Adhesion of human enterotoxigenic *Escherichia coli* to human mucus secreting HT-29 cell subpopulations in culture

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Abstract

Enterotoxigenic *Escherichia coli* (ETEC) bearing the fimbrial colonisation factor antigens CFA/I, CFA/II, CFA/III, and the non-fimbrial antigen 2230 were tested for their ability to adhere to two cultured human intestinal HT-29 mucus secreting cell subpopulations. These populations are referred to as HT29-MTX and HT29-FU, which differ in the amount of secreted mucins and in their gastric or colonic mucin immunoreactivity respectively. Adherence of radiolabelled bacteria to cell monolayers infected apically was assessed. All ETEC strains adhered to the mucus secreting HT29-FU subpopulation, which secretes mucins of colonic immunoreactivity. Visualisation of bacteria by scanning electron microscopy showed that ETEC bound to the HT29-FU cells possessing a brush border, but not to the mucus and that ETEC binding developed as a function of cell differentiation. The adhesion of ETEC to cells possessing a brush border and to mucus secreting cells was also analysed by indirect immunofluorescence in HT29-MTX cells, which secrete mucins of gastric immunoreactivity. Fluorescein isothiocyanate labelling using specific anti-CFA/I antibody was used to show ETEC; rhodamine isothiocyanate labelling using a monoclonal antibody (designated M1) against purified human gastric mucus was used to detect secreted mucins, and rhodamine isothiocyanate labelling using a monoclonal antibody (designated 4H3) against human dipeptidylpeptidase IV was used to show cells possessing a brush border. Binding of bacteria colocalised with dipeptidylpeptidase IV of enterocytes and not with mucins.

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Adherence of bacteria to epithelial intestinal cells is an important prerequisite for colonisation by micro-organisms and virulence manifestations.^{1,2} To remain associated with the gut mucosa and to withstand the flow of the intestinal chyme, bacteria have developed several mechanisms of association. In particular, pathogenic bacteria form close associations with the intestinal mucosa and their inability to do so results in rapid elimination from the gut. Moreover, bacteria trapped in the surface mucus, coupled

with peristalsis, results in the rapid expulsion of bacteria from the intestine.

Two major cell phenotypes – that is, enterocytes and goblets cells – are represented in the intestinal mucosa. The brush border of enterocytes is the target of many pathogenic bacteria.² The human small intestinal mucosa has a mucus coating at the surface. In vivo, for some pathogenic bacteria the mucus gel could serve at least two functions.³ Firstly, it might be a source of nutrients for bacterial growth, thus influencing positively the intestinal colonisation by the adhering bacteria, which have the ability to survive in and multiply in the outer areas of the mucus layer. Secondly, the mucus coat overlying the microvillous surface contributes to host defence by preventing bacterial adhesion or invasion and toxin binding to the mucosal surface.⁴

The role of mucus in enterotoxigenic *Escherichia coli* (ETEC) colonisation is an important question to investigate. In this study, we describe the use of the recently described mucus secreting HT29-MTX⁵ and HT29-FU⁶ cell subpopulations as human cell models to study in vitro adhesion of enterotoxigenic *E coli* expressing colonisation factor antigens CFA/I, CFA/II, CFA/III or 2230 antigen adhesive factors. Binding assay of ¹⁴C-radiolabelled ETEC was used to assess adhesion. Attachment of ETEC onto mucus secreting cells was visualised by scanning electron microscopy and by indirect immunofluorescence labelling with polyclonal antibodies directed against purified CFA factors, whereas intestinal mucus was shown using a monoclonal antibody directed against human mucins.

