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ENDOCYTOSIS-INDUCER ADHESINS PRODUCED BY ENTEROPATHOGENIC SEROGROUPS OF *ESCHERICHIA COLI* PARTICIPATE ON BACTERIAL ATTACHMENT TO INFANT ENTEROCYTES

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Enteropathogenic E. coli *(EPEC) infection of Hep-2 cells proceeds through bacterial attachment to cell surface and internalization of adhered bacteria. EPEC attachment is a prerequisite for cell infection and is mediated by adhesins that recognize carbohydrate-containing receptors on cell membrane. Such endocytosis-inducer adhesins (EIA) also promote EPEC binding to infant enterocytes, suggesting that EIA may have an important role on EPEC gastroenteritis.*

Key words: *Escherichia coli* – infantile diarrhea – bacterial adhesiveness – bacterial pathogenicity

Some enteropathogenic serogroups of *Escherichia coli* (classic or EPEC) are important causal agents of enterocolitis especially among infants. Children two or more years old and adults are infrequently affected (Neter, 1959; Gurwith et al., 1978; W. H. O., 1980). EPEC enteropathogenicity seems unrelated to enterotoxin production or to the invasive properties which have been described in the *Shigella* or enteroinvasive *E. coli*. Levine et al. (1983) described that EPEC would adhere intimately to the enterocyte surface, effaced or dissolved the microvilli on the infected cells, promoting absortive disturbances and diarrhea.

Scaletsky et al. (1984) described that the adhesive pattern was typical for EPEC, and the term "localized adherence" was proposed. There were several clear-cut microcolonies of bacteria on the surface of HeLa or Hep-2 cells. Our own observations indicated that "localized adherence" to Hep-2 cells did not involve type 1 or mannose-resistant pili and that non-piliated adhesins with affinity for glycoproteic receptors on the cell surface were functionally apparent and related to "localized adherence" (Andrade & Santa Rosa, 1986b). The bacterial nests typical of "localized adherence" actually were sites of infolded cell membrane accompanied by intense internalization of attached bacteria. The endocytic process seemed to be followed by replication of bacteria inside the infected cells (Andrade & Santa Rosa, 1986a). Recent work (Andrade & Santa Rosa, data to be published) suggest that EPEC infection of Hep-2 or HeLa cells include at least two different steps: (i) bacterial attachment to cell receptors blocked by galactose, glucose and rhamnose and (ii) the internalization of adhered bacteria by an active endocytic process, inhibited by cytochalasin B.

In the present report we described that the same kind of adhesins responsible for the EPEC internalization into cultured cells also allows bacterial attachment to enterocytes from infant small intestine.

MATERIALS AND METHODS

Bacterial strains and human cells – EPEC strains were obtained from both recent cases of infantile enterocolitis or from older isolates kept as stock cultures. Non-EPEC strains were isolated from feces of normal individuals. Production of adhesins, cytotoxins and virulent properties for guinea-pig eyes (Serény test) and rabbit ileal loops of these strains have been described (Andrade et al., 1984; J. R. C. Andrade & E. C. da Cunha Rodrigues – unpublished observations) and are listed in Table I. Human enterocytes were obtained from the proximal jejunum of a eight-month-old dead male infant. Cells were gently scrapped from the intestinal mucosa, washed thrice in Dulbecco's phosphate-buffered saline (pH 7.2) (PBS-D) and adjusted with PBS-D plus 10% (v/v) dimethyl sulfoxide-10% (v/v) calf serum to 10^6 cells/ml. Known volumes of the cell suspension were frozen at -25ºC and were thawed once for each experiment. It was previously determined that cell freezing in such conditions did no allow to modification of bacterial attachment.

