheat germ agglutinin 10 µg/ml, phytohaemagglutinin 1.0 µg/ml and Concanavalin A 0.1 µg/ml. Higher concentrations of phytohaemagglutinin or Concanavalin A caused lysis of guinea-pig RBCs.

Treatment of bacteria

Bacteria (10⁹ cfu) were treated with: (1) enzymes for 4 h at 37°C—trypsin (Flow Laboratories, Herts) 100 µg/ml, proteinase K (Sigma) 100 µg/ml; (2) rabbit sera for 1 h at 37°C—normal serum and homologous and heterologous antisera against whole bacterial cells;¹⁴ (3) heat, at 56°C or 100°C for 1 h.

Extraction of bacterial components

Lipopolysaccharide (LPS) of *S. flexneri* strain Z-611, *S. dysenteriae* type 1 strain Z-24623, and EIEC strain 2 were extracted with hot phenol-water¹⁵ and purified by ultracentrifugation at 100 000 *g* for 4 h. Nucleic acid and protein were removed by treatment with DNAase and RNAase, and proteinase K 100 µg/ml, respectively. Bacterial cells were depleted of LPS by treating cells with edetic acid. Outer-membrane protein (OMP) was prepared by water extraction.¹⁶

LPS and OMP were freeze-dried and reconstituted in PBS. Protein was estimated by dye-binding¹⁷ and

carbohydrate in LPS was estimated by the method of Dubois *et al.*¹⁸ CH activity of LPS and OMP was tested at concentrations up to 10 mg/ml. Bacterial cells depleted of OMP and LPS were also assayed for CH activity.

Results

Influence of growth medium on CH activity

Bacteria were grown with shaking in TSB, CYE and MM, and their CH activity was assayed with sheep RBCs (fig. 1). For *S. flexneri*, TSB was the best medium, whereas *S. dysenteriae* and EIEC exhibited maximal activity when grown in CYE; on the other hand, CYE provided only poor CH activity by *S. flexneri*, *S. sonnei* and *S. boydii*. CH activity was reduced or undetectable when EIEC and shigellae were grown in static broth or on agar plates of these media.

CH activity on RBCs of different animal species

RBCs from guinea-pig, rabbit, rat, mouse, monkey, man (blood group O), sheep and chicken were compared (fig. 2). All the bacterial species exhibited maximal CH activity against guinea-pig RBCs; hence, the latter were used for subsequent experiments. Except with *S. flexneri* and EIEC, sheep RBCs were poorly lysed.

The influence of growth medium was re-tested with guinea-pig RBCs: TSB was the most suitable medium for production of CH by shigellae and CYE for EIEC (fig. 1).

Influence of pH of growth medium on CH activity

For *S. flexneri*, *S. dysenteriae* type 1 and *S. sonnei*, neutral or slightly alkaline pH (7.3–8.5) was suitable for CH production. However, with *S. boydii*, the activity was reduced as the pH was increased from 7.3 to 9.0, and there was not much reduction in CH activity as the pH decreased from 7.3 to 6.0. EIEC showed a marked optimum at pH 7.3, and this pH was also near the optimal for shigellae (fig. 3).

Influence of duration and temperature of growth on CH activity

CH activity was examined in bacterial cultures grown for 4–48 h. It was maximal at 6–10 h, but beyond 24 h bacteria exhibited little haemolytic activity.

CH activity of both shigellae and EIEC was greatly dependent on growth temperature: strains which were strongly positive when grown at 37°C showed reduced activity when grown at 30°C. Activity could be partially restored by changing the growth temperature to 37°C for 2 h before incubation with RBCs, but

Fig. 1. Influence of growth medium on CH activity of *Shigella* spp. and EIEC. Bacteria grown in TSB (▨), CYE (▩) and MM (▤) were tested with sheep RBC. Blank bars (□) to the right of the marked bars indicate CH activity of bacteria grown in the same medium but tested with guinea-pig RBC. OD₄₉₀ was measured with an ELISA reader. *S. flex* = *S. flexneri*; *S. dys 1* = *S. dysenteriae* type 1.

Fig. 2. CH activity of *Shigella* spp. and EIEC against RBCs of different animal species: guinea-pig, ■; rabbit, ▨; rat, ▩; mouse, ▤; monkey, ▥; man, ▦; sheep, ▧; chicken, ▨ and see footnote to fig. 1.

extension of this period to 4 h did not result in further increase of CH activity.

No CH activity was shown when the assay was performed at 4°C, even with bacteria grown optimally at 37°C.

Association of large virulence plasmid with CH activity

Plasmid profiles of several virulent strains, both shigellae and EIEC, and of their avirulent counterparts

Fig. 3. Influence of pH of growth medium on CH activity of *Shigella* spp. and EIEC: pH 6.0, □; 6.5, ▤; 7.3, ■; 8.0, ▨; 8.5, ▩; 9.0, ▪ and see footnote to fig. 1.

were analysed to study association of the 120–140-Mda plasmid with CH activity. Avirulence was determined by the inability to bind the dye Congo-red,¹⁹ to produce keratoconjunctivitis in the guinea-pig,¹³ and to invade HeLa cells.⁶ In all the species, CH activity appeared to be plasmid mediated, because loss of the large plasmid, or reduction in size by deletion, resulted in almost complete absence of CH activity (table I).

