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Synergistic effect of anti-*Helicobacter pylori* urease immunoglobulin Y from egg yolk of immunized hens and *Lactobacillus johnsonii* No.1088 to inhibit the growth of *Helicobacter pylori in vitro* and *in vivo*

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ABSTRACT

Helicobacter pylori is a pathogenic bacterium that infects the stomach, causing chronic gastritis; and it is also considered to be related to the occurrence of gastric cancers. Although some eradication regimens including multiple antibiotics have been developed, the emergence of resistance to antibiotics becomes problematic. Therefore, other approaches to compensate or augment the effects of standard regimens are needed. In this study, we examined the possible synergistic effects of anti-*H. pylori* urease IgY and *Lactobacillus johnsonii* No.1088 (LJ88) both *in vitro* and *in vivo*. Anti-*H. pylori* urease IgY was purified from egg yolks laid by the hens immunized with urease purified from *H. pylori*. LJ88 is a unique strain of lactic acid bacterium isolated from human gastric juice, and it has been reported to inhibit *H. pylori* both *in vitro* and *in vivo*. The *in vitro* mixed culture study showed that anti-*H. pylori* urease IgY augmented the anti-*H. pylori* activity of LJ88 against both clarithromycin-sensitive and -resistant *H. pylori* strains. In a germ-free mice infection model, combined administration of daily anti-*H. pylori* urease IgY and weekly living LJ88 significantly reduced *H. pylori* infections, whereas either monotherapy did not. In an *in vivo* human gut microbiota-associated mice model, not only daily administration of living LJ88 but also heat-killed one significantly reduced an *H. pylori* infection in the stomach when combined with anti-*H. pylori* urease IgY. The extent of reduction of the stomach *H. pylori* by such a combination therapy was larger than that reported for LJ88 monotherapy. These results taken together revealed a synergistic effect of anti-*H. pylori* urease IgY and living or heat-killed LJ88, thus suggesting that such a combination might be a promising therapy to possibly compensate and/or augment standard anti-*H. pylori* regimens.

1. Introduction

Helicobacter pylori (*H. pylori*) is a pathogenic bacterium that infects the stomach and causes chronic gastritis [1–3]. Recent studies revealed that most patients with stomach cancer are infected with *H. pylori* [4], suggesting that eradication of an *H. pylori* infection is crucial not only to ameliorate chronic gastritis but also to prevent suffering from stomach cancer. The standard eradication therapy for an *H. pylori* infection, called triple therapy, consists of 2 antibiotics and 1 proton-pump inhibitor [5]; but the emergence of resistance to antibiotics is becoming problematic [6]. Especially in countries where the use of antibiotics is not well-controlled, the rate of successful eradication of *H. pylori* infections is reported to be relatively low [7,8]. Although replacement of traditional antibiotics with newly developed ones, addition of strong anti-bacterial compounds (e.g., bismuth compound), and/or usage of novel proton-pump inhibitors have improved the eradication rate of *H. pylori* [8–10], strong side effects (especially in the case of bismuth compounds) and cat-and-mouse chase-like emergence of new types of resistance to novel antibiotics demand the development of novel anti-*H. pylori* therapy strategies.

Immunoglobulin Y (IgY) is a type of immunoglobulin found in birds and is comparable to mammalian immunoglobulin G (IgG). By immunizing hens, it is possible to obtain specific IgY in egg yolk;

Abbreviations: H. pylori, *Helicobacter pylori*; IgY, immunoglobulin Y; LJ88, *Lactobacillus joshnsonii* No.1088; IgG, immunoglobulin G; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate poly acrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; MRS, De Man, Rogosa and Sharpe; BHI, Brain-Heart Infusion.

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and because hens lay eggs every day over a long time period, we can harvest large amounts of IgY without sacrificing the animals [11]. IgYs specific for *H. pylori* have been raised against whole cells/lysates of *H. pylori* [12–14], against specific proteins such as urease [15,16], and some recombinant or synthesized *H. pylori* proteins (urease subunits [17,18], urease peptides [19], OipA [20], VacA [21], etc.); and their anti *H. pylori* activities have been examined both *in vitro* and *in vivo*. However, in most cases these IgYs failed to completely eradicate *H. pylori* in experimental animal models.

