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Effects of traditional medicines on the adherence of intestinal bacteria to rat enterocytes

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Abstract

The adherence of *Bifidobacterium adolescentis* and pathogenic *Escherichia coli* to the rat enterocytes and the effect of traditional medicines on their adherent functions were investigated. Experimental results showed that both bacteria were able to adhere to rat enterocytes where *E. coli* was more effective than *B. adolescentis*. However, when they were incubated with the enterocytes taken from the animals which were administered a compound prescription of traditional medicine (Shikunshi-to, Si-Jun-Zi-Tang), the adherent pattern was inverted : the adherence of *B. adolescentis* increased and that of the pathogenic *E. coli* decreased to a certain degree. This suggests that the prescription enhanced the adherence of *B. adolescentis*, an important member of the normal intestinal flora.

Key words bacterial adherence, intestinal microflora, Shikunshi-to (Si-Jun-Zi-Tang), rat enterocyte.

Introduction

Bacterial adherence to mucosal epithelial cells has been recognized as a common phenomenon and is generally assumed to be a prerequisite for successful initial colonization.[1,2] There is evidence that the adherence of enterotoxigenic *Escherichia coli* to the mucosa of the small intestine is an inevitable early event in its colonization.[3] The colonization is necessary for the microorganism to multiply there and produces sufficient quantities of enterotoxin to cause disease.[4] Early studies have revealed that *E. coli* is suppressed by the obligatory anaerobic bacteria which dominate the intestinal microflora.[5,6] Many experiments revealed that the anaerobic flora in humans (such as *Bifidobacterium*) and in animals are closely adhered or bound to the epithelium or mucous membrane of the intestine. Thus, one of the mechanisms of bacterial antagonism may be ascribed to competitive adherence. The aim of the present study is to explore the effects of traditional medicines on the adherence of *Bifidobacterium* and *E. coli*. The results are expected to reveal the possibility that traditional medicines can influence the bacterial adherence and the antagonistic activities beween normal intestinal flora and the pathogenic bacteria.

In the study of bacterial adherence and the effects of various factors on it, both *in vitro*, and *in vivo* assay methods have been developed.[7] An *in vitro* assay method using rat enterocytes as the model was employed in this study.

Materials and Methods

Animals : Female Wistar rats of seven weeks old (SLC), maintaned on CLEA CE-2 chow, were used throughout the experiments.

Bacteria and culture : *Bifidobacterium adolescentis* (JCM strain 1275) was used as a representa-

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tive of the normal flora. The strain was maintained in this laboratory on GAM blood agar (Nissui) at 4°C. The enterotoxigenic *Escherichia coli* (Toyama *E. coli* 896, serotype 06, colonization factor antigen II, heat labile- and stable toxin positive, a clinical isolate from the patient with travelers' diarrhea) was kindly provided by Toyama Institute of Health. The strain was maintained on trypticase soy agar (BBL) slant. These organisms were grown in broths overnight (*E. coli* in trypticase soy broth, *B. adolescentis* in GAM broth) aerobically and anaerobically respectively at 37°C. For adherence assay, the broth cultures were centrifuged at 10,000×g for 10 min, and the bacterial cells were washed twice with phosphate-buffered saline (PBS, pH 7.0). The resulting suspension was diluted and followed by measuring turbidity with a spectrophotometer (Shimadzu, UV-160). The number of the bacteria in the suspension was adjusted to 1×10^8 colony forming units (CFU)/ml with PBS according to the result of the preliminary experiments.

Preparation of the rat enterocytes: Rat enterocytes were isolated by EDTA chelating method as described by Weiser.[8] In brief, rat small intestine was excised and trimmed of fat and mesentery. The intestine segments were longitudinally split open and treated repeatedly with the chelating agent at 37°C. The dispersed enterocytes were centrifuged at 900 × g for 10 min, and washed twice with PBS. The resulting cell pellet was suspended in PBS and the cell number was measured microscopically with a hemocytometer to adjust the final suspension to contain 1.0×10^5 cells per ml in PBS.

