BACTERIAL PATHOGENICITY

Studies of the effect of *Clostridium butyricum* on *Helicobacter pylori* in several test models including gnotobiotic mice

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The interaction between *Clostridium butyricum* and *Helicobacter pylori* was examined *in vitro* and *in vivo*. The culture supernate of *C. butyricum* MIYAIRI 588 inhibited the growth of *H. pylori* even when its pH was adjusted to 7.4. The bactericidal effect of butyric acid on *H. pylori* was stronger than that of lactic, acetic or hydrochloric acids. Flow cytometric analysis showed that pre-incubation of gastric epithelial (MKN45) cells with *H. pylori* and *C. butyricum* inhibited the adhesion of *H. pylori* to the cells. Persistent infection with *H. pylori* in the gastric mucosa of germ-free mice was observed for 5 weeks. Cure of persistent infection with *H. pylori* in the gnotobiotic mice was demonstrated following infection with *C. butyricum*. The probiotic agent, *C. butyricum* MIYAIRI 588 may have some beneficial effects on *H. pylori* infection.

Introduction

The isolation of *Helicobacter pylori* in 1984 by Marshall and Warren [1] ushered in a new era in gastric microbiology. Although spiral organisms had been observed in the gastric mucosal layer many times in the preceeding century, the isolation of *H. pylori*, in conjunction with an increased interest in the pathogenesis of gastroduodenal diseases, has led to important breakthroughs in medical care [2]. Many investigators have reported that eradication of *H. pylori* by antibiotics and a proton pump inhibitor resulted in the cure of chronic gastritis and the decreased recurrence of peptic ulcer diseases. However, eradication therapy induces side-effects, especially antibiotic-associated diarrhoea [3], and the appearance of antibiotic-resistant *H. pylori* strains [2].

Clostridium butyricum is a butyric acid-producing gram-positive anaerobe which is found in the soil and in the intestines of healthy animals and people. The MIYAIRI 588 strain of *C. butyricum* was isolated from soil and its phenotypic characteristics have been

described previously [4]. The strain has been used as a probiotic for treating and preventing non-antimicrobial-induced diarrhoea as well as antimicrobial-associated diarrhoea in man and animals [5–7]. The mechanism by which *C. butyricum* controls diarrhoea is based on several properties. For example, *C. butyricum* has been shown to have an antagonistic interaction against *Candida albicans*, *C. difficile*, enterotoxigenic *Escherichia coli*, *Klebsiella* spp., *Salmonella* spp. and *Vibrio* spp. [5–7]. Butyric acid also has a proliferative effect on mucosal cells in the intestine [8], suggesting that butyric acid may have a therapeutic effect on inflammatory bowel disease. The purpose of this study was to examine the interaction between *H. pylori* and *C. butyricum* in several test models, including gnotobiotic mice.

Materials and methods

Mice

Germ-free mice (IQR/jic strain, 8 weeks old, female) were obtained from Japan Clea (Tokyo, Japan). The animals were housed in a sterilised vinyl isolator and fed a sterilised diet and water *ad libitum*.

Micro-organisms and culture conditions

Standard strains of *H. pylori* (ATCC 43526 and NCTC 11637) and three clinical strains (TK1029, TK1401 and

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TK1402) were used [9]. Strain TK1029 was isolated from a patient with a gastric ulcer and a submucosal tumour. Strains TK1401 and TK1402 were isolated from patients with gastroduodenal ulcers. *H. mustelae* strain NCTC 12032 was also used in this study. These strains were cultured under micro-aerobic conditions (O₂ 5%, CO₂ 10%, N₂ 85%) at 37°C on Brain Heart Infusion Agar (Difco) containing horse blood 7% (B-BHI) for 3 days as described previously [10]. The probiotic agent, *C. butyricum* strain MIYAIRI 588 used in this study was obtained from Miyarisan Pharmaceutical Co. Ltd, Tokyo, Japan. This strain was isolated from soil and its biochemical characteristics have been described elsewhere [4–7]. BL (blood) agar (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) was used for routine culture of *C. butyricum*.

Effect of *C. butyricum* supernate on the growth of *H. pylori* in vitro

After anaerobic incubation of *C. butyricum* at 37°C for 12 h in BHI broth, the supernate of a stationary phase culture was harvested after centrifugation at 6000 rpm for 20 min and used to examine its effect on the growth of *H. pylori*. In some experiments, the pH (natural pH 4.8) of the culture supernate was adjusted to 7.2 with 1 N NaOH. The growth of *H. pylori* was examined under micro-aerobic cultivation in BHI broth supplemented with fetal calf serum 10% (F-BHI) with or without the addition of *C. butyricum* supernate at final concentrations of 25, 50 and 100%. The quantification of *H. pylori* was performed by colony counting on B-BHI agar.