The combination of different agents having anti-microbial effects is known to enhance therapeutic efficacy against infections. Certain probiotic strains of lactic acid bacteria have been reported to increase the eradication rates of *H. pylori* by antibiotics and proton-pump inhibitors [22]. In a previous study, hyperimmune bovine colostrum obtained by immunizing pregnant cows with human strains of rotavirus combined with probiotic *Lactobacillus rhamnosus* GG has shown clear synergistic prophylactic effects against rotavirus diarrhea in a mouse model [23]. *Lactobacillus johnsonii* No.1088 (LJ88) is a unique lactic acid bacterium that was isolated from the gastric juice of a healthy Japanese, and it has anti-*H. pylori* activity in both living and heat-killed forms [24–26]. Since LJ88 is thought to attack the *H. pylori* cell surface physically and to destroy cellular structure [25], this bacterium could be effective against *H. pylori* strains having resistance to antibiotics. In this study, we examined the combinatorial effect of anti-*H. pylori* urease IgY and LJ88 on *H. pylori* proliferation both *in vitro* and *in vivo*; and the results demonstrated synergistic inhibition of *H. pylori* activity.

2. Materials and methods

2.1. Preparation of IgY against H. pylori urease

IgY specific for *H. pylori* urease was produced according to the method reported previously [27]. In brief, for purification of *H. pylori* urease as the immunogen, *H. pylori* No.130 was lysed and fractionated by DEAE-Sepharose ion-exchange column, after which the active fractions were applied onto a Sephacryl S-300 size-exclusion column. The purified urease was eluted with phosphate-buffered saline (PBS), and its purity was examined by sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE), resulting in the observation of 2 single bands of 32 kD and 60 kD, corresponding to the urease subunits A and B, respectively. The purified *H. pylori* urease (1 mg/mL) emulsified with Freund's incomplete adjuvant (1:1) was injected into both breast muscles of female white leghorns of approx. 18 weeks of age, and a booster with the same immunogen emulsion was given 6 weeks later. Eggs laid 2 weeks or later after the booster were used as the source for purifying IgY. Egg yolks were pooled (1 vol), well mixed with the same volume of PBS (1 vol), and then mixed and agitated with the same volume of chloroform as "egg yolk + PBS" (2 vol). After centrifugation, the aqueous fraction of the egg yolk was obtained. The IgY contained in it was purified by thrice repeated precipitation with saturated ammonium sulfate solution. The titer of the resulting IgY solution, determined by performing an enzyme-linked immunosorbent assay (ELISA), was about ×50,000, and its protein concentration was about 10 mg/mL. As for the control IgY, egg yolks laid by hens without immunization were used as the source for IgY purification.

2.2. ELISA for anti-H. pylori urease IgY

Anti-*H. pylori* urease immunoreactivity was determined by a sandwich ELISA method using purified *H. pylori* urease. Each well of a 96-well micro titer plate was coated with purified *H. pylori* urease subunits A + B (1:1) at a concentration of 5 μg/mL for 18 h at 4 °C, washed 3 times with PBS containing 0.05% Tween 20 (PBS-Tween), blocked with 2% skimmed milk, and then washed 3 times with PBS-Tween. Samples diluted with PBS-Tween were added to the wells and incubated for 60 min at 37 °C. The amounts of IgY bound to the wells were monitored by using HRP-rabbit-anti-chicken IgY (H + L) (Invitrogen, Carlsbad, CA, USA) as the reporter along with o-phenylene diamine dichloride solution containing hydrogen peroxide as the substrate. The reaction was stopped with 3N sulfuric acid and quantified with a microplate reader at 490 nm.

2.3. Lactobacillus johnsonii No.1088 (LJ88)

LJ88 is a unique lactic acid bacterium strain isolated from a healthy Japanese individual [24]. LJ88 was cultured in De Man, Rogosa and Sharpe (MRS) medium for 18 h at 37 °C. For the *in vitro* study and single-dose *in vivo* study, LJ88 was used after having been washed with PBS. For repeated-dose *in vivo* studies, the bacterial bodies were washed with PBS, after which the cell number was counted microscopically with hemocytometer and adjusted to be 5×10^9 cells/mL with PBS. For preparation of a stock of living LJ88 cells, the bacterial solution was aliquoted (10 mL each) and then directly frozen at −80 °C. The number of living cells was determined after thawing to be 4.1×10^9 cfu/mL (viability was 82%; dose 0.25 mL/mice; 10^9 cfu/mice). For preparation of heat-killed LJ88 stocks, the bacterial solution was aliquoted (10 mL each) after autoclaving at 115 °C for 15 min (dose 0.25 mL/mice; 1.25×10^9 cells/mice). All stock samples were kept at −80 °C until use.

2.4. Evaluation of the effect of IgY and/or LJ88 on the proliferation of H. pylori in vitro

The in vitro anti-*H. pylori* activity of IgY and LJ88 was determined by using the method reported previously [24]. About 10^6 cfu/mL of *H. pylori* No.130 (genotype: cagA+; vacA m1/s1a) and TK007 (genotype: cagA+; vacA m1/s1a), both of which had been isolated at Tokai University Hospital, were cultured in Brain-Heart Infusion (BHI) broth supplemented with 5% horse serum at 37 °C under microaerobic conditions up to 48 h with or without anti-*H. pylori* urease IgY (1 mg/mL) and/or living LJ88 (about 10^6 cfu/mL). The number of living *H. pylori* was determined at 0, 12, 24, and 48 h after the start of cultivation, as described previously [24,25]. For comparison, 3 other *H. pylori* strains which also had been isolated at Tokai University Hospital, named TI3 (genotype: cagA+; vacA m1/s1a), TI6 (genotype: cagA+; vacA m1/s1a), and U-12 (genotype: cagA+; vacA m1/s1a) were used under the same experimental setting.

