Original Article

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The Inhibitory Effect of Ileal Mucosal Media Originated from FVB/N mice strain on *Escherichia coli* LF82 Invasion

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ABSTRACT

Objective: The aim of this study was to investigate the effect of healthy mucosa on adhesive and invasive properties of AIEC reference strain *Escherichia coli (E. coli)* LF82. For this purpose, we had designed special medias that contained cell culture medium and mucosal content obtained from different regions (colon and ileum) of the digestive tract.

Methods: We tested the infecting ability of AIEC reference strain *E. coli LF82* on I-407 cells in the presence of mucosal media (Muc-M) under *in vitro* conditions. Muc-M composed of certain rates of cell culture medium or M63 minimal medium and mucosal contents obtained from different part of intestine were designed for cell-infection experiments and biofilm-formation assays.

Results: The result showed that the mucosal media decreased the infection percentage of *E. coli* LF82 strain when compared with control group. It was seen that the mucosal media originating from ileum almost completely inhibited the invasion of LF82 strain. On the other hand, it was observed that the mucosal media prepared from colon) reduced the bacterial invasion only in half the rate when compared with control.

Conclusion: The findings showed that these medias obtained from different regions of the intestinal tract affected LF82 invasion at different rates. Therefore, this study provided crucial information that could contribute to the future studies on the localization of bacteria

Keywords: *Escherichia coli* LF82, Crohn's disease, biofilm, pathobiont, adhesion, invasion

Introduction

Crohn's disease (CD) is a chronic inflammatory bowel disease that can affect various parts of the intestinal tract and complex genetic and environmental factors are involved in its development. On the other hand, the intestinal flora plays an important role in the development of this disease because there are some strong findings about the role of the intestinal flora in the development of Crohn's disease. One of these is the detection of the significant inflammatory process in response to the contact of luminal content with terminal ileum, shown after surgical operations in Crohn's patients. However, when the fecal flow is directed to another side, the signs of healing are observed in these regions (1). And the others are the positive results of antibiotic treatment in some Crohn's patients, the reduction of ulceration symptoms following antibiotic treatment in multiple animal experiments and no findings about colitis in germ-free animals (2).

On the other hand, scientific studies performed about the relationship between intestinal flora and CD have shown the presence of a significant increase in the amount of *Escherichia coli (E. coli)* in individuals with the ileal type of CD when compared to normal individuals (3-5). After some detailed studies, a new *E. coli* member, adherent-invasive (AIEC), was isolated from the inflamed tissue of an individual with ileal type of CD (6). *In vitro* studies have shown that this new strain can adhere to and invade intestinal epithelial cells. Similarly, it has been determined by in vitro methods that this strain can live and multiply in

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macrophages and cause a high amount of tumour necrosis factor-alpha (secretion. These bacteria was also shown to cause high-level expression of carcino-embryonic antigen-related cell adhesion molecule-6 molecules, a kind of adhesion receptor in patients with CD (7).

Despite all the research done with AIEC, it is still unclear how these bacteria play a role in the development of Crohn's disease. Furthermore, it is not possible to determine the location of these bacteria in the intestinal flora because these bacterial specific molecules have not yet been identified. The intestinal mucus covering the intestinal epithelium along the digestive tract serves as a barrier to pathogenic microorganisms reaching the epithelial cells as well as hosting many commensal bacteria (8).

The basic structure of the mucus is composed of mucins which are a family of high molecular weight, heavily glycosylated proteins (glycoconjugates) produced by epithelial tissues in most animals. Typically, these mucins are glycoproteins containing 80% carbohydrate and consisting of proline, serine and threonine amino acid repeats (referred to as peroxisomal targeting signal sequence) (8,9). Although there are several types of mucin glycoproteins that form the mucous membrane throughout the digestive tract, goblet cells secrete MUC-2 mucin in the colon and small intestine of the intestinal tract. However, the same mucin exhibits different behaviors in the small intestine and the colon (10-12). Studies suggest that this difference originates from the differences in glycan epitopes in MUC-2. It is emphasized that these oligosaccharide units present in the mucus may also play an important role in the distribution of the intestinal flora in the digestive tract (13). Therefore, the mucin glycoprotein in different compartments of the digestive tract may have a key role in studies on the localization of AIEC bacteria, or on the detection of its specific molecules.

