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• BASIC RESEARCH •

Competitive inhibition of adherence of enterotoxigenic *Escherichia coli*, enteropathogenic *Escherichia coli* and *Clostridium difficile* to intestinal epithelial cell line Lovo by purified adhesin of *Bifidobacterium adolescentis* 1027

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Abstract

AIM: To observe competitive inhibition of adherence of enterotoxigenic *Escherichia coli* (ETEC), enteropathogenic *Escherichia coli* (EPEC) and *Clostridium difficile* (*C. difficile*) to intestinal epithelial cell line Lovo by purified adhesin of *Bifidobacterium adolescentis* 1027 (B. ado 1027).

METHODS: The binding of bacteria to intestinal epithelial cell line Lovo was counted by adhesion assay. The inhibition of adherence of ETEC, EPEC and *C. difficile* to intestinal epithelial cell line Lovo by purified adhesin of B. ado 1027 was evaluated quantitatively by flow cytometry.

RESULTS: The purified adhesin at the concentration of 10 μg/mL, 20 μg/mL and 30 μg/mL except at 1 μg/mL and 5 μg/mL could inhibit significantly the adhesion of ETEC, EPEC and *C. difficile* to intestinal epithelial cell line Lovo. Moreover, we observed that a reduction in bacterial adhesion was occurred with increase in the concentration of adhesin, and MFI (Mean fluorescent intensity) was decreased with increase in the concentration of adhesin.

CONCLUSION: The purified adhesin of B. ado 1027 can inhibit the adhesion of ETEC, EPEC and C. difficile to intestinal epithelial cell line Lovo in a dose-dependent manner.

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INTRODUCTION

It is well known that enterotoxigenic *Escherichia coli* (ETEC) and enteropathogenic *Escherichia coli* (EPEC) are major cause of diarrhoea in neonates and travelers. The gastrointestinal tract appears to be a reservoir for *E. coli* which are able to translocate across the intestinal mucosa. *Clostridium difficile* (*C. difficile*), a gram-positive spore-forming anaerobic bacillus, is the most common cause of infectious diarrhoea in hospitalized patients[1]. Adherence of bacteria to intestinal epithelium is known to be a prerequisite for colonization and infection of the gastrointestinal tract by many gastrointestinal pathogens[2-4]. The intestinal epithelium is the primary site of contact for pathogens with host cells and plays an important role in the cross-talk between epithelial cells, luminal micro-organisms and immune cells. Therefore, inhibition of bacterial adhesion to the intestinal surface may prevent enteropathogens from translocating across the intestinal mucosa.

Bifidobacterium is known to be a predominant constituent of the human intestinal microflora[5]. The presence of bifidobacteria in the human intestine has been reported to contribute to human health and well being[6,7]. Adherence of bifidobacteria to intestinal mucus is regarded as one of the prerequisites for successful colonization[8], antagonistic activity against enteropathogens[9,10], modulation of the immune system[11]. In addition, mucosal adhesion has been proposed as one of the main selection criteria for probiotic strains[12-14]. Recently, studies have focused on its anti-infectious effects, particularly the inhibition activity against enteropathogens for protecting human[15-17]. As the production and preservation of live bifidobacterium are difficult, so its application is restricted. Bernet *et al*.[18] observed that the occurrence of bifidobacteria adhering to the human intestinal cells by a mechanism of adhesion which involved a proteinaceous component. Fujiwara *et al*.[19,20] clarified that bifidobacterium longum SBT2928 produced a proteinaceous factor which prevented the binding of ETEC to the binding receptor gangliotetraosylceramide (GA1). Zheng *et al*.[21] extracted and purified a protein with a molecular weight of 16 ku from spent culture supernatant of *Bifidobacterium adolescentis* 1027 (B. ado 1027). In the present study, competitive inhibition of adherence of ETEC, EPEC and *Clostridium difficile* to intestinal epithelial cells by purified adhesin of B. ado 1027 was observed by adhesion assay and flow cytometry assay.