Methods

CELL CULTURE

We used two mucus secreting HT-29 cell subpopulations selected from the parental mainly undifferentiated HT-29 cell line⁷ by growth adaptation to, (a) methotrexate (10⁻⁶ M) referred to as HT29-MTX⁵, and (b) 5-fluoruracil (10⁻⁵ M) referred to as HT29-FU⁶ (A Zweibaum, INSERM Unité 178 Différenciation Cellulaire Intestinale, 94807 Villejuif, France). The two subpopulations remain stable when they are subcultured under standard conditions – that is, in standard glucose containing medium.⁵ Cells were routinely grown in Dulbecco modified Eagle's minimal essential medium

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(25 mM glucose) (Eurobio, Paris, France), supplemented with 10% inactivated (30 minutes, 56°C) fetal bovine serum (Boehringer GmbH, Mannheim, Germany).⁸ Monolayers of cells were prepared on glass coverslips, which were placed in six well tissue culture plates (Corning Glass Works, Corning, NY). For bacterial binding assay, HT-29 cells were grown in 24 well tissue culture plates (Corning Glass Works, Corning, NY). Cells were seeded at a density of 2×10^4 cells per cm^2 . For maintenance purposes, cells were passaged weekly using 0.25% trypsin in $\text{Ca}^{2+}\text{Mg}^{2+}$ free phosphate buffered saline (PBS) containing 0.53 mM EDTA. Experiments and maintenance of the cells were carried out at 37°C in a 10% $\text{CO}_2/90\%$ air atmosphere. The culture medium was changed daily. Unless otherwise stated, cultures were used at late post-confluence – that is, after 20 days in culture.

BACTERIAL STRAINS, GROWTH CONDITIONS, AND RADIOLABELLING

The strains of ETEC used were: H10407 expressing colonisation factor antigen I (CFA/I), Pb176 expressing CFA/II (Cs1 and Cs3), 1373 expressing CFA/III, and 2230 expressing the non-fimbrial 2230 antigen.⁹ The bacteria were stored in CFA glycerol broth at -80°C . Before adherence assays, all strains were grown on 2% CFA agar containing 1% casamino acids (Difco Laboratories, Detroit, MI), 0.15% yeast extract, 0.005% magnesium sulphate, and 0.0005% manganese chloride for 18 hours at 37°C.

Bacteria were metabolically labelled by the addition of ^{14}C -acetic acid (Amersham, 94 mCi/mmol; 100 $\mu\text{Ci}/10$ ml tube) as previously described.¹⁰

ADHERENCE ASSAY

The method used for determination of adherence of ETEC to HT-29 cells was that described elsewhere for Caco-2 cells.^{9 10} Briefly, HT-29 monolayers were gently washed twice with PBS before infection. ^{14}C -radiolabelled *E. coli* cells were suspended in the culture medium and 2 ml (10^8 CFU/ml) of this suspension was added to each well of the tissue culture plate. The plates were incubated at 37°C in 10% $\text{CO}_2/90\%$ air for one hour. The monolayers were then washed gently five times with sterile PBS. Adhering bacteria and HT-29 cells were dissolved in a one tenth normal NaOH solution. The amount of bacterial adhesion was evaluated by liquid scintillation counting (background less than 0.05%). Each adherence assay was conducted in triplicate on three successive HT-29 cell passages.

ANTIBODIES

A pool of mAbs reactive with mucin M1 epitopes of gastric mucins¹¹ has also been reported to recognise gastric mucins secreted

by the colonic HT29-MTX cells.¹² A sample of this mAb pool was obtained from Dr J Bara (INSERM U55, Paris). mAb 4H3 against the human dipeptidylpeptidase IV (EC 3.4.14.5) was obtained from Dr S Maroux (Centre de Biochimie et de Biologie Moléculaire, Marseille). ETEC whole cells were shown using polyclonal rabbit antibodies raised against purified ETEC CFA/II¹⁰ obtained from Dr A Darfeuille-Michaud (Laboratoire de Microbiologie, Université Clermont I).

IMMUNOFLUORESCENCE

Adhering *E. coli*, mucins, and dipeptidylpeptidase IV were shown in unpermeabilised cell layers by indirect immunofluorescent labelling. Cell layers were fixed (15 minutes at room temperature) in either paraformaldehyde (3.5% in PBS $\text{Ca}^{2+}\text{Mg}^{2+}$ free) or ethanol, as described by Lesuffleur *et al.*^{5 6} Fluorescein and rhodamine coupled goat antiglobulins were from Institut Pasteur Productions (Paris, France). Immunolabelling was examined with a Leitz Aristoplan microscope with epifluorescence. All photographs were taken on Kodak T-MAX 400 black and white film (Eastman Kodak Co, Rochester, NY).