The aim of current study was to investigate the effect of healthy mucosa on adhesive and invasive properties of AIEC. For this purpose, we designed special medias that contained cell culture medium and mucosal content obtained from different regions (colon and ileum) of the digestive tract.

Methods

Bacterial Strain

The AIEC reference strain, *E. coli* LF82 isolated from an ileal lesion of a patient with CD was kindly provided by Dr. Nicolas Barnich and Dr. Elisabeth Billard (Universite´ d'Auvergne, Clermont-Ferrand, France). Bacteria were grown routinely in the Luria-Bertani (LB) broth overnight at 37 °C and without shaking.

Cell Line and Cell Culture Procedure

The intestine-407 (I-407) cell line (The Global Bioresource Center, chemokine (C-C motif) ligand 6, Manassas, VA, USA) was also kindly provided by Dr. Nicolas Barnich and Dr. Elisabeth Billard (Universite ´ d'Auvergne). The cells were maintained in an atmosphere containing 5% CO_2 at 37 °C in Eagle minimum essential medium (MEM) (MEM; Sigma-Aldrich, MO, USA) supplemented with 10% (v/v) heat- inactivated fetal bovine serum [Fetal bovine serum (FBS); Life Technologies, CA, USA], 1% nonessential amino acids (Life Technologies), 1% L-glutamine (Life Technologies), 100,000 units/L penicillin, 100 mg/L streptomycin, 25 mg/L amphotericin B and 1% MEM vitamins solution X-100 (Life Technologies).

Mice Strain

FVB/N female mice were housed under specific pathogen-free conditions in the animal care facility of Universite ´ d'Auvergne (Clermont-Ferrand, France). Animal protocols were performed according to the local ethical rules during ERASMUS+ Traineeship Program between 23th and June 19th 2015 in Universite ´ d'Auvergne (Clermont-Ferrand, France).

Isolation of Ileum & Colon and Preparation of Mucosal Media (Muc-M)

Mucosal media (Muc-M) was designed as an artificial medium, which was isolated from the 8- to 10-week-old female healthy FVB/N mice intestine. Muc-MIR is originated from ileum regions and Muc-MCR is originated from colon regions of healthy mice intestines. The 8- to 10-week-old female FVB/N mice were euthanized by cervical dislocation. The mouse was opened by ventral midline incision under 70% ethanol anesthesia. Colon was isolated as the distance (~5 cm) from rectum to cecum. Then, the ileum was isolated from cecum to (~8 cm) superior region of cecum. The colon and ileum regions of intestine were separately collected in sterile Petri dishes containing physiological water. The colon was brought into the open using scissors and the inner surface of the colon scrapped by a glass slide. This procedure was repeated also for ileum. Scrapped contents obtained from three mice were collected in a sterile 2 mL eppendorf tube for each region.

Cell infection procedure and biofilm assays were performed in different times. For cell-infection experiments, the tubes were labelled then, 1 mL sterile MEM added into tubes. Samples were treated for 15 min at +4 °C at a maximum speed of the disruptor. After disruption, samples were centrifuged for 10 min at +4 °C in 10,600x g. Supernatant was collected and transferred into falcon tubes separately for colonic regions and ileal regions.

Sterilization was performed by filtration method in the laminar air-flow cabinet. Samples were passed through filter with 0.45μm to new sterile falcons, then the filtrate was passed through filter with 0.20-μm pore diameter to another new sterile falcons and finally, it was diluted with sterile MEM as half of their total volume. The mentioned procedures were similarly performed for biofilm-formation assays. The only difference between two methods is that M63 minimal medium supplemented with 8 g/L (0.8%) glucose was used instead of MEM for biofilm-formation assay. To test the sterility of these media, Muc-M were inoculated onto LB and Mac Conkey (Oxoid, Basingstoke, UK) agar plates, and afterward, it was seen no colony forming on both plates after overnight incubation at 37 °C.

By the way, the effects of Muc-M alone on I-407 cell monolayers were also tested for cell infection experiments with a similar experiment performed by Aygun et al (14). Briefly, I-407 cell monolayers in the cell culture medium including 20% of Muc-M was incubated for 3-4 h at 37 °C with 5% CO_2. After incubation period, it was seen that the cell culture medium including 20% of Muc-M had no negative or deleterious effects on cell monolayers. Finally, Muc-M was ready for cell infection and biofilm-formation assays.