MATERIALS AND METHODS

Bacterial strains

B. ado 1027 was isolated from healthy infants feces and identifiedby API-20A and TAB system (British). *B. ado* 1027 was cultured in sulfglycolic acid salt broth at 37 ℃ for 48 h under anaerobic conditions. ETEC and EPEC were obtained from Department of Epidemiology, First Military Medical University. ETEC and EPEC were cultured in agitation, nutrient broth at 37 ℃ for 48 h. A toxin-producing *C. difficile* strain (VPI10463) was obtained from Lanzhou Institute of Biological Products (China). *C. difficile* strain was grown in brain-heart infusion broth at 37 ℃ for 48 h under anaerobic conditions. All bacteria were harvested from the broth culture by centrifugation at 2 500 r/min for 10 min, followed by resuspension of the pellet in phosphate buffered saline (PBS, pH 7.4) to a concentration of 1×10^8 colony forming units (cfu)/mL.

Cell line

Human intestinal epithelial cell line Lovo was obtained from American type culture collection (ATCC) and was maintained in RPMI-1 640 (Gibco) supplemented with 2 mmol/L *L*-glutamine and 100 mL/L FCS at 37 ℃ in a humidified atmosphere containing 50 mL/L CO_2. For the adhesion assay, monolayer of Lovo cell was prepared on glass coverslips which were placed in six-well tissue culture plates (Corning Glass Works, Corning, N.Y.).

Extraction and purification of adhesin

The adhesin of bifidobacterium was extracted and purified as reported earlier by Zheng *et al*[21]. In short, the adhesin of bifidobacterium was isolated and purified by Superdex 75 gel filtration and Q-Sepharose FF ion exchange chromatography, and the adhesin was analyzed by SDS-PAGE. It was a protein with a molecular weight of 16 Ku , stored at -20 ℃.

In vitro adhesion assay

The adherence of bacteria strains to Lovo cells was examined as described previously[22-24]. Briefly, the Lovo monolayer prepared on glass coverslips which were placed in six-well tissue culture plates, was washed twice with PBS. The adhesin (1 μg/mL, 5 μg/mL, 10 μg/mL, 20 μg/mL, and 30 μg/mL) of bifidobacterium was added to each well of the tissue culture plate, and the plate was incubated at 37 ℃ in a humidified atmosphere containing 50 mL/L CO_2. After 30 min of incubation, the monolayer was washed one time with PBS. The suspensions of bacteria (1mL) were added to each well, respectively. After 3 h of incubation at 37 ℃ atmosphere, the monolayer was washed twice with sterile PBS, fixed with methanol, stained with Gram stain, and examined microscopically. For each monolayer on a glass coverslip, the number of adherent bacteria was evaluated in 30 random microscopic areas. Adherence was evaluated by two different technicians to eliminate bias. Bifidobacterium+bacteria and alone bacteria group were used as controls of adhesion.

Mean fluorescent intensity (MFI) of human intestinal epithelial cell with adherent bacteria were detected by flow cytometer

The fluorochrome FITC (Fluorescein isothiocyanate) was used in this study. All bacteria were washed 2 times in bicarbonate buffer (0.1 mol/L, pH 9.2), and labelled with fluorochrome FITC (1.5 mg/mL) by incubating 1×10^8 bacteria/mL at 4 ℃ for 1 h. Excess fluorochrome was removed by washing 5 times with PBS at 1 500 *g*. All bacteria were resuspended in PBS which contained B. ado 1027 (1×10^8/mL), adhesin of different concentrations and non-adhesin. Fluorescently labelled bacteria (1×10^8) were incubated with 1×10^5 epithelial cells for 2 h at 37 ℃. After incubation, cells were washed three times with PBS to remove non-adherent bacteria. MFI (wave-length of excitation: 488 nm, wave-length of emission: 575 nm) of human intestinal epithelial cells with adherent bacteria was measured in a FACS-420 flow cytometer (Coulter, U.S). A total of 10 000 cells was acquired and the data were analysed with the Cell Quest software program from Coulter.

Statistical analysis

Values were expressed as mean±SD. Statistical comparisons between the means were made with students *t* test by SPSS 10.0 version. A P value of <0.05 was assumed for statistical significance.