2.5. Evaluation of the effect of IgY on the binding of H. pylori urease to porcine gastric mucin

Porcine mucin, purchased from Sigma Aldrich (St. Louis, MD, USA), was dissolved in adhesion medium (10 mM phosphate, 150 mM NaCl, 0.05% Tween20; pH4.0) at a concentration of 2.5 μg/mL, dispensed into each well of a 96-well micro titer plate (100 μL/well), and incubated at 37 °C for 1 h to be adsorbed onto the surface of the wells. After 5 washings with the adhesion medium, anti-*H. pylori* urease IgY or control IgY (0.5, 0.05, 0.005, 0.0005, and 0.0005 mg/mL) was added to the wells and mixed with 2.5 μg/mL purified *H. pylori* urease in dilution medium, and then incubation was carried out at 37 °C for 60 min. Thereafter each well was washed 3 times with adhesion medium, and the urease enzyme activity was measured according to Nagata et al. [28].

Because our anti-*H. pylori* urease IgY did not inhibit *H. pylori* urease activity at the concentration range of this experiment (data not shown), the urease activity detected was thought to be proportional to the amount of bound urease.

2.6. Evaluation of the anti-H. pylori effect in mouse models

Anti-*H. pylori* effect of LJ88 and/or anti-*H. pylori* urease IgY was examined by using both germ-free mouse and human gut microbiota-associated mouse models as previously described [24,25]. Since *H. pylori* cannot persistently infect in specific pathogen-free mice [29], which bears intrinsic gastrointestinal tract microbiota, we employed either of germ-free or human gut microbiota-associated mouse model. The latter was used to evaluate anti-*H. pylori* activity under more similar microbial microenvironment condition to human than that of germ-free one [30]. *H. pylori* strain No.130 was used in the *in vivo* studies, because this strain had already been used in other *in vivo* studies by our group and variety of results to be compared had been published [24–26,29]. For the germ-free mouse model, male germ-free BALB/c mice (4 weeks of ages) raised in Trexler-type flexible-film plastic isolators with sterile food and water were orally administered 10^9 cfu of *H. pylori* No.130 for 3 or 4 consecutive days; and after 4 weeks from the first administration, sample administration was started. Detailed time schedules for each experiment are described in the Results section. As for the human gut microbiota-associated mouse model, male germ-free BALB/c mice (4 weeks of age) were orally administered human feces obtained from a healthy Japanese male (0.5 mL of about 10 mg feces/mL in PBS); and then after 4 weeks *H. pylori* No.130 (10^9 cfu) was administered once a day for 4 consecutive days. Then, after another 4 weeks sample (IgY and/or LJ88) administration was started. In all experiments, samples suspended in 0.5 mL of PBS were orally administered via feeding needles. Mice were sacrificed before and after the sample administration period, and the number of viable *H. pylori* in each stomach was determined according to the method described previously [24,25]. In brief, the excised stomach was homogenized in PBS, and the number of viable *H. pylori* was determined by counting colonies on BHI agar plates containing 7% horse serum, 25 μg/mL tetrazolium chloride, 2.5 units/mL polymyxin B, and 2 μg/mL vancomycin B after spreading the homogenates at differential dilutions on the plates and culturing for 3 days at 37 °C under microaerobic condition. All animal experiments were carried out in accordance with the institutional guideline of Tokai University.

2.7. Statistics

Statistical significances of viable numbers of *H. pylori* in the stomachs between test groups were determined by Student *t*-test (two groups) or Tukey's honestly significant difference test (multiple groups) by using KaleidaGraph ver4.5 software. Statistical significances of *H. pylori* eradication rates between multiple groups were determined by a pairwise Fisher's exact test (P adjusted by Holm) with R statistical software [31].