SCANNING ELECTRON MICROSCOPY

For scanning electron microscopy, cells were grown on glass coverslips. After the bacterial adhesion assay, cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 24°C for one hour. After five washes with phosphate buffer, cells were post-fixed for 30 minutes with 2% osmium tetroxide, washed three times with the 0.1 M phosphate buffer, and dehydrated in a graded series (30 to 100%) of ethanol. Cells were dried after substitution with liquid carbon dioxide (Balzers CPD030, Hudson, NH) and coated with gold. The specimens were then examined with a scanning Jeol JSM 25S electron microscope.

Results

CHARACTERISTICS OF THE HUMAN MUCUS SECRETING HT29-MTX AND HT29-FU CELL SUBPOPULATIONS

Figures 1 and 2 show the morphological characteristics of the two mucus secreting HT-29 cell subpopulations – that is, HT29-MTX and HT29-FU cells – used here to study the cell association of enterotoxigenic *E. coli*.

Visualisation of the cell surface of the HT29-FU cells at late confluency (20 days of culture) by scanning electron microscopy (Fig 1) shows that this cell subpopulation is largely composed of enterocyte like cells with an apical brush border (Fig 1A) and of randomly distributed goblet cells that secrete mucus blebs (Fig 1A and B). The brush border of fluid transporting cells in HT29-FU cells resembles that seen in differentiated enterocyte like HT-29 cells.^{8 13} As previously reported,⁶

Figure 1: Scanning electron micrographs of the cultured human intestinal HT29-FU cells. (A) Low magnification showing fluid transporting cells with a brush border and of randomly distributed goblet cells (arrow); (B) high magnification showing mucus secreted by the goblet cells.

the mucins secreted by the HT29-FU cells react with antibodies against colonic mucins (not shown).

Examination by scanning electron microscopy of the cell surface of the goblet cells HT29-MTX at late confluency (20 days of culture) shows that the cells produce comparatively large amounts of mucus (Fig 2). Higher magnification shows a dense mucus gel (Fig 2A) with numerous mucus buds (Fig 2B). This mucus gel is labile, however, and after extensive washes a brush border is seen at the cell surface (Fig 2C). As previously reported,^{5 12 14} the brush border of these cells is endowed with intestinal differentiation associated hydrolases such as dipeptidylpeptidase IV, and mucins are of gastric immunoreactivity (see Fig 5).

ATTACHMENT OF ETEC TO THE HUMAN MUCUS SECRETING HT-29 CELL SUBPOPULATIONS

The HT29-FU cell line has the advantage of closely mimicking in vitro the situation present in vivo where the two cell phenotypes – that is, enterocyte like and mucus secreting – are present. Human *E. coli* strains harbouring CFA/I, CFA/II (Cs1 + Cs3), CFA/III, and 2230 antigen adhesive factors were incubated with cell monolayers to evaluate their abilities to adhere to the fluid transporting and the mucus secreting cells. Quantitative binding assay entailing the incubation of a fixed concentration of radiolabelled ETEC bacteria (10^8 CFU/ml) with cultured cells was performed. Bacterial adhesion was expressed as the percentage of the incubated bacteria remaining associated with the adhering microorganisms and the cells after washing. All

Figure 2: Scanning electron micrographs of the cultured human intestinal HT29-MTX cells. (A) Low magnification showing the dense mucus gel at the cell surface; (B) high magnification showing budding of mucus; (C) high magnification showing brush border after elimination of mucus by extensive washes.

ETEC strains bind to the HT29-FU cell subpopulation. Low values (mean (SD)) of adhesion were seen for ETEC strains bearing CFA/III (1.82 (0.09)) or 2230 antigen (1.46 (0.05)), compared with adhesion of ETEC harbouring CFA/I (4.10 (0.30)) or CFA/II (3.78 (0.45)). As disclosed by scanning electron microscopy, no binding occurred to the colonic mucus secreted by the HT29-FU goblet cells (Fig 3A), whereas ETEC bound to the cells possessing a brush border (Fig 3B).