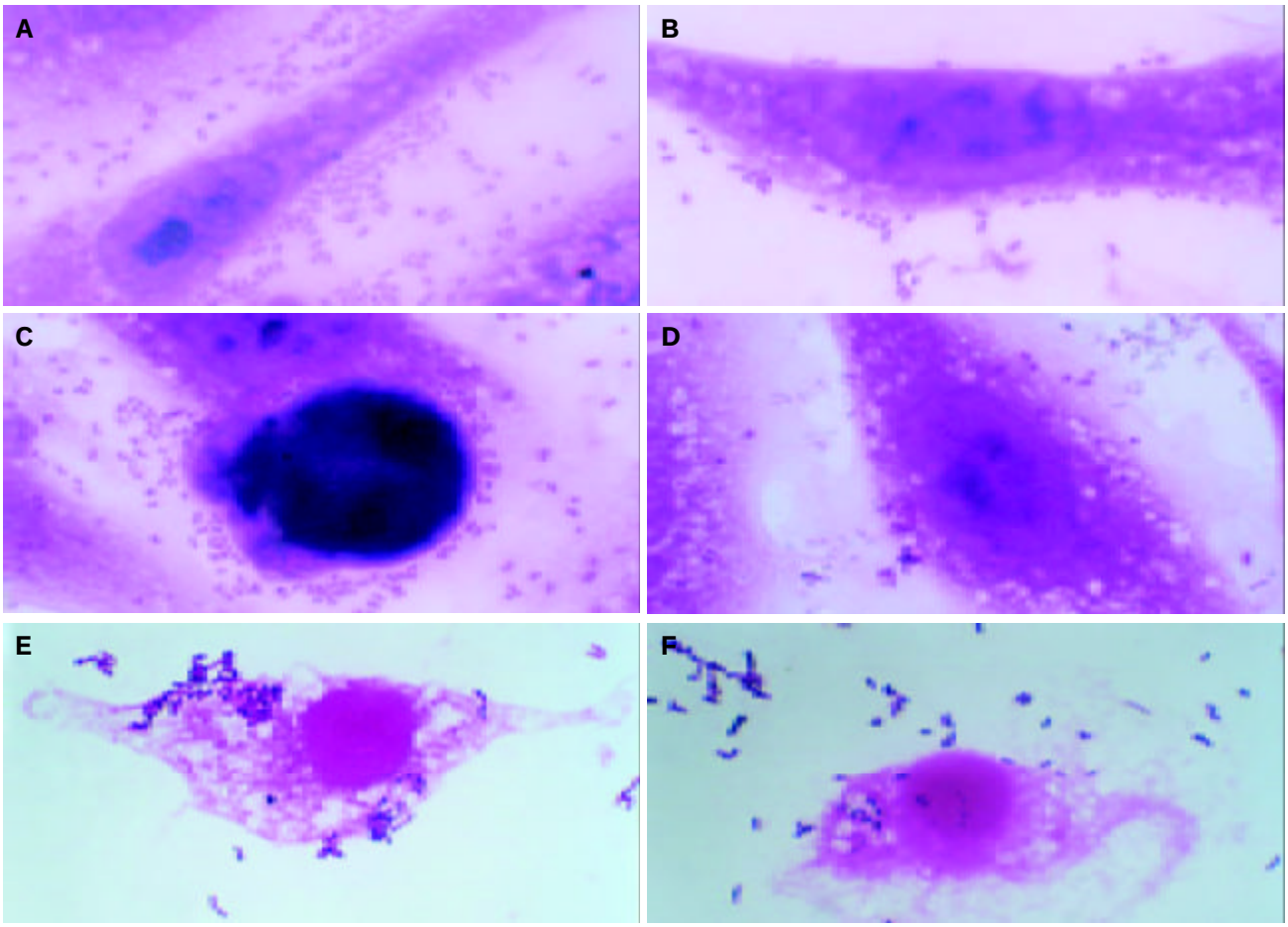

Figure 1 Gram staining of ETEC, EPEC and *Clostridium difficile* (×1000). Competitive inhibition of adherence of ETEC, EPEC and *Clostridium difficile* to intestinal epithelial cells by purified adhesin of B. ado 1027 was observed by adhesion assay. A: adherence of ETEC to intestinal epithelial cells without adhesin; B: adherence of ETEC to intestinal epithelial cells with adhesin; C: adherence of EPEC to intestinal epithelial cells without adhesin; D: adherence of ETEC to intestinal epithelial cells with adhesin; E: adherence of *C. difficile* to intestinal epithelial cells without adhesin; and F: adherence of *C. difficile* to intestinal epithelial cells with adhesin.