3. Results

3.1. Effect of anti-H. pylori urease IgY and/or LJ88 on the proliferation of H. pylori in vitro

Fig. 1 shows the results of mixed culture evaluation of living LJ88 and/or anti-*H. pylori* urease IgY on the viability of 2 different *H. pylori* strains, e.g., clarithromycin-sensitive No.130 (minimum inhibitory concentration = 0.031 μg/mL) and -resistant TK007 (minimum inhibitory concentration = 8 μg/mL). As shown in Fig. 1A and B, living LJ88 largely reduced the viability of both strains of *H. pylori*. On the contrary, anti-*H. pylori* urease IgY only slightly affected the viability of *H. pylori*. When both living LJ88 and anti-*H. pylori* urease IgY were added to the culture system, a higher inhibitory effect on the viability of *H. pylori* was observed. To know whether the same results could be obtained with respect to different *H. pylori* strains, other clarithromycin-sensitive (TI6) and -resistant (TI3 and U-12; their minimum inhibitory concentrations = 16 μg/mL) strains were also examined. The result at 24 h culture is summarized in Fig. 1C, representing the average values

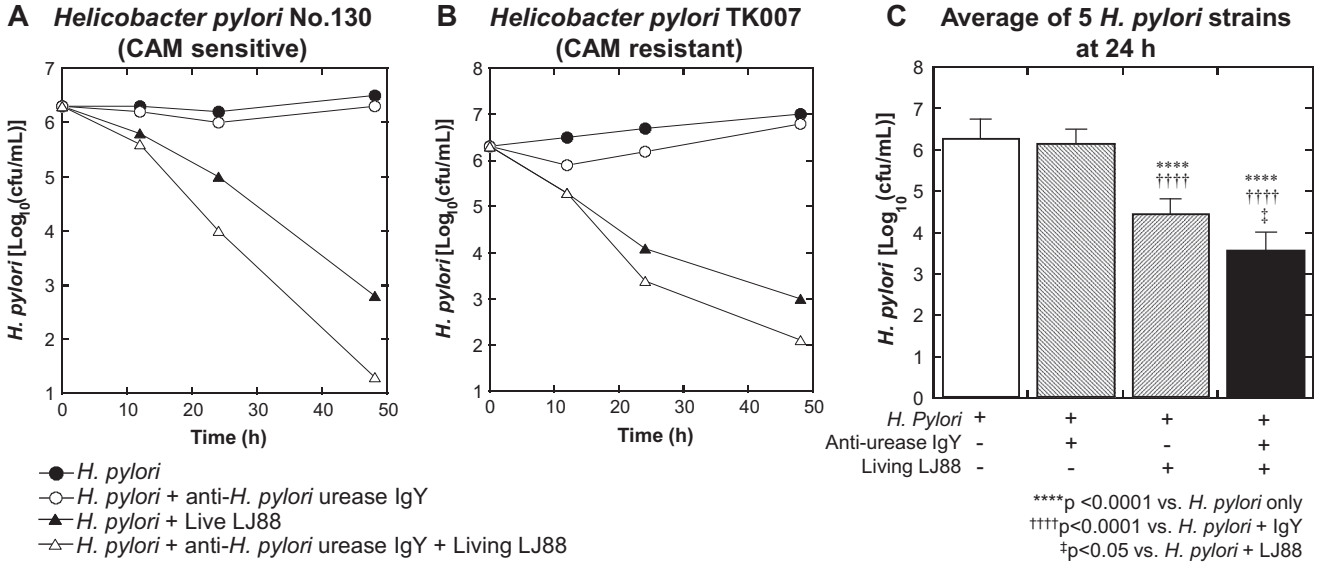

Fig. 1. Effects of anti-*H. pylori* IgY and living LJ88 on the proliferation of *H. pylori in vitro.* Clarithromycin (CAM)-sensitive (A) and -resistant (B) strains of *H. pylori* (about 10^6 cfu/mL) were cultivated in the presence or absence of anti-*H. pylori* IgY (1 mg/mL) and/or living LJ88 (about 10^6 cfu/mL) at 37 °C for 48 h under microaerobic conditions. The number of live *H. pylori* was determined at 12, 24, and 48 h of cultivation. Closed circle, open circle, closed triangle, and open triangles represent *H. pylori* only, with anti-*H. pylori* urease IgY in solo, with living LJ88 in solo, and with both anti-*H. pylori* urease IgY and living LJ88 in combination, respectively. The data for *H. pylori* No.130 is representative of 3 independent experiments, whereas that for TK007 is from the single one. (C) Average values with standard deviations obtained from 5 different strains of *H. pylori* (No.130, TI3, TI6, TK007, and U-12) at 24 h of cultivation at the same condition. The statistical significances between groups were determined with Tukey's honestly significant difference test. $^{****}p < 0.0001$ vs. *H. pylori* only, $^{††††}p < 0.0001$ vs. *H. pylori* + IgY, and $^{‡}p < 0.05$ vs. *H. pylori* + LJ88.

with standard deviations obtained from all 5 different *H. pylori* strains. As shown in this figure, although anti-*H. pylori* urease IgY could not affect the viability of *H. pylori* in solo, its addition significantly augmented anti-*H. pylori* effect of living LJ88 ($p < 0.05$).