Cell Infectivity and Adhesion&Invasion Experiments

The cell infectivity, adhesion and invasion experiments were performed according to Darfeuille-Michaud method (15). I-407 cells were seeded in 24-well tissue culture plates (Sigma-Aldrich) at a density of $4x10^5$ cells per well and incubated for 20 h. The cell monolayers were washed twice with phosphate-buffered saline (PBS) (pH 7.2). 200 µL of Muc-M was prepared with MEM, was added to monolayers and brought to 1 mL with 10% FBS-supplemented MEM for each regions. 10% FBS-supplemented MEM was only designed and used as control. Each monolayer was infected with a multiplicity of infection (MOI) of 10 bacteria per epithelial cell (MOI: 10). In 3 h incubation period, it was performed at 37 °C with 5% CO_2.

For adhesion assay, after 3 h incubation period at 37 °C with 5% CO_2, the monolayers were washed three-times with PBS and the epithelial cells were then lysed with 1% Triton X-100 (Sigma-Aldrich) in deionized water. The samples were diluted and plated onto LB agar plates to determine the number of colony forming unit (CFU), and the mean number of bacteria per cell was determined. Adhesion assays were performed in triplicate.

For invasion assay, after 3 h incubation period at 37 °C with 5% CO_2, the monolayers were washed three-times with PBS. One hundred µL/m fresh culture medium containing gentamicin (Sigma-Aldrich) was added to cell media to kill the extracellular bacteria and 1h incubation was performed. After incubation for an additional hour, monolayers were washed with PBS and 1% Triton X-100 in deionized water placed in the wells to lysis the eukaryotic cells for 5 min. The samples were diluted and plated onto LB agar plates to determine the number of CFU. Invasive ability of LF82 with I-407 cell lines were expressed as the percentage of intracellular bacteria compared with the initial inoculum, taken as 100%. Invasion assays were performed in triplicate.

Statistical Analysis

One-way ANOVA followed by Dunnet T3 test was used for the assessment of the numerical data. P values ≤ 0.05 were considered statistically significant. All assays were performed at least three-times in separate experiments. Microsoft Excel and SPSS version 24.0 (IBM, NY, USA) programs were used for statistical analysis.

Results

We evaluated the effects of sterile mucosal media, derived from different parts of healthy intestines of FBV/N mice strain, on LF82 adhesion and invasion to human intestinal epithelial cell line (I-407) by adding each Muc-M (20; Muc-MCR or Muc-MIR) to the cell culture medium before infection and during 3 h infection period for adhesion/invasion assays.

After incubation period, it was seen that the cell culture medium including 20% of Muc-M had no negative or deleterious effects on cell monolayers (Figure 1, Figure 1a, Figure 1b, Figure 1c and Figure 1d).

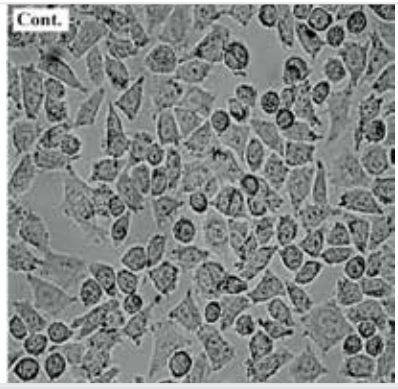

Figure 1. The apperance of cells in normal cell culture medium.

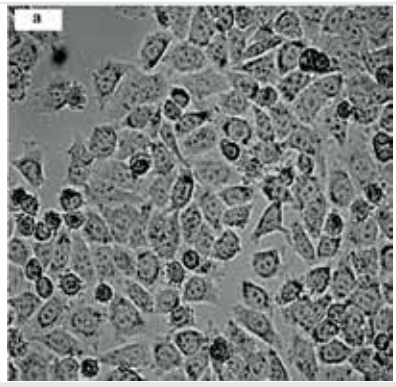

Figure 1a: Micrographs of crystal violet stained biofilms in the different mucosal medias originated from colon or ileum. The appearance of cells in mucosal media (Muc-MCR) after 3 hour incubation

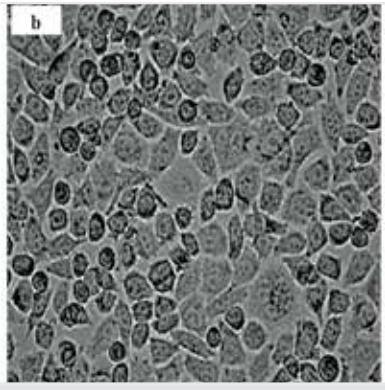

Figure 1b. The apperance of cells following 3- hour incubation, after replacement mucosal media (Muc-MCR) with fresh cell culture medium