Effect of butyric, acetic and lactic acids on growth of *H. pylori* in vitro

Various concentrations (50 mM, 12.5 mM and 3.125 mM) of butyric, acetic and lactic acids were used to examine their effect on the growth of *H. pylori*. *H. pylori* strains (10⁶ cfu/ml) were inoculated in to the acids, and the numbers of bacteria were counted 5, 30, 60, 120 and 180 min after inoculation.

Effect of *C. butyricum* on adhesion of *H. pylori* to MKN45 cells

An adhesion assay was performed by flow cytometric analysis as described previously [9, 11]. *H. pylori* or *C. butyricum* cells were suspended in 500 µl of labelling buffer (Zynaxis Cell Sciences, Phoenixville, USA). Fifty µl of the suspension were mixed with 500 µl of the labelling buffer, then 8 µl of PKH-2 lipophilic dye (Zynaxis Cell Sciences) for *H. pylori* or PKH-26 lipophilic dye (Zynaxis Cell Sciences) for *C. butyricum* were added. After incubation at room temperature for 10 min, the reaction was stopped immediately by adding 5 ml of PBS containing bovine serum albumin 0.5% (PBS-BSA). The labelled micro-organisms were washed once with PBS-BSA and suspended in 3 ml of Hanks's Balanced Salts Solution (HBSS) containing gelatin 0.1% (HGS). MKN45 cells (2.5 × 10⁶ cells) were pre-incubated with the labelled *C. butyricum* (10⁷ or 10⁹ cfu) at 37°C for 1 h and then further incubated with the labelled *H. pylori* (2.5 × 10⁸ cfu) at 37°C for 1 h with gentle shaking. Non-adherent bacteria were removed by centrifugation at 1000 rpm for 10 min with 9 ml of sucrose 15%

Fig. 1. Inhibitory effect of the supernate of *C. butyricum* on the growth of *H. pylori* TK1402 in vitro; —○—, BHI broth-10% FCS (pH 6.8); —△—, 25% *C. butyricum* culture supernate (pH 5.8); —□—, 25% supernate (pH 7.2); —●—, 50% supernate (pH 5.2); —▲—, 100% supernate (pH 7.2); —■—, 100% supernate (pH 4.8); ND, not detectable.

solution. The cells were washed four times with HGS, then resuspended in 300 μ l of HGS for flow cytometry. A flow cytometer (FACS Vantage, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) was used for the measurement of fluorescence intensity. Fluorescence data were obtained in a logarithmic mode on a 1024 channel scale.

Infection of germ-free mice with *H. pylori* and *C. butyricum*

H. pylori TK 1402, (2.5×10^8)–(1.0×10^9) cfu/mouse, was inoculated daily into 13 germ-free mice *per os* for 3 days as described by Kabir *et al.* [12]. One gnotobiotic mouse was killed 1 week after the inoculation. Gastric mucus (100 μ l) and duodenal

mucus (100 μ l) were harvested and suspended in 400 μ l of HBSS. Each sample was mixed and diluted with HBSS and cultured on BHI-blood agar at 37°C under micro-aerobic conditions [13]. *H. pylori* was identified by positive results for urease, oxidase and catalase tests and morphological examination by Gram's stain.

In the first experiment, *C. butyricum* spores (5×10^8 cfu/mouse) or vegetative cells (1×10^8 cfu/mouse) were inoculated daily *per os* for 3 days into gnotobiotic mice mono-associated with *H. pylori* for experimental eradication. *C. butyricum* was incubated anaerobically in PYG broth (peptone 1%, yeast extract 1%, glucose 1%) at 37°C for 6 h and a sample of the culture was used as a vegetative inoculum. The

Fig. 2. Inhibitory effect of butyric and lactic acids on *H. pylori* strains: (a), TK1029, (b) TK1401, (c) TK1402. --□--, lactic acid (pH 3.7); --○--, lactic acid (pH 7.2); —●—, butyric acid (pH 7.2); —■—, butyric acid (pH 4.2); ND, not detectable.