RESULTS

Effect of purified adhesin of B. ado 1027 on adherence of ETEC, EPEC and C. difficile to intestinal epithelial cells

The adhesion assay was performed after Lovo cell and different concentrations adhesin were incubated at 37 ℃ for 30 min. As shown in Table 1, the purified adhesin at the concentration of 10 μg/mL, 20 μg/mL and 30 μg/mL except at 1 μg/mL and 5 μg/mL could significantly inhibit the adhesion of ETEC, EPEC and clostridium difficile to intestinal epithelial cell line Lovo. Adhesion of ETEC, EPEC or *Clostridium difficile* to intestinal epithelial cell was significantly decreased when the concentration of purified adhesin was 10 μg/mL, 20 μg/mL or 30 μg/mL ($P<0.05$, $P<0.01$). Moreover, we observed that the ability of inhibition was enhanced with increase in the concentration of adhesin. These results indicated that purified adhesin of *B. ado* 1027 was involved in the adhesion of bacteria. The adhesion of ETEC and EPEC to intestinal epithelial cell was linear (Figures 1A, B, C and D). The adhesion of *C. difficile* to intestinal epithelial cell was like cluster (Figures 1E and F). The number of adherence of ETEC, EPEC and clostridium difficile to intestinal epithelial cells with adhesin was less than that of without adhesin. Inhibition of adherence of ETEC, EPEC and *Clostridium difficile* to intestinal epithelial cell by purified adhesin of B. ado 1027 was not significantly different between adhesin (30 μg/mL) and bacteria+ *B. ado* 1027 group (1×10^8/mL).

Table 1 Effect of purified adhesin of B. ado 1027 on adhesion of ETEC, EPEC and *C. difficile* to intestinal epithelial cell line Lovo

Group	Bacteria / cell		
	ETEC	EPEC	*C.difficile*
Adhesin (0 μg / mL)	108.83±16.34	110.27±13.12	110.00±16.91
Adhesin (1 μg / mL)	106.00±14.46	107.77±13.28	108.07±14.54
Adhesin (5 μg / mL)	102.97±7.65	104.20±10.82	104.23±9.62
Adhesin (10 μg / mL)	81.37±7.18[b]	81.73±9.01[b]	78.83±6.23[b]
Adhesin (20 μg / mL)	41.23±7.13[b]	42.37±7.54[b]	38.77±7.82[b]
Adhesin (30 μg / mL)	11.97±4.42[b]	11.33±4.35[b]	11.40±4.20[b]
B. ado 1027	10.87±3.22	10.07±3.32	11.73±3.66

[b]$P<0.01$ *vs* adhesin (0 μg / mL).

Table 2 Mean fluorescent intensity (MFI) of human intestinal epithelial cell with adherent ETEC, EPEC and *C. difficile*

Group	MFI		
	ETEC	EPEC	*C.difficile*
Adhesin (0 μg / mL)	5.76±0.43	5.26±0.29	5.14±0.21
Adhesin (1 μg / mL)	5.55±0.44	5.06±0.32	5.06±0.14
Adhesin (5 μg / mL)	5.10±0.24	4.83±0.28	4.84±0.15
Adhesin (10 μg / mL)	3.91±0.20[b]	3.67±0.35[b]	3.25±0.32[b]
Adhesin (20 μg / mL)	2.23±0.43[b]	2.21±0.32[b]	2.19±0.26[b]
Adhesin (30 μg / mL)	1.41±0.23[b]	1.25±0.18[b]	0.89±0.14[b]
B. ado 1027	1.25±0.23	0.98±0.13	0.79±0.19

[b]$P<0.01$ *vs* adhesin (0 μg / mL) group

Flow cytometric analysis of bacteria adherence to intestinal epithelial cell

ETEC, EPEC and a toxin-producing C. difficile strain (VPI10463) were assessed for their ability to adhere to human intestinal epithelial cell line Lovo (Table.2). There was no significant difference in MFI of ETEC, EPEC or *C. difficile* when the concentrations of adhesin were 1 μg/mL and 5 μg/mL. However, MFI was significantly decreased when the concentrations of adhesin were 10 μg/mL, 20 μg/mL and 30 μg/mL ($P<0.05$, $P<0.01$). MFI was decreased with increase in the concentration of adhesin. There was no significant difference in MFI between adhesin (30 μg/mL) and bacteria+ *B. ado* 1027 group (1×10^8/mL).