It has been reported that for intestinal cell lines established from the parental HT-29 cell line,⁷ the appearance of a brush border and the apical expression of brush border components are a growth related phenomenon.¹⁵ We therefore examined the adhesion of ETEC as it relates to the intestinal differentiation of HT29-FU cells. No adhesion appeared during the first days of culture. Adhesion of ETEC appeared after confluency and increased during subsequent days (Fig 4). As ETEC bind in preference to the brush border of the enterocytic cells in the HT29-FU cell subpopulation, this result suggests that the brush border expression of functional ETEC binding sites is a growth related phenomenon. This result is consistent with our previous finding showing

Figure 3: Scanning electron micrographs of HT29-FU cell monolayers infected apically with enterotoxigenic *E coli* (ETEC). (A) Low magnification micrograph of HT29-FU cells showing adhesion of ETEC harbouring CFA/I adhesin to cell possessing brush border, whereas no adhesion occurs to mucus secreted by the goblet cells (arrow); (B) high magnification of adhesion of ETEC CFA/I on brush border in fluid transporting cells. Bacterial binding to the microvilli takes place without damage to the underlying cell population.

that brush border ETEC binding to the human cultured enterocyte like HT-29 and Caco-2 cells develops as a function of cell differentiation.^{9 10}

ETEC binding was also examined using the HT29-MTX cells. Because at post-confluent culture (20 days) the cell monolayer is covered by a large amount of mucins that cover the brush border, these cells were examined at an earlier period of the culture (seven days) – that is, when all the cells express an apical brush border but only a limited amount of mucus is secreted on the cell surface.^{12 14} Immunofluorescence study of unpermeabilised HT29-MTX cells at seven days in culture showed the presence of mucus secretion (Fig 5B) and dipeptidylpeptidase IV (Fig 5E). Colocalisation comparisons were conducted – that is, ETEC (Fig 5A) and mucins (Fig 5B) – as well as ETEC (Fig 5D) and dipeptidylpeptidase IV (Fig 5E). ETEC binding clearly did not colocalise with mucins (Fig 5C), whereas it did colocalise with dipeptidylpeptidase IV (Fig 5F).

Discussion

A number of studies have been performed using in vitro models to study the role of the mucus layer in bacterial growth, adhesion, and colonisation. These models consist of crude or purified mucus, which have been used to study positive adhesion of human pathogenic bacteria, such as the enterohaemorrhagic *E coli* strain CL-49,^{16 17} *Yersinia enterocolitica*,^{18 19} and *Campylobacter jejuni*.²⁰ Fresh and formalin fixed isolated human ileal segments containing

goblet cells producing mucus and purified human mucus have been used to study the adhesion of *Helicobacter pylori*,²¹ enterovirulent *E coli*,^{22 23} and *Pseudomonas aeruginosa*.²⁴ Human tracheobronchial mucins have been used to study adhesion of *Pseudomonas aeruginosa*^{24 25} and *cepacia*.²⁶

Studies concerning the development of pathogenicity of human ETEC require appropriate cellular models that closely mimic the cells participating in vivo in intestinal infection. Indeed, ETEC that attach to the epithelium by means of specific fimbrial or non-fimbrial adhesions,²⁷ exhibit high tissue and host specificities.^{9 10} Adhesion of human ETEC has been studied using human, freshly isolated enterocytes.²⁸ These enterocytes, however, are unstable in the laboratory. In the absence of normal cultured intestinal cells, various investigators have started to use cultured human intestinal cell lines to circumvent this problem. The parental mainly undifferentiated HT-29 cell population (HT-29 Std)⁷ contains a small proportion of enterocyte like^{5 10} and mucus secreting⁵ cells. Several laboratories have isolated from HT-29 Std cells various differentiated HT-29 cell subpopulations and clones that can express in culture the differentiation characteristics of columnar absorptive cells.^{8 13 29 30} Enterocyte like HT-29 cell subpopulations have been extensively used to study the organisation and function of human intestinal cells.¹⁵ Moreover, they closely mimic in vitro infection conditions by pathogens involved in human diarrhoea such as ETEC,^{9 10 31–33} diffusely adhering *E coli*,³⁴ and virus.^{35 36}