Adherence assay method: Adherence assay was performed by mixing equal volumes of enterocyte and bacterial suspensions in a ratio of approximately 1 cell to 1,000 bacteria and the resulting mixture was placed in an ice bath. An aliquot of the mixture was filtered through a polycarbonate membrane filter (pore size 5 μm, Millipore). The filtrate was kept in an ice bath. The remaining mixture was then incubated with gentle agitation for 1 h at room temperature. After incubation the mixture was filtered again as above. The filtration procedure effectively separated the cell bound bacteria from free bacteria. The two filtrates were diluted serially and inoculated respectively onto agar plates. *E. coli* was inoculated on eosin-methylene blue agar (EMB) and *B. adolescentis* on BL blood agar (Nissui). The inoculated plated were incubated and the bacterial colonies from the filtrate before (B) and after (A) incubation were counted. The result of the bacterial adherence was expressed as the adherence rate, i.e., the proportion of the bacterial number being adhered to enterocytes to the bacterial number before incubation: $(B-A)\times100/B$.

After each filtration, the cell surface of the filter membrane was stuck on a glass slide so the cell could adhere on the slide. After allowing it to air dry, the slides were stained with hematoxylin-eosin and examined microscopically under oil immersion (×1,000).[9]

Experiment on antagonism of the two bacteria: For antagonistic study, the broth cultures of the two bacteria were mixed. The mixture was then added to the rat enterocyte suspension. After incubation, the filtrate which contained both *B. adolescentis* and *E. coli* was inoculated on BL blood agar and EMB agar plates simultaneously. The bacterial colonies were counted after cultivation.

Treatment of animals with the prescription: The prescription used for administering animals was Shikunshi-to (Si-Jun-Zi-Tang, granules, Tsumura Co.) which is believed to have the functions of improving the digestive conditions in the traditional theory. According to the dosage that the manufacturer formulated, each rat (average body weight 170 g) was given 0.1 g of the preparation per day which is analogous to five times the dosage of a human adult (supposing the average body weight of an adult to be 65 kg). The drug suspension was prepared by suspending the granules in distilled water and administered orally twice a day for seven days.

Decontamination of the rats: The decontamination was carried out by administering the animals with an antibiotic mixture orally for three days to eliminate all the bacteria in the intestine. The mixture is composed of chloramphenicol(8.75 mg/ml), mycostatin (16.75 mg/ml), streptomycin

(10 mg/ml), penicillin G (0.61 mg/ml), and erythromycin (5 mg/ml).

Results

When *B. adolescentis* and *E. coli* suspensions were incubated individually with rat enterocytes *in vitro* for one hour, both two strains of the test organisms were capable of adhering to the enterocyte preparations from normal animals. *E. coli* adhered more intensely than *B. adolescentis* did.

Table I Effect of Shikunshi-to treatment on the adherence of *B. adolescentis* and *E. coli* to the enterocytes.

	Enterocytes	
	Untreated (%)	Treated (%)
B. adolescentis	34.9±15.7 [9]*	44.3± 9.7 [4]
E. coli	45.7±15.3 [13]	21.9±13.3 [4]

*The figures in the parenthesis denote the numbers of experiments.

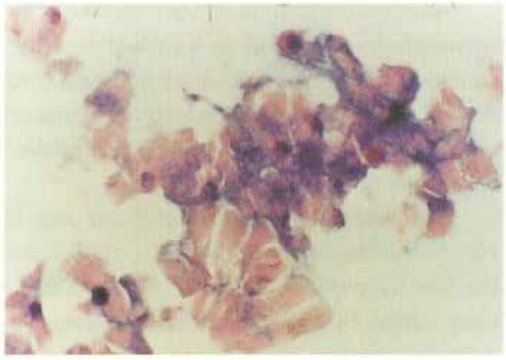

Fig. 1-A

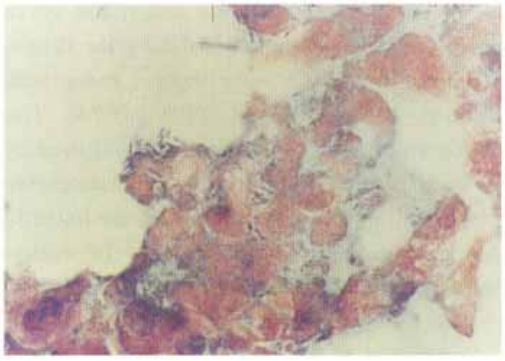

Fig. 1-B

Fig. 1-A Rat enterocytes before incubation with *B. adolescentis*.
1-B *B.adolescentis* adhered on the rat enterocytes after incubation.

