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Lactobacillus acidophilus Inhibits the *Helicobacter pylori* Adherence

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Abstract *Lactobacillus acidophilus* is known to have an inhibitory activity on growth of *Helicobacter pylori* and this activity has been attributed to lactic acid and antibacterial agents produced by *Lactobacillus*. Since every *Lactobacilli* produces lactic acid, an another factor must exist for *L. acidophilus* to inhibit *H. pylori* growth. In this work, the inhibitory activity of *L. acidophilus* on *H. pylori* adherence was studied. An immunoabsorbent assay using TLC plate was developed and used for screening the inhibitory activity of various *Lactobacilli* on *H. pylori* adherence. Glycolipid, the attachment site for *H. pylori*, was isolated from blood type O red blood cells and spotted on a TLC plate. The *H. pylori* adherence increased linearly with increasing amounts of glycolipid spotted on the TLC plate. Various *L. acidophilus* strains, but not *L. casei*, appeared to inhibit *H. pylori* adherence to glycolipid, and the adherence decreased linearly as the concentration of the *Lactobacillus* increased. The results show that the inhibitory activity of *L. acidophilus* on *H. pylori* adherence is an another factor for *L. acidophilus* to inhibit *H. pylori* growth.

Key words: *Helicobacter pylori*, *Lactobacillus acidophilus*, glycolipid, TLC, adherence, enzyme-linked immunoabsorbent assay

Helicobacter spp. are common inhabitants of gastrointestinal tract of both humans and animals [17]. The type species of the genus, *Helicobacter pylori*, causes chronic gastritis and peptic ulcers in humans and has recently been linked to the development of gastric adenocarcinoma and gastric mucosa-associated lymphoma [6].

It was recently demonstrated that probiotic *Lactobacilli* can exhibit an antagonistic activity against human pathogens including *H. pylori*. *Lactobacilli* can inhibit the growth of *H. pylori in vitro* and exhibit antagonistic activity against *H. pylori in vivo* [5]. For example, *L. salivarius* has been suggested as a probiotic for *H. pylori* because of its ability to produce large amounts of lactic acid. The validity of *L. salivarius* as a probiotic to suppress *H. pylori* growth and thus reduce the inflammatory response was confirmed *in vivo* by using an *H. pylori*-infected gnotobiotic murine model [1]. *L. salivarius* given after *H. pylori* implantation could eliminate colonization by *H pylori* [9]. In addition to these results, *Lactobacillus* has been reported to reduce reinfection of *H. pylori* when patients underwent gastroscopy with biopsy and took acidophilus milk containing live cells of *L. acidophilus* [5]. The spent culture supernatant (SCS) of *L. acidophilus* also dramatically decreased the viability of *H. pylori in vitro* independent of the pH and lactic acid levels, protected mice against an *H. felis* infection, and inhibited the urease activity of *H. pylori* and *H. felis*. Furthermore, with human volunteers, the SCS of the human *L. acidopilus* strain LA1 was active against *H. pylori*. This result suggests a possibility of *Lactobacillus* as a probiotic agent against *H. pylori* in humans too. Besides producing lactic acid and antibacterial agents [15], there may be an another factor in *Lactobacillus* that inhibits *H. pylori*, including inhibition of *H. pylori* adherence to gastric mucosa.

In the present study, an enzyme-linked immunoabsorbent assay method was developed using a TLC plate spotted with glycolipid, which is a receptor for *H. pylori* and is expressed on both gastric mucosa and O-type red blood cells (RBC) [2, 3, 8, 11, 12]. Using this method. several Korean strains of *L. acidophilus* and *L. casei* were tested for their inhibitory activity on the attachment of *H. pylori*.

Materials and Methods

Bacteria and Reagents

H. pylori ATCC43504 was grown for 48 h on a Brucella solid medium [Brucella broth, fungizone (2.5 g/ml amphotericin B), and Skirrow's supplement (0.016 mg/ml polymyxin B, 0.5 mg/ml vancomycin, 0.25 mg/ml trimethoprim)] supplemented with 10% horse serum under

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5-10% CO_2. Cells were collected by scraping, washed twice with phosphate-buffered saline (PBS, pH 7.4), and then kept at -20°C until use. *L. acidophilus* HY0404, HY2104, HY7001, and HY7007, and *L. casei* HY2782 were provided by Korea Yakult Co. Ltd. The *Lactobacillus* was grown in MRS broth, collected by centrifugation, washed with PBS, and then kept at -20°C until use. The media were purchased from Difco (Detroit, MICH, U.S.A.) and the TLC plates were purchased from Merck (Kieselgel 60, EM Separations, Gibbstown, NJ, U.S.A.). Other reagents including a second antibody conjugated with alkaline phosphatase, amphotericin B, and Skirrow's supplement were purchased from Sigma (St. Louis, MO, U.S.A.).