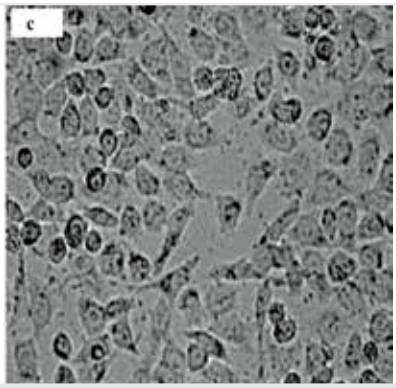

Figure 1c: Micrographs of crystal violet stained biofilms in the different mucosal medias originated from colon or ileum. Cell culture in mucosal media (Muc-MIR) after 3 hour incubation

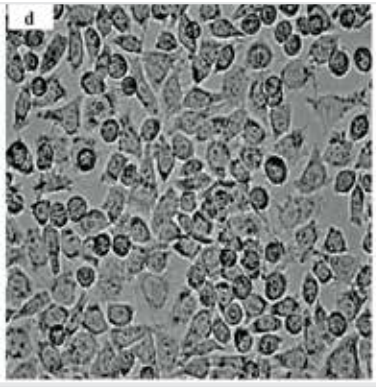

Figure 1d. The apperance of cells following 3- hour incubation, after replacement mucosal media (Muc-MIR) with fresh cell culture medium

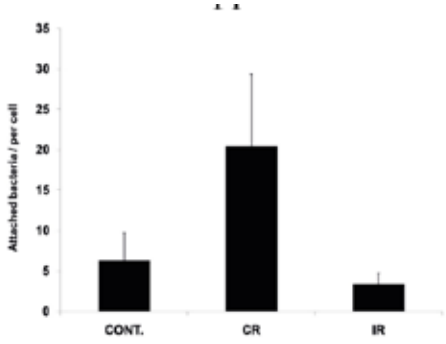

Figure 2. Adhesion abilities of adherent-invasive *Escherichia coli* LF82 in different medias including mucosal media originated from colon or ileum regions. Cell-associated bacteria were quantified after a 3-h infection period. Results are expressed as cell-associated (adherent+intracellular) bacteria per cell. ANOVA was used for multiple comparisons.
CONT.: Control, CR: Colon region, IR: Ileum region

Adhesion results showed that the Muc-MIR medium almost completely inhibited LF82 invasion ($p<0.001$), while the Muc-MCR medium reduced the LF82 invasion by 50% compared to the control, however this decline was not statistically significant ($p>0.001$) as seen in Figure 2. LF82 exhibited a higher adherent

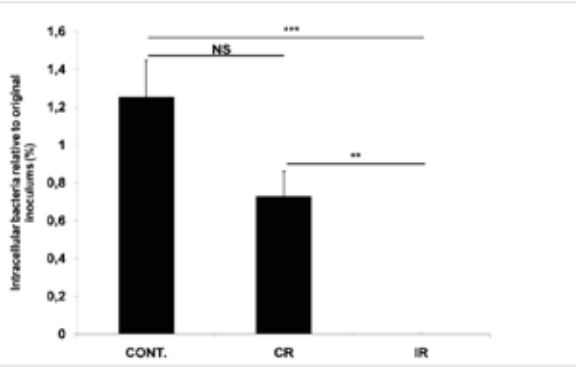

Figure 3. Invasion abilities of adherent-invasive *Escherichia coli* LF82 in different medias including mucosal media originated from colon or ileum regions. Cell-associated bacteria were quantified after a 3-h infection period. Results are expressed as cell-associated (adherent+intracellular) bacteria per cell. ANOVA was used for multiple comparisons.
CONT.: Control, CR: Colon region, IR: Ileum region

ability to I-407 cell monolayers in cell culture medium including Muc-MCR than those including Muc-MIR ($p<0.01$; as attached bacteria per cell, control: 7±3; Muc-MCR: 21±7; Muc-MIR: 4±1; Figure 2). On the other hand, after gentamicin treatment following extra 1 h incubation period, LF82 invasion significantly decreased ($p<0.05$) in the cell culture media including 20% of Muc-MCR or Muc-MIR.