Fig. 3. Inhibitory effect of butyric, hydrochloric and lactic acids on the growth of (a) *H. pylori* ATCC43526, (b) *H. pylori* NCTC11637, (c) *H. mustellae* NCTC12032. —■—, 50 mM butyric acid (pH 4.2); —●—, 50 mM butyric acid (pH 7.2); —□—, 50 mM lactic acid (pH 3.7); —○—, 50 mM lactic acid (pH 7.2); —▲—, hydrochloric acid (pH 4.0); --△--, PBS (pH 7.2); ND, not detectable.

harvested culture (5 ml) was heated at 60°C for 10 min and centrifuged at 10 000 rpm for 10 min, and the pellet was resuspended in the same volume of PBS to prepare the *C. butyricum* spore inoculum. The mice were killed 24 h and 1, 2 and 3 weeks after the last inoculation with *C. butyricum*.

In the second experiment, the combined effect of *C. butyricum* spores and a proton pump inhibitor (PPI) was examined. All 36 germ-free mice were infected with *H. pylori* TK1402 as described above. *C. butyricum* spores (5×10^8 cfu/mouse) or PPI (lansoprazole, 0.6 mg/mouse, or both, ($n = 9$ in each group) were inoculated daily into the gnotobiotic mice mono-associated with *H. pylori per os* for 7 days for experimental eradication. Representative mice ($n = 3$ in each group) were killed immediately, or at 3 or 7 weeks after the last inoculation with *C. butyricum* or administration of PPI only.

Histopathological examination

Stomachs of the gnotobiotic mice mono-associated with *H. pylori* or associated with both *H. pylori* and *C. butyricum* were fixed with formalin 10%. Then the tissue was stained with haematoxylin and eosin and pathological changes were observed by light microscopy.

Results

Inhibitory effect of the culture supernate of *C. butyricum* on the growth of *H. pylori* in vitro

The effect of the supernate culture of the *C. butyricum* on the growth of *H. pylori* TK1402 was examined (Fig. 1). The supernate without pH adjustment had a strong inhibitory effect on *H. pylori* with no growth of *H. pylori* at 24 h. Even after pH adjustment to 7.2, the supernate of *C. butyricum* still had an inhibitory effect

on *H. pylori*. The addition of the culture supernate at final concentrations of 25% and 50% did not inhibit the growth of *H. pylori* significantly by 72 h. Complete inhibition was demonstrated at 144 h, although no significant growth inhibition was demonstrated at a final concentration of 25% of culture supernate adjusted to pH 7.2.

Inhibitory effect of butyric, acetic and lactic acids on the growth of *H. pylori* in vitro

The inhibitory effect of butyric and lactic acids was demonstrated *in vitro* (Fig. 2). The inhibitory effect of lactic acid was detected only when its pH was not adjusted. On the other hand, inhibition of the growth of *H. pylori* by butyric acid was demonstrated in a dose-dependent manner even when the pH was adjusted to 7.2, suggesting the butyric acid has an antibacterial property in addition to its acidity. The bactericidal activity of butyric acid on *H. pylori* was stronger than that of hydrochloric acid, and butyric acid also had an inhibitory effect on *H. mustellae* (Fig. 3).

Inhibitory effect of *C. butyricum* on the adhesion of *H. pylori*

The effect of *C. butyricum* on adhesion of *H. pylori* to human gastric cancer cells (MKN45) was examined by flow cytometry (Fig. 4). The mean fluorescence intensity of MKN45 cells with adherent pH2-labelled *H. pylori* was 157.2. After pre-incubation of MKN45 cells with 10^7 cfu and 10^9 cfu of *C. butyricum*, the mean fluorescence intensities of MKN45 cells with adherent *H. pylori* decreased to 74.4 and 43.5, respectively, showing an inhibition of adhesion of *H. pylori* to MKN45 cells by pre-incubation with *C. butyricum*.

Fig. 4. Effect of *C. butyricum* on the adhesion of *H. pylori* to MKN45 cells as determined by flow cytometry. ▨, MKN45 cells only; —, adhesion of *H. pylori* (10^8 cfu) to MKN45 cells; ···, adhesion of *H. pylori* (10^8 cfu) after incubation with *C. butyricum* (10^7 cfu); ---, adhesion of *H. pylori* (10^8 cfu) after incubation with *C. butyricum* (10^9 cfu).