Preparation of Antiserum Against *H. pylori*

H. pylori cells were fixed with formaldehyde and injected into rabbits (New Zealand White Rabbit, male) with two boosts. Blood was obtained by bleeding from ears. The serum was then divided into aliquots and stored at -20°C.

Glycolipid Isolation from RBCs

Glycolipid was isolated from human O-type red blood cells (RBCs), as described elsewhere with a slight modification [3]. The human O-type RBCs were dispersed in a minimum volume of water and extracted with 20 volumes of a chloroform-methanol mixture (2:1, v/v). The lipid layer (the lower phase) was collected and dried in a rotary vacuum evaporator. The residue was dissolved in chloroform containing 2% methanol and loaded on a column (bed volume = 20 ml) which had been equilibrated with silicic acid. The column was then eluted with single volumes of chloroform, acetone-methanol (3:1, v/v), and methanol. The resulting methanol fraction was then dried in a rotary vacuum evaporator, dissolved in a minimum volume of methanol, and finally stored at -70°C.

Assay of *Helicobacter* Attachment to TLC plate

The extracted glycolipid (200 ng in 5 μl) was spotted on a thin layer chromatography (TLC) plate. The plate was soaked in 100 mM Tris (pH 7.6) containing 3% gelatin for 2 h at 37°C to prevent unspecific binding. The plate was then rinsed twice with the same buffer and incubated in a 10 ml buffer containing *Lactobacillus* (2.4×10^8 CFU) for 2 h at 37°C. After being washed 3 times with the same buffer, the plate was transferred to a 10 ml buffer containing *H. pylori* (7.5×10^8 CFU) and gently agitated at 37°C. After 2 h, the plate was rinsed 3 times with the same buffer for 10 min each time, then the rabbit antiserum raised against *H. pylori* was added to the buffer (1:600) and further incubated for 2 h at room temperature with gentle shaking. After the plate was washed to remove the first antibody, the second antibody conjugated with alkaline phosphatase was added to the reaction solution (1:1,000) and the plate was incubated for 1 h at room temperature. A chromogenic reaction was performed by adding 5-bromo-4-chloro-3-indolyl phosphate disodium salt/nitro blue tetrazolium chloride (BCIP/NBT).

Assay of Color Developed on TLC Plate

The silica in the colored region was scraped off the TLC plate with a razor blade and dispersed in 2% SDS. The color was extracted from the resin by boiling in a boiling water bath. The intensity was recorded at 405 nm by a spectrophotometer.

RESULTS AND DISCUSSION

Adherence is a prerequisite for the microbial colonization of epithelial surfaces and is mediated by molecules on the bacterial surface adhesins that recognize proteins or glycoconjugates on the surface of the eukaryotic cells [2, 10, 13, 17]. The best way to assay *H. pylori* attachment to gastric mucosa may be to use a gastric segment which has been developed to assay the antimicrobial activity of chemicals on *H. pylori* [14]. However, it is impossible to obtain human gastric mucosa and even swine stomachs are difficult to procure. Such gastric segments also need to be prepared freshly for each experiment which requires an expensive culture medium and takes several days to produce a result.

To develop an easier assay method, we attempted to use glycolipid isolated from blood type O RBCs and then test it to assay *H. pylori* adherence. The attachment site of *H. pylori* is generally recognized as the Lewis antigen B, expressed on the surface of the RBCs of blood type O [4, 7, 8, 11, 12], even though there is a contrary report on the effect of expression of the ABO blood group antigen on the attachment of *H. pylori* [16].

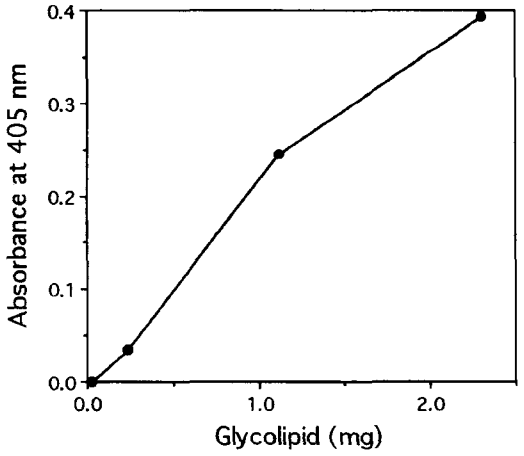

Fig. 1. *Helicobacter* attachment to glycolipid spotted on a TLC plate.
A TLC plate spotted with glycolipid was incubated with 7.5×10^7 CFU/ml of *H. pylori*. Attached *H. pylori* was detected as described in Materials and Methods.