Cultural conditions and adherence tests – *E. coli* strains were cultured in nutrient broth for stimulating type 1 pili (Andrade et al., 1984), in Eagle minimal essential medium (MEM, Gibco) for 2.5 h at 37ºC for stimulating EIA (Andrade & Santa Rosa, 1986b) and in CFA agar (Evans & Evans, 1978) at 37ºC for inhibiting production of both adhesins (Andrade & Santa Rosa, 1986b). Bacterial suspen-

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sions (10^8 CFU/ml) were prepared in PBS-D for adherence tests. Equal volumes (0.2 ml) of intestinal cell suspension and bacterial suspensions were mixed and incubated in 24-well flat-bottom tissue culture plates (Costar) at 37°C for 15 min with circular agitation (160 rpm). For some tests 10mg/ml of galactose, D-glucose or L-rhamnose were added to promote EIA blocking. The intestinal cells were then washed thrice at 200 xg and the sediment of the last centrifugation was extended on a glass slide, dried, heat-fixed and stained with Gram's crystal violet. Thirty intestinal cells were observed at random and the attached bacilli were counted. Adherence index was obtained from the total number of attached bacilli counted in duplicate samples and divided by 60. Cells incubated in PBS-D were used as a control of the indigenous bacteria eventually attached to the cells. For each group of bacterial strains was obtained the mean and the standard deviation of all adherence indexes and comparisons between groups were done by Student's t test.

RESULTS

Table I shows that most of epidemic EPEC produced type 1 pili besides EIA. EPEC strains did not produce enterotoxins or cytotoxins or were invasive by the Serény test while EIA-positive strains seems related to positive results in rabbit ligated intestinal loops. Freshly isolated EPEC strains attached better to enterocytes than EIA-negative stock strains or fecal non-EPEC *E. coli* ($p < 0.02$; Table II). EIA seems to participate in bacterial adherence to infant enterocytes as non-piliated, EIA-positive strains had higher adherence indexes when cultured in MEM than in CFA agar, where EIA production is strongly inhibited. Cooperation between two adhesins for enterocyte binding is suggested from the higher adherence indexes obtained when the tested strains were both type 1-piliated, EIA-positive. Tabel III shows a highly significative decrease ($p < 0.001$) on bacterial attachment in tests performed with carbohydrates that specifically blocks EIA of non-piliated EPEC strains (Andrade & Santa Rosa, 1986b).

DISCUSSION

Our former observations had indicated that EPEC internalization into Hep-2 cells involved an early step requiring EIA interaction with cell surface receptors. In the present report we observe in addition that such adhesins also

TABLE I

Adhesins and virulence traits of EPEC strains and non-EPEC *E. coli*

Origin	Serogroup	Strain	Adhesins			Virulence properties			
			t–1	MR	EIA	Toxins(*)	Hemolysins	Serény Test	Rabbit loops
EPEC Outbreaks	0111:K58	386	+	–	+	–	–	–	+
		410	+	–	+	–	–	–	ND
	0119:K69	329	–	–	+	–	–	–	ND
		341	+	–	+	–	–	–	ND
	086a:K61	262	+	–	+	–	–	–	ND
EPEC Sporadic cases	0111:K58	54	–	–	+	–	–	–	+
		94	–	–	+	–	–	–	ND
	055:K59	68	–	–	+	–	–	–	+
		1415	+	–	+	–	–	–	ND
	0119:K69	60	–	–	+	–	–	–	ND
EPEC Collection	0111:K58	3943-67	+	–	–	–	–	–	–
	055:K59	ECT-122	+	–	–	–	–	–	–
E. coli Normal feces	ND	F-2	–	–	–	–	–	ND	–
	095	F-11	+	–	–	–	–	ND	ND

t-1: type 1 pili; MR: mannose-resistant pili; EIA: endocytosis-inducer adhesins.
(*) Enterotoxin or cytotoxins: tested in Vero cells (LT and VT) and Hep-2 cells (cytotoxic effect).
ND: not determined.