Effect of C. butyricum on colonisation by H. pylori in germ free mice

The effect of *C. butyricum* on colonisation by *H. pylori* in germ-free mice was examined. Persistent infection with *C. butyricum* in the gastric mucosa was observed in gnotobiotic mice; 10^{6-7} cfu/g mucus were detected in the gastric mucosa 1–3 weeks after inoculation (data not shown). There was no significant difference in the number of *C. butyricum* recovered

between mice inoculated with vegetative and spore cells. After oral inoculation of *H. pylori* into germ-free mice, persistent infection of *H. pylori* was observed in 3 weeks (Table 1); 10^{4-5} cfu/g of mucus were detected from gastric mucosa. Bacteria were not isolated from the gastric mucus of uninfected germ-free mice (data not shown). Although a mild inflammatory change with infiltration of polymorphonuclear leucocytes was observed, no pathological change, such as ulceration or atrophy, was demonstrated (Fig. 5).

Fig. 5. Histopathological examination of stomachs of gnotobiotic mice (A) mono-associated with *H. pylori* and (B) di-associated with *H. pylori* and *C. butyricum*.

The effect of inoculation with *C. butyricum* on persistent infection with *H. pylori* in gnotobiotic mice was examined (Table 1). After inoculation with *C. butyricum* vegetative cells, a rapid decrease in the number of *H. pylori* in the gastric mucosa was detected. There were 3.5×10^2 cfu/g of mucus 1 week after the inoculation of *C. butyricum*. Similarly, inoculation of *C. butyricum* spores resulted in a significant decrease in the numbers of *H. pylori*, and infection with *H. pylori* was completely eradicated 3 weeks after inoculation with *C. butyricum*.

In the second experiment, treatment of *H. pylori* mono-associated gnotobiotic mice with a PPI (lansoprazole 0.6 mg/mouse daily for 1 week) was evaluated (Fig. 6). Administration of the PPI induced no inhibitory effect on colonisation by *H. pylori*. In contrast, when the gnotobiotic mice were treated with *C. butyricum* (10^8 cfu/mouse, daily, three times) with or without PPI, no *H. pylori* was isolated from the gastric mucosa

3 weeks after the treatment. However, low numbers (10^3 cfu/g of mucus) of *H. pylori* were recovered weeks after the gnotobiotic mice were treated with *C. butyricum* and PPI.

Discussion

H. pylori, aetiological agent of gastritis and peptic ulcer disease, may infect the gastric mucosa of over half the world's population [13–17]. It has been reported that the eradication of *H. pylori* reduces the recurrence of peptic ulcer diseases. *C. butyricum* MIYAIRI 588 has been used as a probiotic for both non-antibiotic-induced diarrhoea and antibiotic-associated diarrhoea in man. The results of the present study suggest that there is an antagonistic interaction between *H. pylori* and *C. butyricum* both *in vitro* and *in vivo*. As the culture supernate of *C. butyricum* completely inhibited the growth of *H. pylori*, it is possible that short-chain fatty acids such as butyric acid, acetic acid and lactic acid inhibit the growth of *H. pylori*. Bhatia *et al.* [18] reported inhibition of the growth of *H. pylori* by *Lactobacillus acidophilus* and concluded that the inhibitory effect was dependent on an extracellular secretory product, probably lactic acid. Similarly, Midolo *et al.* [19] reported inhibition of the growth of *H. pylori* by lactic, acetic and hydrochloric acids in a concentration-dependent manner, and showed that lactic acid demonstrated the greatest inhibition. Therefore, inhibition of the growth of *H. pylori* by culture supernate of *C. butyricum* is not specific for *C. butyricum*, and the antagonism presented in this study might be in part attributable to the acidic conditions produced. However, as the pH-adjusted culture supernate still had an inhibitory effect on the growth of *H. pylori*, this inhibition appears to be induced by not only a decreased pH, but also by other inhibitory factors produced by *C. butyricum*.

Table 1. Decrease in the number of *H. pylori* in gastric mucosa following inoculation of *C. butyricum* in gnotobiotic mice mono-associated with *H. pylori*

Mouse no.	Inoculum of <i>C. butyricum</i>	Days after <i>H. pylori</i> infection	Number of <i>H. pylori</i> detected
1	Vegetative	0	7.6×10^4
2	Vegetative	1	1.5×10^3
3	Vegetative	7	3.5×10^2
4	Vegetative	14	1.9×10^3
5	Vegetative	21	5.0×10^2
6	Spore	1	3.1×10^4
7	Spore	7	1.5×10^3
8	Spore	14	1.0×10^2
9	Spore	21	ND
10	None	1	1.5×10^4
11	None	7	7.3×10^4
12	None	14	1.9×10^3
13	None	21	2.3×10^4

ND, not detected.