3.2. Effect of anti-H. pylori urease IgY on the binding of H. pylori urease to porcine gastric mucin

To know whether anti-*H. pylori* urease IgY might affect other functional properties of *H. pylori* than viability, we examined its effects on the binding of *H. pylori* urease to porcine gastric mucin. As shown in Fig. 2, anti-*H. pylori* urease IgY inhibited the binding of *H. pylori* urease to immobilized porcine mucin in a dose-dependent manner, where the inhibition of the binding was over 60% even at 50 ng/mL. On the contrary, IgY isolated from non-immunized hen's eggs (control IgY) did not inhibit the binding up to 0.5 mg/mL. Although no statistical evaluation could be applied to this single replicate result, the effectiveness of anti-*H. pylori* urease IgY appears very clear without statistics.

3.3. Anti-H. pylori effect of anti-H. pylori urease IgY monotherapy in germ-free mouse model

Next, we examined *in vivo* the anti-*H. pylori* effects of anti-*H. pylori* urease IgY monotherapy. Fig. 3 shows the effect of anti-*H. pylori* urease IgY in solo on the number of viable *H. pylori* in the stomach of germ-free BALB/c mice orally infected 3 times with 10^9 cfu *H. pylori*. As shown in Fig. 4, repeated daily administration of anti-*H. pylori* IgY (0.5 mg/day) for 4 weeks significantly reduced the number of viable *H. pylori* in the stomach ($p < 0.05$); and in this group, *H. pylori* was not detected in 2 out of 10 mice. However, no statistical significance was observed.

3.4. Anti-H. pylori effect of anti-H. pylori urease IgY in combination with living LJ88 in germ-free mouse model

Fig. 4 shows the effect of anti-*H. pylori* urease IgY in combination with living LJ88 in a germ-free mouse model orally infected 3 times with 10^9 cfu *H. pylori*. In this run of experiment anti-*H. pylori* urease IgY monotherapy (once a day for 4 weeks) did not significantly affect the number of viable *H. pylori* in the stomach. Moreover, monotherapy with living LJ88 (once a week for 4 weeks) did not significantly reduce the number of viable *H. pylori* in the stomach, too. However, co-administration of anti-*H. pylori* urease IgY (once a day for 4 weeks) and living LJ88 (once a week for 4 weeks) significantly decreased the number of *H. pylori* in the stomach and increased the eradication rate.

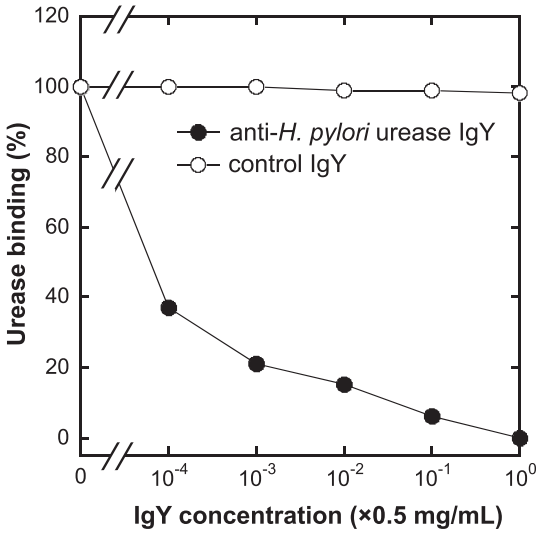

Fig. 2. Effect of anti-*H. pylori* IgY on the binding of *H. pylori* urease to porcine mucin. Urease purified from *H. pylori* (2.5 μg/mL) was incubated in the presence of varying concentration of anti-*H. pylori* urease IgY (closed circle) or control IgY (open circle) in the wells of 96-well microtiter plate coated with porcine mucin. The amount of bound urease was determined by measuring solidified urease activity after incubation.

