Association between cell-bound haemolysin and cell-detaching activity of *Escherichia coli* isolated from children

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Haemolysin α (HlyA) produced by cell-detaching *Escherichia coli*, a putative new class of enteric pathogen, is considered to be the main factor responsible for detachment of cells cultured *in vitro*. HlyA is one of the few *E. coli* proteins actively secreted into the medium during exponential growth. In the present study 27 HlyA-positive *E. coli* isolates, randomly selected from stool specimens, produced a cell-bound haemolysin that was detectable during the exponential and stationary growth phases. The influence of both cell-free and cell-bound haemolysins of the selected isolates on cell-detaching activity of *E. coli in vitro* was determined. The results suggest that cell-bound haemolysin rather than cell-free HlyA was responsible for the cell-detaching activity of *E. coli* strains tested.

INTRODUCTION

Cell-detaching *Escherichia coli* (CDEC) strains were isolated from children with diarrhoea for the first time by Gunzburg *et al.* (1993). In the reversible intestinal tie adult rabbit diarrhoea (RITARD) model, Elliott *et al.* (1998) demonstrated that CDEC strains were able to elicit diarrhoea; however, the studies of Marques *et al.* (1995) and Okeke *et al.* (2002) have not confirmed the association of these strains with diarrhoea in humans. Marques *et al.* (1995), Gomes *et al.* (1995) and Okeke *et al.* (2002) showed that the majority of CDEC were haemolytic on blood agar during 3 h of incubation and hybridized with the DNA probe specific for haemolysin α (HlyA) of *E. coli*. However, these results were not confirmed by Fábrega *et al.* (2002). Moreover, most CDEC isolates tested did not demonstrate the genes for pathogenic *E. coli* virulence factors, such as heat-labile or heat-stable enterotoxins, cytolethal distending toxin (CDT) or Shiga toxins (Stx1 or Stx2), but did show the presence of genes for cytotoxic necrotizing factor 1 (CNF1). However, according to Elliott *et al.* (1998), CNF1 had no influence on cell-detaching activity of *E. coli*.

Since most CDEC isolates studied are HlyA-positive, this haemolysin is considered to be the main factor responsible

for cell detachment *in vitro* (Marques *et al.*, 1995). HlyA is the prototype of the family of calcium-dependent RTX cytolysins and is actively secreted into the medium in the exponential growth phase. However, HlyA is not stable in the external environment and its haemolytic activity decreases to undetectable levels during the stationary phase of growth (Bohach & Snyder, 1985). *E. coli* produces several other haemolysins. Enterohaemolysin of enterohaemorrhagic *E. coli* (EHEC), designated Ehx or Hly_{EHEC}, is very similar to HlyA with regard to its genetic organization and calcium ion dependency although it is cell-bound (Taneike *et al.*, 2002). To our knowledge, the influence of this type of *E. coli* haemolysin, as well as contact haemolysins described among enteroinvasive (EIEC) (Sansone *et al.*, 1986) and enteroaggregative (EAEC) (Haque *et al.*, 1994) *E. coli* strains, on cell-detaching activity has not been studied.

The present study was undertaken to determine the influence of cell-free HlyA and cell-bound haemolysins on the cell-detaching activity *in vitro* of 27 randomly selected haemolytic *E. coli* isolates obtained from human stool specimens.

METHODS

Bacterial strains. Twenty-seven haemolytic *E. coli* isolates randomly selected from faeces of 27 children with a mean age of 18 months, 20 with diarrhoea (20 isolates) and seven without diarrhoea (seven isolates) were examined. *E. coli* K12 strain C₆₀₀ and

Abbreviations: CDEC, cell-detaching *E. coli*; EAEC, enteroaggregative *E. coli*; EHEC, enterohaemorrhagic *E. coli*; EIEC, enteroinvasive *E. coli*; HlyA, haemolysin α .

six non-haemolytic *E. coli* isolates obtained from stool specimens (strains 101–106) were included as negative controls. Two *E. coli* O26 enterohaemolysin-positive strains obtained from children with diarrhoea (strains 738/O26 and 740/O26) and two *E. coli* strains that were haemolytic on blood agar plates after 24 h of incubation (strains 81 and 147) were used as controls in the cell-detaching assay (Table 1).