Fig. 6. Effect of *C. butyricum* and a proton pump inhibitor on colonisation of *H. pylori* in germ-free mice. ■, co-infection with *C. butyricum* (n = 9); ▨, co-infection with *C. butyricum* and administration of lansoprazole (0.6 mg/mouse) (n = 9); □, administration of lansoprazole (0.6 mg/mouse) (n = 9); □, control (n = 9); each bar indicates SD of the means. Data without SD bar 7 weeks after treatment are the mean of two determinations, as no *H. pylori* was isolated from one mouse treated with *C. butyricum* and *H. pylori*. *No *H. pylori* was isolated from the gastric mucus of three treated mice. N.D., not detectable.

The adhesion of *H. pylori* to gastric epithelial cells is a primary event in the development of infection. Bernet *et al.* [20] reported that *L. acidophilus* LA1 inhibits the adhesion of many kinds of pathogenic enterobacteria to intestinal Caco-2 cells. Furthermore, Kabir *et al.* [12] reported an inhibitory effect of *L. salivarius* on the adhesion of *H. pylori* to MKN45 cells. Similarly, the present study showed an inhibitory effect of *C. butyricum* on the adhesion of *H. pylori* to MKN45 cells. However, it is still unclear how lactobacilli or *C. butyricum* inhibited the attachment of *H. pylori* to gastric epithelial cells.

An animal model for *H. pylori* infection could provide much information about the degree of colonisation and pathology in the antrum and gastric body. Kabir *et al.* [12] reported that the gastric tissue of germ-free mice can be colonised with *H. pylori* easily, as described in the present study. These results suggest that the difficulty in colonisation by *H. pylori* in conventional mice may be explained by the large number of other bacteria colonising the gastric mucosa. Although Lee *et al.* [21] reported that the Sydney strain (SS1) of *H. pylori* can colonise C57BL/6 mice in the long-term, quantification of *H. pylori* in gastric mucosa is easier in the germ-free mouse model used in the present study. The number of colonising *H. pylori* was 10^4 – 10^5 cfu/g of mucus in this study. Other workers reported that much higher numbers of *H. pylori* were detected in Swiss mice (10^5 – 10^7 cfu/g of tissue) and human patients (10^5 – 10^7 cfu/g of tissue) infected with *H. pylori* [22,23]. However, the present study used gastric mucus rather than gastric homogenate for quantification colonising of *H. pylori*. The lower numbers of *H. pylori* detected in the present study might be due to the difference in the source of the samples tested.

Histological examination indicated that *H. pylori* TK1402 induced active gastritis, characterised by the infiltration of inflammatory cells. Persistent infection of germ-free mice with *H. pylori* was stable until 12 weeks after inoculation at this laboratory (data not shown). Lee *et al.* [21] reported that Sydney strain SS1 induced chronic active gastritis with severe atrophy in both C68BL/6 and BALB/c mice over a period of 8 months. Van Doorn *et al.* [24] recently reported that inflammation in mouse stomach caused by *H. pylori* depends on the bacterial strain and host mouse strain. Strain TK1402 may not produce a robust infection in germ-free IQI mice, but the murine model used in the present study might be useful for evaluation of antimicrobial agents against *H. pylori*, as colonisation by *H. pylori* persists for a long period.

The eradication of *H. pylori* infection from gastric mucosa by *C. butyricum* spores and vegetative cells of the MIYAIRI 588 strain was demonstrated. The efficacy of eradication by spores was better than that by vegetative cells. It is possible that the germination

process from spore to vegetative cell might be important in the inhibitory effect of *C. butyricum* on the growth of *H. pylori*. Iwahi *et al.* [25] reported that the antibacterial activity of lansoprazole and its related compounds was selective against *H. pylori* *in vitro*; common aerobic and anaerobic bacteria and *Campylobacter jejuni* were not inhibited by lansoprazole 100 mg/ml. However, the present study demonstrated that lansoprazole had no effect on the eradication of *H. pylori* in the gnotobiotic mouse unless *C. butyricum* was co-inoculated. Interestingly, low numbers of *H. pylori* ($c. 10^3$ cfu/g mucus) were detected at 7 weeks when gnotobiotic mice were treated with both *C. butyricum* and PPI. This result implies that inhibition of gastric acid by the PPI may sustain a persistent infection of *H. pylori* in gastric mucosa.

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