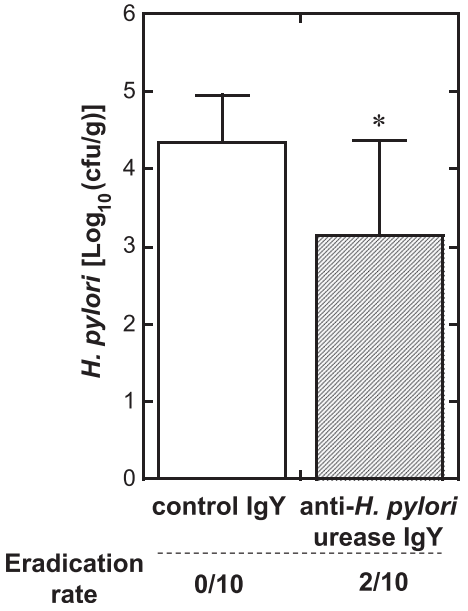

Fig. 3. Effect of anti-*H. pylori* urease IgY monotherapy against *H. pylori* infection in a germ-free mouse model. Male germ-free BALB/c mice were orally administered 10^9 cfu of *H. pylori* once a day for 3 consecutive days. After 4 weeks from the first administration, daily oral administration of anti-*H. pylori* urease IgY (0.5 mg/day) was started up to 4 weeks (n = 10). In the control, the same amount of IgY purified from egg yolks laid by non-immunized hens was administered (n = 10). At the next day of the 4-weeks administration, the mice were sacrificed; and the number of *H. pylori* in the stomach was then determined. When no *H. pylori* was detected, *H. pylori* was considered to have been eradicated. Each bar shows mean with standard deviation. *$p < 0.05$ (control vs. anti-*H. pylori* urease IgY groups; Student *t*-test). No statistical significances were detected with respect to the eradication rate (Fisher's exact test).

3.5. Anti-H. pylori effect of anti-H. pylori urease IgY in combination with heat-killed LJ88 in human gut microbiota-associated mice

Finally, we examined the combinatorial effects of anti-*H. pylori* urease IgY and LJ88 under severer *in vivo* experimental conditions than described above. In this experiment, we employed human gut microbiota-associated mice instead of germ-free mice, in which interference from other bacteria in the stomach might have affected the interaction between *H. pylori* and IgY/LJ88. Furthermore, in this model we infected mice with *H. pylori* 4 times instead of 3 times with 10^9 cfu *H. pylori* to ensure a heavier infection state. As for LJ88, we examined the effect of heat-killed LJ88 in addition to living LJ88 by daily dosing instead of the weekly one.

Under this severer condition, as shown in Fig. 5, anti-*H. pylori* urease IgY alone did not affect the number of *H. pylori* in the stomach of the mice. However, when combined with daily administration of LJ88, this number was significantly reduced; and there was no significant difference between the living and heat-killed LJ88. Regarding the eradication rate, *H. pylori* was not detected in 4 and 5 out of 6 mice in the heat-killed and living LJ88 co-administration groups, respectively, although no statistical significance was noted.

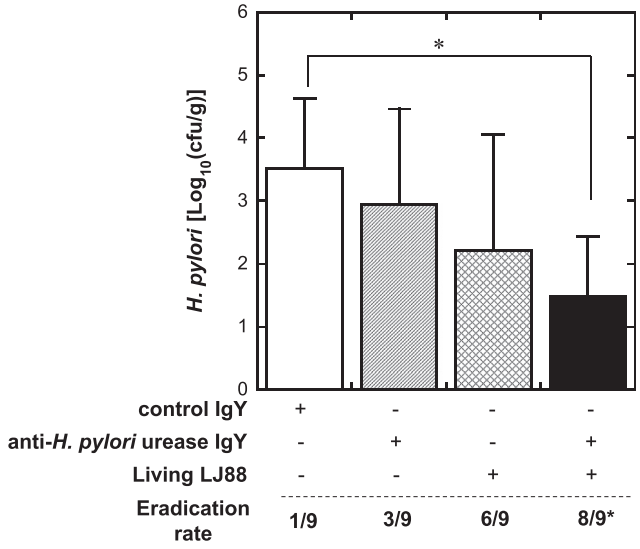

Fig. 4. Effect of anti-*H. pylori* urease IgY and living LJ88 in combination against *H. pylori* infection in germ-free model mice. Male germ-free mice were infected with *H. pylori* as in Fig. 3 (n = 9), and after 4 weeks oral administration of anti-*H. pylori* urease IgY monotherapy (0.5 mg/mice; once a day), living LJ88 monotherapy (10^9 cfu/mice; once a week), or the combination therapy with both of them were started. Then mice were sacrificed, and number of living *H. pylori* in the stomach was determined. Each bar shows mean with standard deviation. Regarding *H. pylori* number, statistical significances between groups were determined with Tukey's honestly significant difference test. Regarding eradication rates, these were determined by using the pairwise Fisher's exact test (P adjusted by Holm). $^{*}p < 0.05$.

