Indian J Med Res [A] 91, September 1990, pp 368-371

Human enterocyte adhesion of enteroadherent *Escherichia coli*

P. Raj, M.K. Bhan, R. Srivastva, R. Kumar*, N. Bhandari & N.K. Arora

*Departments of Paediatrics & *Microbiology, All India Institute of Medical Sciences, New Delhi*

Accepted May 18, 1990

***Esch. coli* strains manifesting localised (17), diffuse (8) or aggregative (17) phenotypes of adherence to HEp-2 cells were tested for their ability to adhere to human enterocytes isolated from duodenal biopsies of adult volunteers to obtain further evidence of their enteropathogenecity. *Esch. coli* strains H10407+ ; CFAI+ and LT+ STp+ STh+, F 294 B; a localised adherent strain positive with entero-adherent factor probe reported previously to attach to small intestinal enterocytes and F 582 C; LT- STp+ STh+ were the positive controls : H10407P (CFAI- mutant of H10407+) and K12 served as negative control strains. Adherence of variable degree was seen with 35.3 per cent of enteroaggregative *Esch. coli* (EAggEc) and with 58.8 per cent of enteroadherent *Esch. coli* localised (EAEC-L); EAEC-diffuse (EAEC-D) did not adhere to the human enterocytes. The possibility that EAgg EC and diffuse phenotypes may adhere better to lower small intestine or the large intestine, needs to be investigated.**

HEp-2 cell adherent *Escherichia coli* has now been proposed as a new class of diarrheagenic *Esch. coli* and termed as enteroadherent *Esch. coli* (EAEC)[1]. By definition, these are negative for production of labile toxin, stable toxin, verotoxin and invasiveness[1,2]. Among the three different phenotypes of HEp-2 cell adherent *Esch. coli* described *viz.*, localised (EAEC-L), enteroaggregative (EAggEC) and diffuse (EAEC-D) (2), there is epidemiological evidence that EAEC-L and EAggEC may be enteric pathogens[2-4]. EAEC-L have been shown to cause brush border effacement and damage to the microvilli[5,6]. EAggEC were epidemiologically found to be associated with diarrhoea in India and Chile but its mechanism of action remains unknown[3,4]. At least in these two studies, the role of EAEC-D in causation of diarrhoea was not suggested as these were excreted with similar frequency among patients and controls.

Adhesion of enteric pathogens to the mucosa of small intestine is an important early event in colonisation and eventual development of diarrhoea[7]. *In vitro* human enterocyte adhesion assay has been developed for enterotoxigenic *Esch. coli* (ETEC) which is shown to be mediated by specific colonisation factor antigens[8]. EAggEC and EAEC-D have not been tested for adherence to human enterocytes. We report *in vitro* adherence of EAEC to human small intestinal enterocytes.

Material & Methods

Esch. coli strains : A cohort of 452 children up to 3 yr of age was followed longitudinally in a rural community in Haryana over 13 months to evaluate the role of HEp-2 adherent *Esch. coli* and other enteric pathogens in causing acute ($\leq$14 days) and persistent diarrhoea ($>$ 14 days)[3]. These included all children in the age group residing in

the study village but excluding those in whom consent was not available. From a total of 72 HEp-2 adherent *Esch. coli* isolated in as many patients during the study, 17 strains each of EAggEC and EAEC-L and 8 of EAEC-D from patients of diarrhoea were randomly selected for enterocyte adhesion assay. All these strains were negative with DNA probes that detect ETEC (LT, STp, STh), EIEC, EAF (EPEC adherent factor) ad EHEC[3]. These strains were kept at -70°C in 20 per cent glycerol broth, revived on CFA agar, and incubated at 37°C overnight, subcultured in trypticase soy broth and incubated for 18 h at 37°C. Bacterial suspensions were prepared by sedimenting bacteria at 3000 × *g* for 15 min, washing thrice with PBS and suspending the pellet in Eagle's MEM to a concentration of ~10^9 bacteria/ml.

Enterocyte isolation : Endoscopic duodenal biopsies from adult patients with peptic ulcer disease or negative endoscopies, were transported to the laboratory in ice-cold Eagle's MEM containing 100 μg gentamicin per ml. Before the intubation, informed consent from patients was obtained for taking biopsies. Enterocytes were separated by an EDTA-chelation procedure as described by Evans and co-workers[9]. Mucosal biospies were placed in 2 ml of EDTA buffer (96 m*M* Nacl, 8 m*M* KH_2PO_4, 5.6 m*M* Na_2HPO_4. 1.5 m*M* KCl, 10 m*M* EDTA, *p*H 6.8) for 5 min at 37°C with gentle shaking and then immediately transferred into fresh ice-cold Eagle's MEM. With the help of a wide bore Pasteur pipette, loosened epithelium was released by repeated suction. Large sheets of released enterocytes were sedimented at 100×*g* for 1 min, washed thrice with MEM, and suspended in fresh Eagle's MEM containing 0.5 per cent D-mannose. All subsequent operations were carried out in medium containing D-mannose. Enterocytes from six duodenal biopsies (10^6 to 10^7 enterocytes) were pooled and suspended in 10 ml of Eagle's MEM. These enterocytes were immediately used for adhesion assay. Viability of enterocytes was determined by trypan blue dye exclusion test.