TABLE II

EPEC adherence to infant enterocytes after bacterial growth in culture conditions favourable or not to the adhesins production

Origin	Strain	Adhesins		Adhesins production in: NB		MEM		CFA	
		t1	EIA	t1+/EIA +		t1−/EIA +		t1−/EIA −	
EPEC outbreaks	386	+	+	2.52[a]	2.17 ±0.24[b]	2.26	1.34 ±0.18	0.32	0.35 ±0.05
	410	+	+	1.49		1.04		0.32	
	329	−	+	ND		0.86		ND	
	341	+	+	1.96		1.39		0.22	
	262	+	+	2.69		1.15		0.52	
EPEC sporadic cases	54	−	+	1.52	2.87 ±0.11	1.04	1.05 ±0.08	0.50	0.29 ±0.06
	94	−	+	ND		0.82		ND	
	68	−	+	2.04		0.84		0.31	
	1415	+	+	1.89		1.32		0.22	
	60	−	+	2.02		1.24		0.12	
EPEC collection	3943-67	+	−	1.99	1.57 ±0.43	0.86	0.87 ±0.03	0.46	0.55 ±0.07
	ECT−122	+	−	1.15		0.89		0.64	
non-EPEC *E. coli*	F-2	−	−	1.34	1.68 ±0.22	0.59	0.74 ±0.10	0.50	0.41 ±0.08
	F-11	+	−	2.01		0.89		0.32	

NB. nutrient broth; MEM: Eagle minimal essential medium; CFA: colonization factor antigen agar;
a: adherence index (see text); b: mean ±SD

TABLE III

Inhibition of EPEC attachment to infant enterocytes by carbohydrates that specifically blocks EIA

Strains	Carbohydrates (10mg/ml)	Adherence indexes: without carbohydrate		with carbohydrate	
68	L-rhamnose	0.84	0.85 ±0.05	0.32	0.31 ±0.03
54	D-glucose	1.04		0.25	
94	galactose	0.82		0.39	
329	D-glucose	0.86		0.26	

participate in the EPEC binding to infant enterocytes.

Moon et al. (1983) established that EPEC binding to the intestinal epithelium resulted in the effacement of the microvilli on the enterocyte at the points of bacterial attachment. We showed that EPEC binding to Hep-2 cells determined an immediate mobilization of cell membrane, leading to the local budding of digitiform projections and the infolding of the membrane to form endocytic vacuoles (Andrade & Santa Rosa, 1986a).

It has been pointed out that cell movements, formation of the endocytic vacuoles and maintenance of microvilli are dependent of an actively sustained actomyosin-like microfilament network (Allison et al., 1971). Cytochalasin B, a microfilament-disrupting agent, was found to decrease EPEC endocytosis by Hep-2 cells (Andrade & Santa Rosa, to be published) suggesting that attached bacteria actively induces cell membrane growth and differentiation.

It might be possible that the microvilli loss found in the EPEC-infected enterocytes could be assigned to the same mechanism initiated by the contact between bacterial adhesins and cell receptors. Our findings indicate that cell receptors similar or identical to those found on Hep-2 cells occurs on enterocytes from the infant small intestine and points toward EIA participation in infant EPEC diarrhoea.

EPEC enterocolitis in susceptible children appears to involve a low infective doses (Neter, 1959). The marked enterocyte-binding

activity shown by type 1-piliated, EIA-positive EPEC strains suggest that such strains may be most efficient in intestinal colonization. Further investigations are need to characterize the EIA receptor (s) and to find out its distribution on enterocytes of older children and adults, leading to a best reasoning of the age-related incidence of EPEC in humans.

RESUMO

A infecção de células Hep-2 por *E. coli* enteropatogênicas (ECEP) implica na aderência bacteriana e posterior interiorização dos microrganismos aderidos por um mecanismo de endocitose. A aderência das ECEP é pré-requisito para a infecção e é mediada por adesinas que reconhecem receptores inibidos por certas oses na membrana celular. Tais "adesinas indutoras da endocitose" (AIE) também promovem a ligação bacteriana a enterócitos obtidos do intestino delgado de lactente, sugerindo que as AIE possam desempenhar algum papel nas diarréias causadas por ECEP.

Palavras-chave: *Escherichia coli* – diarréia infantil – aderência bacteriana – patogenicidade bacteriana.

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