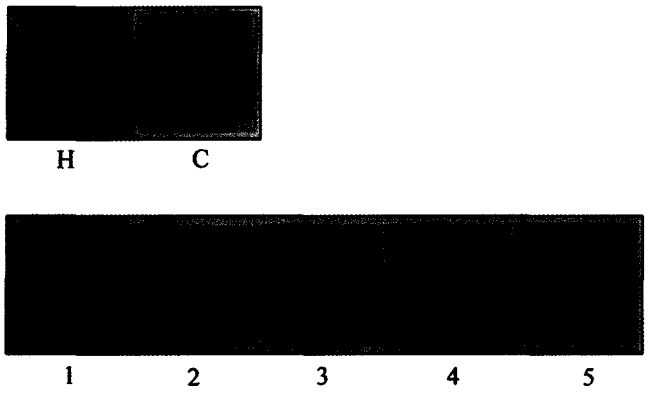

Fig. 2. *Helicobacter* attachment to glycolipid in the presence of various strains of *L. acidophilus*.
TLC plates spotted with glycolipid were incubated in the presence of *L. acidophilus* (2.4×10^8 CFU/10 ml) and the resulting plates were incubated with *H. pylori* (7.5×10^8 CFU/10 ml). The amount of attached *H. pylori* was measured as described in Materials and Methods. TLC plates were incubated with *H. pylori* (H), *L. acidophilus* HY0404 (C), *H. pylori* and *L. acidophilus* HY0404 (1), *H. pylori* and *L. acidophilus* HY2104 (2), *H. pylori* and *L. acidophilus* HY7001 (3), *H. pylori* and *L. acidophilus* HY7007 (4), or *H. pylori* and *L. casei* HY2782 (5).

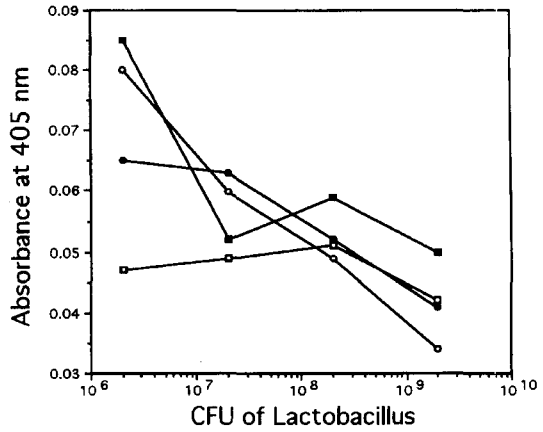

Fig. 3. *Helicobacter* attachment in the presence of various concentrations of *L. acidophilus*.
A TLC plate was spotted with glycolipid and incubated with various cell concentrations of *L. acidophilus*. The resulting plate was then incubated with *H. pylori* (7.5×10^7 CFU/ml). The amount of *H. pylori* attached to the TLC plate was assayed as described in Materials and Methods. The experiment was done in triplicate. Symbols used: ●, *L. acidophilus* HY0404; ○, *L. acidophilus* HY2104; ■, *L. acidophilus* HY7001; □, *L. acidophilus* HY7007.

When different amounts of glycolipid were spotted onto a TLC plate, the amounts of bound *H. pylori* cells increased linearly as the amount of spotted gycolipid increased (Fig. 1). Since a large volume of glycolipid produced a dispersed spot, 5 μl was used as the amount in the following experiments. When various *Lactobacilli* competed with *H. pylori* for glycolipid, all the *L. acidophilus* strains, except for *L. casei,* showed very good inhibitory activities (Fig. 2). The *H. pylori* adherence was used as the positive control, whereas glycolipid with only *Lactobacillus* was used as the negative control. When various strains of *L. acidophilus* were serially diluted and used in competition with *H. pylori,* the increase in the number of *Lactobacillus* caused a decrease in *H. pylori* adherence (Fig. 3). This result shows that *Lactobacillus* produces a real inhibitory activity on *H. pylori* adherence and the different inhibitory activities on the *H. pylori* growth of various *Lactobacilli* [5] might be partially due to the different inhibitory activities on the *H. pylori* adherence. *L. acidophilus* seemed to be a possible probiotic against *H. pylori*, because of its lactic acid producing activity and inhibitory activity on the *H. pylori* adherence.

As the results indicate, this immunoabsorbent assay is a better and easier method to screen a large number of *Lactobacilli* than a binding assay using a swine gastric segment, for the following reasons: (1) The glycolipid required for this method can be prepared immediately and kept for a long period, whereas fresh swine stomachs and especially human stomachs, are difficult to obtain for each experiment: (2) *H. pylori* and *Lactobacillus* do not have to be kept alive for this experiment since *L. acidophilus* kept in a freezer maintained its inhibitory activity. This suggests that frozen or freeze-dried *L. acidophilus* can be used as a probiotic for *H. pylori.*

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