The results of invasion assay showed that both of these medias considerably reduced bacterial invasion (Figure 3). It was quite remarkable that the Muc-MIR medium originating from the ileum inhibited more LF82 invasion than the Muc-MCR medium prepared from the colon region of intestine ($p<0.01$; intracellular bacteria relative to original inoculums (%), control: 1.3±0.2; Muc-MCR: 0.7±0.2; Muc-MIR: 0; Figure 3).

Discussion

Many studies examined the relationship between etiopathogenesis of CD and AIEC strains which are found in high amounts in the inflamed ileal tissues of patients with Crohn's disease. However, the current literature does not have enough evidence about the role of AIEC on Crohn's disease. The same studies showed that although the *E. coli* strains are relatively found in higher amounts in ileal tissues both in healthy and CD patients, the localization of AIEC in digestive tract has not yet been identified (16,17). Therefore, studies to find these bacterial specific molecules will help illuminate the role of AIEC strains in the progression of CD. In the current study, we designed special medias that contained mucosal content obtained from different regions (colon and ileum) of FBV/N healthy female mice digestive tract. Then, we tested the infecting ability of AIEC reference strain *E. coli* LF82 to I-407 cells in the presence of Muc-M originating from the colon (Muc-MCR) and ileum (Muc-MIR) regions, under *in vitro* conditions.

After adding Muc-MCR or Muc-MIR media to the cell culture medium at 20% percentage, we infected the cells with LF82 bacteria. There was no statistically significant difference when we

examined the results of the adhesions after the 3 hour infective period. However, the results of invasion assay showed that both of these media considerably reduced bacterial invasion. According to our results; the Muc-MIR medium almost completely inhibited LF82 invasion ($p<0.001$), while the Muc-MCR medium reduced the LF82 invasion by 50% compared to the control, however this decline was not statistically significant ($p>0.001$). The results we obtained from the current study strongly supported our previous study that performed with Balb/c mouse strain (14). The strong inhibition of bacterial invasion, in particular, may be due to glycosylated oligosaccharide units. Likewise, in a study supporting this result, the role of mannose oligosaccharide in the adhesion and invasion of LF82 bacteria was investigated by cell infection experiments using the I-407 cell line. The results showed that mannose strongly inhibited LF82 invasion and that inhibition largely occurred via type-1 pili/mannosyl interaction (18). In another study, commercially purchased mucin was added to the cell culture medium at a certain rate before it was infected with *E. coli* C25 strain. The results obtained after the infection period showed that mucin inhibited bacterial translocation strongly in both cell lines and the bacterial pili retained by the mucin-bound oligosaccharide units without reaching the cell (19). On the other hand, it was quite remarkable that the Muc-MIR medium originating from the ileum inhibited more LF82 invasion than the Muc-MCR medium prepared from the colon region of intestine. Similarly, goblet cells in both the ileum and the colon secrete the same mucin called MUC-2. Thus, these results support the view that the mucin in the colon and the small intestine may exhibit different properties although they have the same structure (10-12). In addition, it is suggested that specific receptor-ligand interactions with LF82 strain may occur, because medias originated from colon and ileum regions of intestine affect LF82 invasion in different ratios. Also, oligosaccharide units associated with mucin may play a decisive role in the distribution of gut flora in the gut system (13). If these interactions can be identified in further studies, specific molecules can be identified that facilitate the detection of LF82.

Conclusion

In conclusion, this study showed that AIEC LF82 strain has different patterns in terms of the adhesion and invasion capabilities in mucosal medium originated from ileum region of healthy FBV/N female mice strain. Therefore, our data have a potential importance to give new ideas about determining the localization of AIEC bacteria within the healthy intestinal mucosa as in our previous work (14).

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Ethics

Ethics Committee Approval: Animal protocols were performed according to the local ethical rules during ERASMUS+ Traineeship Program between 23th and June 19th 2015 in Universite ´ d'Auvergne (Clermont-Ferrand, France).

Informed Consent: *In vitro* study.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Concept: H.A., F.U., Design: H.A., M.K., Data Collection or Processing: H.A., M.K., Analysis or Interpretation: H.A., F.U., M.K., Literature Search: H.A., F.U., M.K., Writing: M.K.

Conflict of Interest: No conflict of interest was declared by the authors.

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