Haemolysin assay. For detection of haemolytic activity, the *E. coli* isolates were grown on tryptose soy agar with 5% defibrinated sheep blood or 5% sheep erythrocytes washed in saline (0.85%) with or without 10 mM CaCl₂ or 10 mM EDTA added. The blood agar plates were incubated at 37 °C for 4 and 24 h and then examined for the presence of haemolysis zones around bacterial colonies (Beutin *et al.*, 1989).

Production of extracellular HlyA and cell-bound haemolysins. The standardized red blood cell (RBC) suspension in PBS-CM (PBS with 0.01% CaCl₂ and 0.01% MgCl₂; pH 7.4) was defined as the quantity of washed RBCs that gave an absorbance of 1.0 at 550 nm after total lysis in 1 ml distilled water. *E. coli* isolates were grown at 37 °C for 4 h (exponential growth phase) or 24 h (stationary phase) in 2× yeast extract medium with 10 mM CaCl₂. The culture supernatants were filter-sterilized (0.2-µm pore-size), mixed with an equal volume of RBC suspension and incubated at 37 °C for 90 min. The amount of released haemoglobin was determined spectrophotometrically at 550 nm (Bhakdi *et al.*, 1986; Elliott *et al.*, 1998). Bacterial cells of the same cultures were washed twice with PBS-CM and resuspended in the same buffer to a concentration of 3×10^8 cells ml⁻¹ and haemolytic activity of the suspensions was determined as described above. The culture filtrates and the bacterial cells were also incubated at 37 °C for 90 min without RBCs and then tested to determine the influence of the incubation period on haemolytic activity. Haemolytic activity was also measured in filter-sterilized supernatants obtained from bacterial cell suspensions in PBS-CM incubated at 37 °C for 90 min to determine whether cell-free haemolysin was released into the environment during that period. Haemolytic activity was expressed as the mean percentage of haemoglobin released in two independent assays in comparison to total lysis of RBCs in water.

Osmotic protection assay. Standardized RBC suspensions were prepared in PBS-CM containing one of the following substances: 30 mM dextran 4, 30 mM dextran 8, 30 mM sucrose (Bhakdi *et al.*, 1986) or an alcoholic solution of cholesterol (10 mg ml⁻¹) (Figueirêdo *et al.*, 2003). Haemolytic activity of the culture filtrates from exponential and stationary growth phases of the isolates and the corresponding bacterial cell suspensions were then determined as described above.

Influence of UV irradiation and heating on haemolytic activity. Exponential and stationary culture filtrates and bacterial cell suspensions were tested for haemolytic activity as described above after UV irradiation for 10 min and after heating at 56 °C for 10 min (Jorgensen *et al.*, 1976).

Cytolysin ClyA expression assay. The ability of *E. coli* isolates to express cytolysin ClyA was determined on 1% Columbia agar containing 5% horse RBCs washed with PBS as described by Oscarsson *et al.* (2002) except that the medium was supplemented with 10 mM EDTA to inhibit α-haemolysin activity.

Contact haemolysin assay. The assay was performed according to Sansonetti *et al.* (1986).

Detection of CNF, CDT and enterohaemorrhagic *E. coli* (Hly_{EHEC}) enterohaemolysin genes by PCR. The genes were identified as described by Blanco *et al.* (1996), Wieler *et al.* (2000) and Osek & Weiner (2003).

Cell-detaching (CD) assay. CD activity of exponential and stationary culture filtrates and bacterial cell suspensions, as well as bacteria heated at 56 °C for 10 min and UV-irradiated for 10 min, was screened on Int 407 (human jejunum carcinoma) and Caco-2 (human colon carcinoma) cell lines according to Elliott *et al.* (1998). A semiconfluent monolayer of Int 407 or Caco-2 cells in a 96-well tissue culture plate was washed three times with PBS-CM and then 100 µl aliquots of bacterial culture filtrates or bacterial cell suspensions of exponential or stationary growth phase diluted 1:100 with PBS-CM were added to duplicate wells and the plate was incubated at 37 °C for 90 min under 5% CO₂. Monolayers were washed three times with PBS-CM, fixed with 70% methanol for 10 min, stained with 0.13% crystal violet for 10 min and then briefly destained in sterile water. Cell detachment was quantified by eluting crystal violet with a solution of 50% ethanol, 49% water and 1% SDS, and measuring absorbance of the eluate at 590 nm. CD activity was expressed as the mean percentage of cell detached from a monolayer in five independent assays in comparison with intact cell monolayers and *E. coli* strains used as negative controls.