The adherence rate of *E. coli* was 45.7±15.3 % (mean±S.D.), and that of *B. adolescentis* was 34.9 ±15.7 % as shown in Table I. This showed that the adherence activity of *E. coli* is somewhat stronger than that of *B.adolescentis,* but the difference is not significant statistically. The adherence of the bacteria was confirmed by microscopic observation in which the bacteria were adhered to the cell membranes of the enterocytes (Fig. 1-A and 1-B).

In the experiment for observing the antagonism between these two bacteria, both suspensions were mixed so as to contain an equal number of bacteria, i.e., the CFU each was 1.0×10^8/ml. The mixture was added to the enterocyte suspension prepared from normal animals and incubated. The result of the antagonistic experiment showed that *B. adolescentis* was able to adhere enterocyte more intensely than *E. coli*: their adherence rates were 41.4±8.6 % and 27.3± 11.2 %, respectively.

In the experiments for determining the effects of traditional medicines on bacterial adherence, the enterocytes were prepared from the rats which were administered with Shikunshi-to for seven days. To the enterocytes obtained from the treated animals, the adherence rates of the two bacteria were changed, i.e., *B. adolescentis* was superior to *E. coli.* The rates of them were 44.3± 9.7 % for *B. adolescentis* and 21.9±13.3 % for *E. coli* (Table I). The difference is statistically significant ($p<0.05$). This suggests that the prescription enhances 1.3 fold the adherence of *B. adolescentis,* whereas the adherence of the pathogenic *E. coli* to the enterocytes decreased approximately 50 % ($p<0.05$).

There was no difference in the adherence rate of *E. coli* to the enterocytes from normal animals and from antibiotic treated animals.

Discussion

The present results clearly demonstrated that both *Bifidobacterium adolescentis* and *Escherichia coli* can adhere to the enterocytes, thus providing evidence that adherence is a means of bacteria for possession of existence niches no matter if the bacteria is pathogenic or non-pathogenic.

The influence of traditional medicines on intestinal bacteria has been studied in a few works. It has been shown that some of the traditional herbal medicines could change the amounts and relative proportions of various bacterial species to a certain extent.[10] In an *in vivo* study on the effects of Shikunshi-to (Si-Jun-Zi-Tang), the imbalance state of the normal intestinal flora of mice, induced by rhubarb decoction, could be restored to normal conditions by administering the decoction of the prescription. The decreased counts of *Bifidobacterium* and *Lactobacillus* could be increased to a normal level.[11] The present study provided further information that the bacterial adherence may also be affected, and the effect is beneficial to one of the normal intestinal bacteria. At present, we can not explain the reasons how this comes into effect.

A preliminary experiment designed to test the competitive adherence of these two bacteria was conducted. Although the result was encouraging, more experiments remain to be done in order to reach a conclusion.

Compared with the colon, the small intestines of man and animals are less colonized by the bacterial flora.[12] It is well known that there are specific receptors on the epithelial cell surfaces of the intestine. Because the receptors of the small intestine are not fully bounded, it can accept more bacteria to adhere. Perhaps this may be the reason why the decontamination treatment of the rat intestine did not affect the adherence rate of the test microorganisms.

It is important to note that present study might not reflect the interrelation between the two bacteria as they normally occur in the intestinal tract, and moreover. a single bacterial species is hardly representative of the 400 to 500 different bacterial species that normally inhabit the intestinal tract. Nevertheless, the findings presented in this report actually demonstrated what happened between bacteria and showed the effect of traditional medicines on bacterial adherence *in vivo*.

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和文抄録

Bifidobacterium adolescentis と病原性 *Escherichia coli* のラット腸粘膜細胞に対する付着性と漢方方剤の付着に及ぼす影響について検討した。その結果，この二菌種とも腸粘膜細胞に付着したが，特に *E. coli* のほうが *B. adolescentis* より強い傾向を示した。漢方方剤である四君子湯をあらかじめ投与したラットより調製した腸粘膜細胞を用いた時，これら二菌種の付着パターンに変化が認められた。すなわち，四君子湯の投与により *B. adolescentis* の付着力は促進され，病原性 *E. coli* の付着力は抑制された。四君子湯は正常な菌叢の重要な一員である *B. adolescentis* の腸粘膜への付着性を高めることを示唆した。

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