Enterocyte adhesion assay : The enterocyte adhesion assay was done as per the method described previously[8]. Briefly, one part of the enterocyte suspension and two parts of a washed suspension of bacteria (~10^6/ml) in a Bijoux bottle, were incubated for 3 h at 37°C in a shaker water bath. The enterocytes were sedimented at low speed 100×*g* for 1 min followed by six washings by fresh medium containing 0.5 per cent D-mannose to remove nonadherent bacteria. Brush border adhesion of bacteria was quantitatively assessed by phase-contrast microscopy using Carl Zeiss microscope with camera. In each assay, bacteria adhering to the brush border of 50 enterocytes were counted. Only those enterocytes which showed a well preserved columnar morphology and brush border were selected at random by using a low power (10X) objective. Bacteria adhering to the brush border were subsequently counted by using a 40X objective. Enterocytes with laterally adhered bacteria were excluded. Adhesion assays were carried out in duplicate and the adherence was examined blind by an observer who had no knowledge of the bacterial identity.

Table. Mean number of bacteria per brush border of enterocytes with different phenotypes of enteroadherent *Esch. coli* strains

EAggEC strains	Adhesion index	EAEC-L strains	Adhesion index	EAEC-D strains	Adhesion index
F 24 II	2.0	F 201 A	0.5	F 255 A	0.02
F 35 I	1.0	F 255 B	1.9	F 424 A	0
F 417 A	0	F 255 C	3.9	F 424 B	0
F 417 B	0	F 278 C	1.1	F 424 C	0
F 417 C	0	F 279 A	3.3	F 444 A	0
F 482 A	0	F 279 B	2.4	F 453 A	0
F 482 B	0.08	F 279 C	6.2	F 453 C	0
F 482 C	0	F 353 A	0.5	F 555 I	0
F 490 A	0	F 353 B	0		
F 490 B	0	F 531 B	3.6		
F 490 C	0	F 531 C	0.5		
F 510 A	0	F 547 A	0		
F 510 C	0.1	F 547 B	0		
F 575 B	0.5	F 547 C	0		
F 575 C	3.5	F 562 A	0		
F 579 A	1.4	F 562 B	0		
F 579 B	1.5	F 562 C	0		

EAEC, enteroadherent *Esch. coli*; EAggEC, enteroaggregative; EAEC-L, localised; EAEC-D, diffuse
Adhesion index = No. of bact. counted/no. of enterocytes counted

Three positive controls, H10407+ (CFAI+ LT+ STh+ STp+), F 582C (LT-STh+ STp+) and F294B (EAF+), previously shown as adherent to enterocytes by Knutton *et al*[8] and two negative controls, *Esch. coli* K12 and H10407P (a CFAI-mutant of H10407+) previously shown as non-adherent to enterocytes also by Knutton *et al*[8] were run with each batch of the assay. The control strains were supplied by Dr S. Knutton, Birmingham, UK.

Results

The enterocyte adhesion index of enteroadherent *Esch. coli* is shown in the Table. Three positive and two negative controls were run with each batch. The mean adhesion indices for the positive control strains in 10 repeat assays were H10407+ 4.7 (range 4.2 –5.5), F294B 5.05 (range 4.7 - 5.5) and F582C, 2.52 (range 2.3 – 2.8). The negative controls H10407 P (CFAI-mutant of H10407+) and K12 did not reveal a single attached bacterium on ten repeat assays.

Among the 42 EAEC strains tested, 16 (38.1%) showed one to several bacteria adherent to enterocytes. Of the EAggEC strains, 6 (35.3%) and for EAEC-L strains, 10 (58.8%) showed adherence of bacteria to enterocytes. None of the EAEC-D strains tested, adhered to the enterocytes. Among the enterocyte adherent EAEC-L strains, the adhesion index ranged from 0.5-6.2; in the EAggEC adherent strains, the corresponding values were 0.5-3.5. In the enterocyte adherent strains, between 30-40 per cent of the enterocytes had one or more bacteria attached to their brush border. In each positive assay the number of bacteria adhering to the brush border ranged from 1-20 per brush border and occasionally a cluster of bacteria was observed while in negative assay, no bacterium was seen. About 70-80 per cent of the cells were found to be viable as shown by the trypan blue exclusion test.

Discussion

In this preliminary study, we observed *in vitro* human enterocyte adherence of enteroaggregative and localised adherent *Esch. coli* strains but not of diffuse adherent *Esch. coli*. During the process of colonization in the intestine, an initial step is the attachment of pathogen to the mucosal membrane. Adhesion of ETEC to the human small intestinal enterocytes has been shown as an early event which is mediated by specific colonisation factor antigens[8]. Recent studies have demonstrated that EAEC-L attach to the enterocytes of the intestinal mucosa[5,6]. At the site of attachment of bacteria, microvillous border is lost and the cytoskeleton and the epethelial cell membrane are disrupted[5,6,10]. Further, attaching and effacing lesion of EAEC-L in HEp-2 cells have been detected by a very sensitive and specific fluorescence staining with phallotoxin which binds to the actin at the site of lesion[11].