Data analysis. Regression analysis was used to evaluate the relationship between the mean haemolytic activity of bacterial cell suspensions and the mean cell-detaching activity of bacterial cells of the *E. coli* strains examined.

RESULTS AND DISCUSSION

Among 27 *E. coli* isolates examined, two strains (7.4%) showed the presence of genes for CDT 1 and 14 (51.8%) showed the genes encoding CNF1. None of the isolates revealed the genes for Hly_{EHEC}, except for the two *E. coli* O26 strains used as controls. All of the *E. coli* isolates were haemolytic within 4 h of incubation on all blood agar media used except the media containing 10 mM EDTA, indicating that the haemolysin produced was calcium-dependent (Table 1). After 4 h of incubation, 25 (92.6%) of the culture filtrates and all 27 bacterial cell suspensions showed comparable haemolytic activity with mean levels of 35.8 and 37.9%, respectively. Both the cell-free (HlyA) and the cell-bound haemolysins were calcium-dependent and their activity was inhibited in the presence of dextran 4 and dextran 8 but not in the presence of sucrose or cholesterol. UV irradiation inhibited haemolytic activity of 10 (37%) culture filtrates and all bacterial cell suspensions; this loss of activity was associated with killing of bacteria as determined by the lack of growth on MacConkey agar plates. Heating at 56 °C for 10 min inhibited HlyA present in 20 (74.1%) filtrates and haemolytic activity of all 27 bacterial cell suspensions, although it had no influence on growth of the bacteria on MacConkey agar plates. In contrast, after 24 h of incubation, haemolytic activity was detected in three (11.1%) culture filtrates and it was associated with 25 (92.6%) bacterial cell suspensions.

No haemolytic activity was observed in the filtrates obtained from bacteria suspended in PBS-CM and incubated at 37 °C for 90 min without RBCs, indicating that cell-free haemolysin was not released under these conditions. The incubation period influenced neither the haemolytic activity of the bacterial cells nor the cell-free haemolysin. None of the *E. coli* isolates produced contact haemolysins as tested by the contact haemolysin assay. For promoting contact

Table 1. Characters of analysed *E. coli* strains

HAA represents haemolytic activity on agar plates supplemented with whole sheep blood unless indicated.