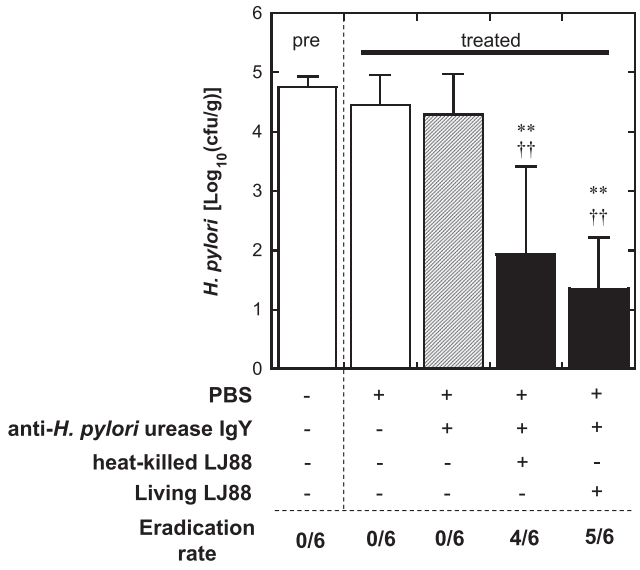

Fig. 5. Effect of anti-*H. pylori* urease IgY in combination with living or heat-killed LJ88 against *H. pylori* infection in human gut-microbiota-associated model mice. Male germ-free BALB/c mice were administered human feces as described in Materials and Methods, and after 4 weeks 10^9 cfu of *H. pylori* was orally administrated once a day for 4 consecutive days. Then, after another 4 weeks, daily oral administration of PBS (0.5 mL/mice), anti-*H. pylori* urease IgY (0.5 mg/mice), anti-*H. pylori* urease IgY (0.5 mg/mice) + 10^9 cells heat-killed LJ88, or anti-*H. pylori* urase IgY (0.5 mg/mice) + 10^9 cfu living LJ88 was started and continued up to 4 weeks (n = 6 for each group). Then the mice were sacrificed, and the number of living *H. pylori* in the stomach was determined. Each bar shows the mean with standard deviation. Regarding *H. pylori* number, statistical significances between groups were determined with Tukey's honestly significant difference test and results obtained after treatment are represented. $^{**}p < 0.01$ vs. PBS group; $^{††}p < 0.01$ vs. anti-*H. pylori* urease IgY group. Regarding eradication rates, these were determined with a pairwise Fisher's exact test (P adjusted by Holm), where no significant differences between groups were detected.

4. Discussion

In this study we examined the synergistic effect of anti-*H. pylori* urease IgY raised against urease purified from *H. pylori* and a lactic acid bacterium, LJ88, both *in vitro* and *in vivo*, and found synergistic effects in both cases. In the *in vitro* mixed-culture study, anti-*H. pylori* urease IgY at 1 mg/mL only slightly reduced the viability and growth of *H. pylori* in both clarithromycin-sensitive and -resistant strains, whereas living LJ88 clearly inhibited the viability of both *H. pylori* strains. This finding is in agreement with that reported previously [24]. Also, we found earlier that LJ88 could decrease the viability of *H. pylori in vitro* even in its heat-killed form [25]; and we suggested its anti-*H. pylori* effects to be attributable to some cell-surface molecule(s) and/or molecular structure (s) on the surface of LJ88 cells interacting with *H. pylori* [25]. Interestingly, addition of anti-*H. pylori* urease IgY augmented the effect of living LJ88 against *H. pylori* viability, as was shown in Fig. 1. The mechanism of this additional effect is not clear, but the presence of IgY molecules on the surface urease of *H. pylori* might have affected the interaction between LJ88 and *H. pylori*. These results suggest that it is better to use LJ88 in combination with anti-*H. pylori* urease IgY to effectively reduce the viability of *H. pylori*, although understanding of the molecular mechanism involved awaits further studies.

In addition to the direct effect of anti-*H. pylori* urease IgY on *H. pylori*, its effect on the colonization of *H. pylori* in the gastric mucosa is thought to be important as well. The urease of *H. pylori* accumulates on the cell surface in addition to intracellular sites and is considered as an essential factor for *H. pylori* colonization of the stomach [32]. In the mucus layer of the stomach a pH gradient exists, which is lower toward the gastric lumen. And for the stable colonization of *H. pylori* in the gastric mucosa, it has been reported that its urease activity, motility, and the taxis toward higher pH are essential [32,33]. *H. pylori* urease has been also reported as a molecule to which mucin binds. We firstly examined whether anti-*H. pylori* urease IgY could inhibit the urease activity of *H. pylori*, but no inhibition was detected up to 1 mg/mL (data not shown). Next we examined the effect of anti-*H. pylori* urease IgY on the binding of *H. pylori* urease to porcine mucin and found that it strongly inhibited the binding compared with the control IgY (Fig. 2). These results suggest that anti-*H. pylori* urease IgY interfered with the stable attachment of *H. pylori* onto the mucus layer and thus reduced its infection rate.