DISCUSSION

Adherence of pathogenic to the intestinal mucus is regarded a prerequisite for prolonged transient colonization and infection of the gastrointestinal tract, and plays an important role in invasion and in yileding and secretion of virulence factor[25]. Bifidobacteria, the predominant bacteria in the human intestinal microflora, are considered to be microorganisms with a great influence on human health[26]. There have been many studies demonstrating that strains of bifidobacteria have anti-infectious properties against enteropathogenic bacteria[18,27-29]. However, there existed a few flaws when live bifidobacteria agents was used in gut barrier dysfunction: live bifidobacteria was difficult to produce and preserve, and also difficult to breed sufficiently due to lack of local gas and growth substrate. Bernet *et al*.[18] and Zheng *et al*.[30] reported the occurrence of bifidobacteria adhering to the human intestinal cells by a mechanism of adhesion which involves a proteinaceous component. Fujiwara *et al*.[31] reported that SBT2928 produced a proteinaceous factor, binding inhibitory factor (BIF), which prevented the binding of the ETEC to GA1 *in vitro*, and the binding of ETEC to the human intestinal epithelial cell line HCT-8 was reduced by BIF treatment in a dose-dependent manner. These results showed there was adhesin component in bifidobacteria.

Adherence assay which can Gram stain and microscopically examine bacteria to human intestinal epithelial has been accepted widely for quantitative and direct visual. However, light microscopic methods, while useful, are tedious and time-consuming and may be prone to observer error. Flow cytometry allows analysis of cell populations by virtue of their physical characteristics and has been used previously to assess the adherence of *Helicobacter pylori*[32]. With this approach, it is possible to distinguish differences in cell populations based on changes in fluorescent intensities for test and control populations. When compared with conventional microscopy, it is also possible to examine much larger numbers of cells in a shorter time period[33]. The present study used Gram stain and flow cytometry to demonstrate competitive inhibition of adherence of ETEC, EPEC and *C. difficile* to intestinal epithelial cell line Lovo by purified adhesin of B. ado 1027. Our study showed that the purified adhesin at the concentration of 10 μg/mL, 20 μg/mL and 30 μg/mL except at 1 μg/mL and 5 μg/mL could significantly inhibit the adhesion of ETEC, EPEC and *C. difficile* to intestinal epithelial cell line Lovo. Moreover, we observed that the ETEC, EPEC and *C. difficile* adhesion were reduced by adhesin treatment in a dose-dependent manner. Inhibition of the binding of ETEC, EPEC and *C. difficile* to intestinal epithelial cell was not significantly different between adhesin (30 μg/mL) and bacteria+ B. ado 1027 group. The results of flow cytometry analysis of bacteria adherence to intestinal epithelial cell also confirmed it. MFI was decreased with increase in the concentration of adhesin. These results indicated that purified adhesin of B. ado 1027 was involved in the adhesion of bacteria. Two basic factors, receptor and adhesin, are required for classical adhesion. One of the mechanisms by which probiotic bacteria can protect epithelial is receptor competition[34]. Our results suggest that adhesin functions by blocking the binding site of ETEC, EPEC and *C. difficile* to intestinal epithelial cell. Adhesin produced by bifidobacterial may play an important role in protecting the host. Future studies need to carry out a survey of the resistance of adhesin against the activities of digestive enzymes in the gastrointestinal tract of human. In addition, it is important to determine whether all strains or all species of bifidobacteria produce adhesin or a similar protein(s)

which prevents the binding of pathogens to receptor in intestinal epithelial surfaces.

We also observed that the adhesive behaviors of bacteria to intestinal epithelial cell line Lovo were different. The adhesion of ETEC and EPEC to intestinal epithelial cell was linear, whereas the adhesion of *C. difficile* to intestinal epithelial cell was like cluster. This suggests that different bacteria may have different distribution of receptor on cell surface.

In conclusion, the purified adhesin of *B. ado* 1027 can effectively inhibit adherence of ETEC, EPEC and C. difficile to intestinal epithelial cell *in vitro*. However, whether adhesin could prevent diseases related with ETEC, EPEC and *C. difficile*, still needs to be proved *in vivo* by human clinical studies.

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