EAggEC have not been tested for adherence to enterocytes. In this preliminary study, we have demonstrated that some EAggEC adhere to human enterocytes *in vitro*. The factors mediating this adherence are not known, but it has been suggested that distinct fimbrial colonization factor antigens may be involved[12]. However, more than half the strains of EAggEC did not adhere to enterocytes from proximal small intestine. We need to determine if these might attach better to the distal small intestine or the colonic cell. The events following colonization that lead to development of diarrhoea by EAggEC are not known. Recently, Vial *et al*[12] have suggested that elaboration of toxin may cause damage to the intestinal mucosa. In our laboratory, EAEC-D strains did not adhere to enterocytes.

Several factors may be responsible for variation in the number of bacteria adhering per brush border within a single enterocyte adhesion assay. These may include individual variations in enterocyte receptors from donor to donor as biopsies were pooled from different donors; difference in enterocyte receptor sites because the cells from different biopses pooled are at different stages of maturation and variation in the number of bacterial fimbriae which could affect their ability to attach to enterocytes. Previously with ETEC, difference in fimbrial number among bacteria, both within the same culture and from culture to culture, have also been observed[8].

In conclusion, in this preliminary study, we have shown that majority HEp-2 cell adherent *Esch. coli* of localised and a smaller proporation of aggregative phenotype adhere to isolated human

enterocytes *in vitro*; diffusely adherent strains did not attach to upper small bowel enterocytes. Future studies are necessary to determine if the aggregative and perhaps diffuse strains might adhere better to enterocytes from lower small intestine or the large intestine.

Acknowledgment

Authors thank Dr Stuart Knutton, Institute of Child Health, Birmingham, UK, for introducing enterocyte adhesion assay in our unit and the Department of Gastroenterology, AIIMS, New Delhi, for providing adult human duodenal biopsies.

References

1. Levine, M.M. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic and enteroaderent. *J Infect Dis* **155** (1987) 377.
2. Nataro, J.P., Kaper, J.B., Robins-Browne, R., Prado, V., Vial, P. and Levine, M.M. Patterns of adherence of diarrheagenic *Escherichia coli* to HEp-2 cells. *Pediatr Infect Dis J* **6** (1987) 829.
3. Bhan, M.K., Raj, P., Levine, M.M., Kaper, J.B., Bhandari, N., Srivastva, R., Kumar, R. and Sazawal, S. Enteroaggregative *Escherichia coli* associated with persistent diarrhea in a cohort of rural children in India. *J Infect Dis* **159** (1989) 1061.
4. Levine, M.M., Prado, V., Robins-Browne, R., Lior, H., Kaper, J.B., Moseley, S.L. Giequelais, K., Nataro, J.P., Vial, P. and Tall, B. Use of DNA probes and HEp-2 cell adhrence assay to detect diarrheagenic *Escherichia coli*. *J Infect Dis* **158** (1988) 224.
5. Moon, H.W., Whipp, S.C., Argenzio, R.A., Levine, M.M. and Giannella, R.A. Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. *Infect Immun* **41** (1983) 1340.
6. Taylor, C.J., Hart, A., Batt, R.M., McDougall, C and McLean, L. Ultrastructral and biochemical changes in human jejunal mucosa associated with enteropathogenic *Escherichia coli* (0111) infection. *J Pediatr Gastroenterol Nutr* **5** (1986) 70.
7. Boedecker, E.C. Enterocyte adherence of *Escherichia coli*: its relation to diarrheal disease. *Gastroenterology* **83** (1982) 489.
8. Knutton, S., Lloyd, D.R., Candy, D.C. and McNeish, A.S. Adhesion of enterotoxigenic *Escherichia coli* to human small intestinal enterocytes. *Infect Immun* **48** (1985) 824.
9. Evans, E.M., Wrigglesworth, J.M., Burdett, K. and Pover, W.R.F. Studies on epithelial cells isolated from guinea pig small intestine. *J Cell Biol* **51** (1971) 452.
10. Tzipori, S., Robins-Browne, R.M., Gonis, G., Hayes, J., Whithers, M. and McCartney, E. Enteropathogenic *Escherichia coli* enteritis: evaluation of the gnotobiotic piglet as a model of human infection. *Gut* **26** (1985) 570.
11. Knutton, S., Baldwin, T., Williams, P.H. and McNeish, A.S. Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infect Immun* **57** (1989) 1290.
12. Vial, P.A., Robins-Browne, R., Lior, H., Prado, V., Kaper, J.B., Nataro, J.P., Maneval, D., Elsayed, A. and Levine, M.M. Characterization of enteroadherent-aggregative *Escherichia coli*, a putative agent of diarrheal disease. *J Infect Dis* **158** (1988) 70.

Reprint requests: Dr M.K. Bhan, Additional Professor of Paediatrics, All India Institute of Medical Sciences Ansari Nagar, New Delhi 110029