Correlation of CH activity with invasion potential of bacteria

S. flexneri serotypes 2a, 5 and Y, *S. dysenteriae* type 1, and *S. sonnei* showed similar invasion potential after incubation for 2 h, reaching 51–70 bacteria/HeLa-cell (table I). However, EIEC type O28 showed a much higher degree of invasion (152 bacteria/cell) than shigellae. Other serotypes of EIEC and *S. flexneri* and strains of *S. boydii* showed lower invasion capability (3–27 bacteria/cell).

Generally, CH activity correlated well with the invasion potential of the organism. However, avirulent strains of *S. dysenteriae* type 1, although non-invasive in HeLa-cell assay, exhibited slightly higher CH activity than some virulent strains of other *Shigella* spp. and EIEC. Also, one virulent EIEC strain (O124) exhibited low CH activity although it was moderately invasive (table I).

Influence of various treatments of RBCs and bacteria on CH activity

Pre-treatment of RBCs with lysozyme resulted in significant reduction (56–75%) in CH activity, but neuraminidase and NANA had minimal inhibitory effect (table II). Of the three lectins, wheat germ agglutinin showed 50–67% inhibition of CH activity, whereas phytohaemagglutinin and Concanavalin A had no effect.

No reduction in CH activity was noted when bacteria were treated with trypsin, but CH activity was completely lost after treatment with proteinase K, indicating that the CH molecule may be a trypsin-resistant protein. Significant reduction of CH activity occurred after incubation of bacteria with homologous antiserum, whereas little or no inhibition was found with heterologous or control serum. Prior heat-treatment of cells at 56°C for 1 h resulted in significant reduction in CH activity, and the activity was completely lost after heating at 100°C for 1 h.

CH activity of bacterial components

The OMP and LPS obtained from *S. flexneri* strain Z-611, *S. dysenteriae* type 1 strain Z-24623 and EIEC strain 2, either separately or combined, did not haemolyse RBCs in concentrations up to 10 mg/ml (table II). However, cells depleted of OMP showed

stationary phase, and little CH activity was detected when bacteria were either incubated with RBCs at 4°C or pre-heated at 56°C for 1 h. Thus, isolated cell components which include a relevant molecule may be non-functional in the absence of metabolically active bacteria.

Decrease in CH activity after treatment of RBCs with lysozyme suggests that the CH molecule interacts with a lysozyme-sensitive moiety on the RBC surface. Treatment with wheat germ agglutinin, specific for the lysozyme substrate of N-acetyl-D-glucosamine oligomers of three units in length (*N,N',N''*-triacylchitotriose),²³ also caused significant reduction in CH activity. On the other hand, treatment with phytohaemagglutinin and Concanavalin A, which do not interact with the lysozyme substrate chitotriose, failed to reduce CH activity. The minimal inhibitory effect of neuraminidase and NANA on CH activity, probably by non-specific interaction, renders the involvement of sialic acid unlikely. All this evidence suggests a chitotriose-like moiety as the probable receptor for CH.

Studies with bacteria harbouring an intact plasmid and a modified plasmid, and with plasmidless derivatives, showed a correlation between CH activity and HeLa cell invasiveness.⁶ Similar results were obtained in the present study, with two exceptions (table I): avirulent *S. dysenteriae* type 1 strains had relatively high CH activity but were non-invasive, and virulent EIEC (O124) had low CH activity but was invasive. The reason for this lack of correlation is not apparent; but it is noticeable that, within each species, virulent strains always had higher CH activity and invasion potential than their avirulent counterparts. Our results showed that certain EIEC strains, especially O28, were even more invasive than those of *S. flexneri* and *S. dysenteriae*, and *S. boydii* was moderately invasive.

Other investigators also reported the invasive nature of EIEC in HEp-2 cells.^{24,25} EIEC (O28) has also been found to possess high cell-surface hydrophobicity, similar to *S. dysenteriae* type 1 (unpublished observation). The absence of CH activity in EIEC and *S. boydii* noted by Clerc *et al.*⁷ could be due to their use of sheep RBCs (see above) or of different strains of bacteria.

Reduced CH activity by bacterial cells grown at 30°C, and partial restoration after changing from 30°C to 37°C, indicate that the activity might be thermoregulated, as are other virulence properties of *Shigella* spp.—invasion of epithelial cells *in vitro*,^{6,7} expression of peptides involved in invasion,⁵ binding of the dye Congo red,¹⁹ and haemagglutination.²⁰ The CH activity in *S. flexneri* has been shown to be critical for lysis of the phagocytic vacuole after invasion of HeLa cells, which permits efficient intracellular multiplication.⁶ In this study, we have demonstrated CH activity and invasion of HeLa cells by EIEC; it is possible that CH activity of EIEC may also be essential for intracellular multiplication.

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