Strain/serogroup	PCR assays			Agar assays		Bacterial filtrate assays at 4 h/24 h				Bacterial cell assays at 4 h/24 h		
	<i>cnf</i>	<i>cdt1</i>	<i>hly</i> _{EHEC}	ClyA	HAA 4 h/24 h	HA	CD		HA	CD		
							Caco-2	Int 407		Caco-2	Int 407	
Children with diarrhoea												
88	+	−	−	−	+ / +	+ / −	+ / −	− / −	+ / +	+ / +	+ / +	
K57	+	−	−	−	+ / +	+ / +	− / −	− / −	+ / +	+ / +	+ / +	
148	+	−	−	−	+ / +	− / −	− / −	− / −	+ / +	+ / +	+ / +	
62	+	−	−	−	+ / +	+ / −	− / −	− / −	+ / +	+ / +	+ / +	
K70	+	−	−	−	+ / +	+ / −	− / −	− / −	+ / +	+ / +	+ / +	
124	+	−	−	−	+ / +	+ / −	− / −	− / −	+ / +	+ / +	+ / +	
144	+	−	−	−	+ / +	+ / −	− / −	− / −	+ / +	+ / +	+ / +	
P17-4	+	−	−	−	+ / +	+ / −	+ / −	− / −	+ / +	+ / +	+ / +	
29	+	−	−	−	+ / +	+ / −	− / −	− / −	+ / +	+ / +	+ / +	
19-1	+	−	−	−	+ / +	+ / −	− / −	− / −	+ / +	+ / +	+ / +	
23-5	+	−	−	−	+ / +	+ / −	− / −	− / −	+ / +	+ / +	+ / +	
57-1	+	−	−	−	+ / +	+ / −	− / −	− / −	+ / +	+ / +	+ / +	
10-4	+	−	−	−	+ / +	+ / −	− / −	− / −	+ / +	+ / +	+ / +	
212/O127	−	−	−	−	+ / +	+ / +	− / −	− / −	+ / +	+ / +	+ / +	
38-1	−	−	−	−	+ / +	+ / −	− / −	− / −	+ / +	+ / +	+ / +	
12-1	−	−	−	−	+ / +	+ / +	− / −	− / −	+ / +	+ / +	+ / +	
48-1	−	−	−	−	+ / +	− / −	− / −	− / −	+ / +	+ / +	+ / +	
22-1	−	−	−	+	+ / +	+ / −	− / −	− / −	+ / +	+ / +	+ / +	
38	−	−	−	−	+ / +	+ / −	− / −	− / −	+ / +	+ / +	+ / +	
205/O18	−	−	−	−	+ / +	+ / −	− / −	− / −	+ / −	− / −	− / −	
Healthy children												
70-1	+	−	−	−	+ / +	+ / −	− / −	− / −	+ / +	+ / +	+ / +	
51-4	−	+	−	−	+ / +	+ / −	− / −	− / −	+ / +	+ / +	+ / +	
66-1	−	−	−	−	+ / +	+ / −	− / −	− / −	+ / −	+ / −	− / −	
63-1	−	−	−	+	+ / +	+ / −	− / −	− / −	+ / +	+ / +	+ / +	
60-1	−	−	−	−	+ / +	+ / −	− / −	− / −	+ / +	+ / +	+ / +	
50-4	−	−	−	−	+ / +	+ / −	− / −	− / −	+ / +	+ / +	+ / −	
69-2	−	+	−	−	+ / +	+ / −	− / −	− / −	+ / +	+ / +	+ / +	
CD assay control strains												
738/O26	−	−	+	−	− / + *	− / −	− / −	− / −	− / −	− / −	− / −	
740/O26	−	−	+	−	− / + *	− / −	− / −	− / −	− / −	− / −	− / −	
81	−	−	−	−	− / +	− / −	− / −	− / −	− / −	− / −	− / −	
147	−	−	−	−	− / +	− / −	− / −	− / −	− / −	− / −	− / −	
Negative control strains												
101	−	−	−	−	− / −	− / −	− / −	− / −	− / −	− / −	− / −	
102	−	−	−	−	− / −	− / −	− / −	− / −	− / −	− / −	− / −	
103	−	−	−	−	− / −	− / −	− / −	− / −	− / −	− / −	− / −	
104	−	−	−	−	− / −	− / −	− / −	− / −	− / −	− / −	− / −	
105	−	−	−	−	− / −	− / −	− / −	− / −	− / −	− / −	− / −	
106	−	−	−	−	− / −	− / −	− / −	− / −	− / −	− / −	− / −	
K12 C ₆₀₀	−	−	−	+	− / −	− / −	− / −	− / −	− / −	− / −	− / −	

*Haemolytic activity on agar plates supplemented with washed sheep erythrocytes.

haemolysin production, close contact between bacteria and RBCs (as is achieved by centrifugation) is important. No contact haemolysin activity is detected when bacterial suspensions are simply mixed with sheep erythrocytes and

incubated. Cell-bound contact calcium-dependent haemolysin was described by Haque *et al.* (1994) among EAEC. According to their study, this haemolysin was produced by aggregative *E. coli* isolates cultured only in Casamino

Fig. 1. Scatter plots of relationships between the mean haemolytic and cell-detaching activities of bacterial cells of 27 *E. coli* isolates examined on Int 407 (a, b) and Caco-2 (c, d) cell lines after 4 h (exponential phase; a, c) and 24 h (stationary phase; b, d). Open squares show the predicted CD activity, whereas filled diamonds show the measured values. Mean $\pm$ SEM CD activities in each panel are: $61.0 \pm 9.4\%$ (a), $58.5 \pm 11.6\%$ (b), $59.4 \pm 10.3\%$ (c) and $58.7 \pm 9.6\%$ (d). Mean $\pm$ SEM haemolytic activities in each panel are: $37.9 \pm 1.9\%$ (a), $25.2 \pm 2.2\%$ (b), $37.9 \pm 1.9\%$ (c) and $25.2 \pm 13.8\%$ (d).

acids/yeast extract broth supplemented with 1 mM CaCl_2 and demonstration of the activity required close contact between bacteria and RBCs. In contrast, the cell-bound haemolysin detected among *E. coli* isolates examined in the present study was produced by bacteria cultured in various liquid media, e.g. Luria–Bertani broth, tryptose soy broth or yeast extract medium (data not shown). Furthermore, haemolytic activity of the cell-bound haemolysin was detected in bacterial suspensions only mixed with erythrocytes and incubated at 37°C . Another contact haemolysin demonstrated by Sansonetti *et al.* (1986) among EIEC, although calcium-independent, also required the close contact between bacteria and RBCs achieved by centrifugation. ClyA cytolysin that is calcium-independent and inhibited by cholesterol was released upon contact with mitomycin C by two (7.4%) of the 27 haemolytic *E. coli* tested as well as by the *E. coli* K12 strain.