Table 1 summarizes the results of *in vivo* studies examining anti-*H. pylori* effects of LJ88 and/or anti-*H. pylori* urease IgY done by our group including this study and also published studies. As was shown in Fig. 3 and Table 1, monotherapy with anti-*H. pylori* IgY against *H. pylori* infection in a germ-free mouse model significantly reduced the number of *H. pylori* in the stomach. However, in the other *in vivo* experiments consisting of more than 3 test groups, no significant reduction in the number of *H. pylori* in the stomach was observed (Fig. 4, Table 1). Especially in the model employing a heavier *H. pylori* infection condition (4 instead of 3 times) and coexistence of other gut bacteria (human gut microbiota-associated mice instead of germ-free ones), no reduction in the number of *H. pylori* in the stomach was detected (Fig. 5, Table 1). Such a discrepancy might be related to the differences in the number of test animals in each test group, the number of groups examined in a given experiment, and/or the severity of *H. pylori* infection. These results suggest that even though there exists the tendency for an anti-*H. pylori* effect in the case of anti-*H. pylori* urease IgY monotherapy, such an effect may not be strong enough.

As was shown in Fig. 4 and summarized in Table 1, although either monotherapy daily with anti-*H. pylori* IgY or weekly with living LJ88 did not significantly reduce the number of *H. pylori* in

Table 1
Anti-*H. pylori* effect of LJ88 and anti-*H. pylori* urease IgY in mice models.

Mice model	Treatment type	*H. pylori* infection	LJ88			anti-*H. pylori* urease IgY		*H. pylori* reduction (fold)	p	Ref.
			Type	Dose	Schedule	Dose	Schedule			
Germ-free	Monotherapy	10^9 cfu × 3 days	–	–	–	0.5 mg	Daily for 4 weeks	14.5	<0.05	Fig. 3
		10^9 cfu × 3 days	–	–	–	0.5 mg	Daily for 4 weeks	3.63	NS	Fig. 4
		10^9 cfu × 3 days	Living	10^9 cfu	Weekly for 4 weeks	–	–	19.5	NS	Fig. 4
		10^9 cfu × 4 days	Heat-killed	6.8×10^8 cells	Daily for 3 weeks	–	–	7.76	<0.01	Ref. [25]
	Combination therapy	10^9 cfu × 3 days	Living	10^9 cfu	Weekly for 4 weeks	0.5 mg	Daily for 4 weeks	107	<0.05	Fig. 4
Human microbiota-associated	Monotherapy	10^9 cfu × 4 days	–	–	–	0.5 mg	Daily for 4 weeks	1.48	NS	Fig. 5
		10^9 cfu × 4 days	Living	10^9 cfu	Daily for 4 weeks	–	–	126	<0.01	Ref. [24]
	Combination therapy	10^9 cfu × 4 days	Heat-killed	10^9 cells	Daily for 4 weeks	0.5 mg	Daily for 4 weeks	331	<0.01	Fig. 5
		10^9 cfu × 4 days	Living	10^9 cfu	Daily for 4 weeks	0.5 mg	Daily for 4 weeks	1259	<0.01	Fig. 5

the stomach, their combination therapies successfully reduced it in the germ-free mouse model. In a chronic and severe *H. pylori* infection model, not only living LJ88 but also heat-killed one could significantly reduce the number of *H. pylori* in the stomach (Fig. 5; Table 1). These results clearly suggest that anti-*H. pylori* urease IgY and LJ88 (both living and heat-killed types) synergistically inhibited the growth of *H. pylori in vivo*. As was earlier reported, monotherapy daily with living LJ88 against an *H. pylori* infection in the same experimental setting reduces the number of *H. pylori* in the stomach by about 100-fold (Table 1) [24]. Also, monotherapy daily with heat-killed LJ88 against *H. pylori* in a germ-free mouse model reduces it significantly but by less than 10-fold (Table 1) [25]. However in this study, as was shown in Fig. 5 and Table 1, the combination therapy of anti-*H. pylori* IgY with heat-killed LJ88 or living LJ88 resulted in a greater reduction of *H. pylori* in the mouse stomach (>100-fold or about 1000-fold, respectively). This fact indicates that anti-*H. pylori* IgY and LJ88, in both living or heat-killed forms, had synergistic effects when used together.

In conclusion, the combination of anti-*H. pylori* urease IgY with LJ88 (either living or heat-killed type) might be a promising approach to inhibit *H. pylori in vivo* without the risk of emergence of resistance to antibiotics. However, even with such combination therapy, complete eradication of *H. pylori* may be difficult. Therefore, it might be appropriate in a future study to examine whether such combination therapies might improve the eradication rates of standard triple therapy or other new and forthcoming anti-*H. pylori* therapies employing broad and narrow-spectrum antibiotics, especially in the case of greater resistance to antibiotics.

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Authors' contribution

Y.A., K.U., and S.R. collected data, contributed to discussion, and reviewed manuscript. Y.K. and S.V.N. contributed to discussion and wrote manuscript. All authors contributed to the design of the study and had full access to the data used in the study. Y.K. is the guarantor of this work and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Conflict of interest

Y.A. and Y.K. are employees of Snowden Co., Ltd.; and K.U. and S. R., employees of EW Nutrition Japan K.K. S.V.N is the president of EW Nutrition Japan K.K.

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