In vivo ETEC can attach to carbohydrate structures acting as receptors on the thick mucus layer, composed of mucins and a large number of smaller associated proteins, that cover the intestinal cell surface.^{3 37} The role of the mucus gel in promoting bacterial colonisation has been shown for non-pathogenic and pathogenic animal and human *E coli* using animal models.⁴ Recently, Sajjan and Forstner showed that enterohaemorrhagic *E coli* serotype O157:H7 strain CL-49 bound to isolated

Figure 4: Development of adhesion of enterotoxigenic *E coli* harbouring CFA/II (●) and CFA/III (▲) adhesive factors as a function of the days in culture of HT29-FU cells. Mean per cent of total radiolabelled ETEC bacteria adhering to the cell monolayer. Adherence assays were conducted in duplicate with three successive cell passages. Vertical bars indicate standard deviations.

Figure 5: Cell localisation of ETEC CFA/I binding to cultured human intestinal HT29-MTX cells. Binding of ETEC whole cells (A) shown by indirect immunofluorescence staining using anti-CFA/I antibody and costaining of the cells with monoclonal antibody directed against gastric like mucins (B). In C, comparison between ETEC whole cells binding (black area) seen in A and mucus staining (hatched area) seen in B. Note that ETEC binding occurs in an area of the cell layer without positive mucus reactivity. Binding of ETEC whole cells (D) shown by indirect immunofluorescence staining using anti-CFA/I antibody and costaining of the cells with monoclonal antibody directed against dipeptidylpeptidase IV (E). In F, comparison between ETEC whole cells binding (black area) seen in D and dipeptidylpeptidase IV staining (hatched area) seen in E. Note that ETEC binding occurs in an area of the cell layer with positive dipeptidylpeptidase IV reactivity.

human mucus.^{16 17} To our knowledge, no reports have shown the adhesion of ETEC to cultured human mucus secreting cells. As for enterocyte like cells lines, several groups have recently acquired by selection pressure mucus secreting cell subpopulations^{5 6 29 30 38 39} from the parental HT-29 Std cells. These goblet cells lines are being used to study the biochemical composition of intestinal mucus,^{40 41} the neurohormonal control of intestinal mucus secretion,^{42 43} the regulation of mucus production by cytokines,⁴⁴ and the mechanisms and factors that control the commitment of normal intestinal cells to the alternative types of differentiation, namely goblet or absorptive cells.⁴⁵ Moreover, Micots *et al*⁴⁶ recently were first to report the attachment of an enteropathogen – that is, *Helicobacter pylori*, to cultured human mucus secreting HT-29 Cl 16E cells. We examined here the attachment of ETEC to different subpopulations of HT-29 cells containing cells possessing a brush border and goblet cells, which secrete mucins of gastric or

colonic immunoreactivity. Our results show that human ETEC bound in preference to the brush border of the enterocytic cells rather than to mucus. These results agree with those of Yamamoto *et al*^{25 26} who reported that the enterocytes on formalin fixed human ileal segments are the preferential target of ETEC with CFA/I or CFA/II adhesins, whereas no adherence occur to M cells and goblet cells. Moreover, we saw that ETEC binding developed as a function of the cell differentiation in HT29-FU cells. It is of interest to note that the expression of receptors for the ETEC virulence factors – that is, the colonisation factor antigens^{9 10 31} and the heat stable cytotoxic enterotoxin⁴⁷ was associated with cell differentiation of the cultured human intestinal cells.

Taken altogether, the findings of Yamamoto *et al*^{25 26} and our results suggest that intestinal mucus does not influence ETEC colonisation. As our experiments, however, only explored the binding of ETEC to cells having gastric and colonic immunoreactivity, and as *in vivo* ETEC colonise the small intestine,^{1 2} it will be necessary to complete our study by examining the binding of ETEC to normal small intestinal mucins. It is relevant in this context that brush border glycoproteins have been reported to serve as the receptors for human ETEC.^{31 33} This favours the idea that enterocytes rather than goblet cells or mucins are the preferred receptor. Further studies will attempt to confirm this idea and will include a comparison of mucins from HT29-MTX and HT29-FU cells with those of normal human small intestine and colon.

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