In the cell-detaching assay only 2 (8%) of the 25 exponential culture filtrates containing HlyA had cell-detaching activity for Caco-2 cells and none affected Int 407 cells. In contrast, cell-detaching activity was mostly associated with the bacterial cells (Table 1). A scatter plot to show the relationship between the haemolytic activity of bacterial cells and the cell-detaching activity of *E. coli* isolates was constructed and analysed by the regression method. This analysis indicates a close relationship between the haemolytic and the Caco-2 cell-detaching activities of *E. coli* cells as shown in Fig. 1. None of the control *E. coli* strains showed cell-detaching activity.

In the present study, most haemolytic *E. coli* showed HlyA production, and all the isolates demonstrated cell-bound haemolytic activity during the exponential growth phase. However, according to the RAPD (random amplified

polymorphic DNA) assay, all the haemolytic *E. coli* strains exhibited distinct patterns, indicating the presence of different genetic groups (data not shown). The mean haemolytic activity of the filtrates was comparable to that of the bacterial cell suspensions. HlyA present in the culture filtrates was stable and active during the incubation period and the bacterial cells did not release any cell-free haemolysin during the same time of incubation. The cell-bound haemolysin was similar to HlyA with regard to calcium-dependency and lack of the activity in the presence of dextran 4 and dextran 8, but not in the presence of sucrose or cholesterol. In contrast to cell-free HlyA, the cell-bound haemolysin was detected in *E. coli* cultures incubated for 24 h. Moreover, inactivation of the cell-bound haemolytic activity by heating or UV irradiation of the *E. coli* cells tested was associated with loss of cell-detaching ability. The influence of other *E. coli* haemolysins such as Hly_{EHec} or EIEC and EAEC contact haemolysins on cell-detaching activity was excluded. Similarly, there was no association of cytotoxin ClyA with haemolytic or cell-detaching activity of the isolates examined. The six non-haemolytic *E. coli* strains used in the CD assay as negative controls were found to be non-cytotoxic for the cultured cells. The two enterohaemolysin-producing *E. coli* O26 and the isolates that were haemolytic on blood agar plates after 24 h of incubation had no ability to detach cells *in vitro*, in agreement with Marques *et al.* (1995).

Smith (1963) differentiated two main *E. coli* haemolysins, cell-free haemolysin α and cell-associated haemolysin β . The cell-bound haemolysin β was very similar to haemolysin α , but it was not neutralized by antiserum to haemolysin α and its activity was associated only with intact bacterial cells (Short & Kurtz, 1971). Gadeberg *et al.* (1983) showed that washed intact haemolytic *E. coli* cells and the supernatants of the same *E. coli* cultures containing HlyA were cytotoxic for leukocytes. In our study the cell-detaching effect was associated with viable bacteria but not with culture filtrates containing HlyA. The *E. coli* strains examined in the present study were isolated from stool specimens, so it is possible that epithelial intestinal cells are more sensitive to cell-bound haemolysin than to HlyA. As the contact haemolysin of EIEC is considered to contribute to their virulence, it seems possible that the cell-bound haemolysin of *E. coli* strains isolated from stool specimens of patients with diarrhoea in our study may be associated with pathogenicity. On the other hand, the cell-bound haemolysin-producing *E. coli* isolates were recovered from healthy children as well as from those with diarrhoea. This suggests that the haemolysin is not associated with pathogenicity of these strains or it may be a virulence factor but only under particular *in vivo* conditions. The association of the cell-bound haemolysin with virulence of *E. coli* strains remains to be determined.

The results obtained in the present study provide evidence that haemolysin β or an unknown cell-bound haemolysin rather than cell-free HlyA was associated with *in vitro* cell-detaching activity of *E. coli* isolates examined.

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