Use of Egg Yolk-Derived Immunoglobulin as an Alternative to Antibiotic Treatment for Control of *Helicobacter pylori* Infection

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Received 19 February 2002/Returned for modification 17 May 2002/Accepted 12 June 2002

The present study evaluated the potential use of immunoglobulin prepared from the egg yolk of hens immunized with *Helicobacter pylori* (immunoglobulin Y [IgY]-Hp) in the treatment of *H. pylori* infections. The purity of our purified IgY-Hp was 91.3%, with a yield of 9.4 mg of IgY per ml of egg yolk. The titer for IgY-Hp was 16 times higher than that for IgY in egg yolk from nonimmunized hens, and IgY-Hp significantly inhibited the growth and urease activity of *H. pylori* in vitro. Bacterial adhesion on AGS cells was definitely reduced by preincubation of both *H. pylori* (106 CFU/ml) and 10 mg of IgY-Hp/ml. In Mongolian gerbil models, IgY-Hp decreased *H. pylori*-induced gastric mucosal injury as determined by the degree of lymphocyte and neutrophil infiltration. Therefore, in this experimental model, *H. pylori*-associated gastritis could be successfully treated by orally administered IgY-Hp. The immunological activity of IgY-Hp stayed active at 60°C for 10 min, suggesting that pasteurization can be applied to stabilize the product. Fortification of food products with this immunoglobulin would significantly decrease the *H. pylori* infection. In conclusion, the IgY-Hp obtained from hens immunized by *H. pylori* could provide a novel alternative approach to treatment of *H. pylori* infection.

*Helicobacter pylori* is the most common cause of gastritis and gastric ulcers and plays a pivotal role in the development of gastric carcinomas (3, 21). Often a significant portion of those infected do not show symptoms, although chronic infection increases the risk of the development of *H. pylori*-associated gastric disease. There have been tremendous efforts to evaluate numerous therapeutic regimens for eradication of *H. pylori* infections. Successful treatment of *H. pylori* infections most often employs antibiotic therapy, consisting of some combination of metronidazole, amoxicillin, clarithromycin, and either bismuth or a proton pump inhibitor (8, 12). However, antibiotic therapy fails in 10 to 15% of cases due to the development of antibiotic resistance (5, 22). Increasing occurrence of antibiotic resistance would further complicate the treatment of *H. pylori* infections. Consequently, it is important to seek new therapies for a wider means of treating, suppressing, or preventing *H. pylori* infection without drug resistance problems.

The concept of protecting a host with passively derived antibodies is not new. It has been shown that oral administration of antibacterial or antiviral immunoglobulins, through infant formulae or other diet, is effective in preventing intestinal infection (4, 23). However, oral administration of antibodies is prohibitively expensive when large amounts of antibodies are required (14).

Recently, chicken egg yolk was recognized as an inexpensive, alternative antibody source, and the usefulness of egg yolk immunoglobulin Y (IgY) has been assessed for therapeutic application by passive immunization therapy through oral ingestion of IgY, as in fortified food products for prevention or control of intestinal infections, such as those caused by enterotoxigenic *Escherichia coli* (20), *Salmonella enterica* serovar Typhimurium (25), and rotavirus (9, 17, 23). These studies, taken together, provide the potential advantage of using IgY with specificity to *H. pylori* (IgY-Hp) for controlling *H. pylori*-associated gastric disease and subsequently prevent disease resulting from chronic infection. Nevertheless, there has been no report so far on the use of IgY in the prevention and treatment of *H. pylori* infections.

Furthermore, for the practical application of IgY-Hp, together with food or pharmaceutical materials to prevent *H. pylori*-associated disease, the stability of IgY toward heat, acid, and pepsin should be ensured. The aim of this study was to evaluate the potential use of IgY-Hp in the prevention and treatment of *H. pylori* infections. To achieve this objective, we studied, in vitro and in vivo, the activity of IgY-Hp against *H. pylori*. The effect of IgY-Hp on gastric mucosal injury induced by *H. pylori* was evaluated in vivo using Mongolian gerbils. The stability of IgY-Hp was also investigated.

**MATERIALS AND METHODS**

*H. pylori* preparation and immunization. *H. pylori* (ATCC 43504) was cultured in brucella broth (Difco Laboratories, Detroit, Mich.), supplemented with 5% (vol/vol) bovine calf serum (PAA Laboratories Inc., Parker Ford, Pa.) and antibiotics (ampicillin B, 2.5 μg/ml; vancomycin, 10 μg/ml; trimethoprim, 5 μg/ml; and polymyxin B, 2.5 IU/ml; all were from Sigma Chemical Co. [St. Louis, Mo.]) at 37°C under 10% CO2 at 200 rpm. The *H. pylori* was harvested by centrifugation at 12,000 × g for 10 min and disrupted by sonication. Cellular material was removed by centrifugation, and the supernatant was collected (*H. pylori* whole-cell lysate). The protein concentrations were determined by bicinchoninic acid methods (Pierce, Rockford, Ill.).
Brown Leghorn hens (25 weeks old; n = 15) were immunized intramuscularly with 60 μg of whole-cell lysate (200 μg/ml of protein) using an equal volume of Freund's complete adjuvant (Difco Laboratories). Each hen was injected at four different sites (250 μl per site) of the leg muscle. Three booster injections, with Freund's incomplete adjuvant, were given at 2-week intervals following the first injection. One month after immunization, the eggs laid were collected daily for 1 month and stored at 4°C. The egg yolk was separated, pooled, and frozen prior to purification of IgY.

**Isolation and purification of IgY-Hp.** Isolation of IgY-Hp was carried out by the method described by Akita and Nakai (1) with modification. Egg yolk was separated from the white, and the yolk preparation was mixed with an equal volume of distilled water for 30 min, followed by the addition of 0.15% (wt/vol) λ-carrageenan (Wako Pure Chemical Laboratory, Osaka, Japan). After centrifugation at 10,000 × g at 20°C for 30 min, the water-soluble fraction (WSF) was collected and filtered through a Whatman filter paper (no. 1) to remove solid lipid materials. The resulting IgY-containing filtrate was further purified by salt precipitation (19% [wt/vol] sodium sulfate) and ultrafiltration (UF) using a UF membrane (Millipore Corp., Bedford, Mass.) with a molecular weight cutoff of 100 kDa. Purity and yield of IgY were monitored at various stages by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The IgY content was measured by its absorbance at 280 nm.

**SDS-PAGE.** According to the method of Laemmli (18), 10% PAGE was done with a Mini-PROTEAN II Cell (Bio-Rad Laboratories, Hercules, Calif.). Under nonreducing conditions, samples were diluted 1:4 with sample buffer (6.26 mM Tris-HCl, pH 6.8, 25% [vol/vol] glycerol, and 2% SDS [vol/vol]). Under reducing conditions, samples were diluted with sample buffer containing 5% (vol/vol) β-mercaptoethanol and heated for 5 min at 100°C. Fifteen microliters of the samples was loaded into each well (3 μg of protein per well). Prestained SDS-PAGE standards (Bio-Rad Laboratories) and standard chicken IgY (Promega, Madison, Wis.) were used as molecular weight markers.

**ELISA.** To assess the antibody activity of IgY-Hp to H. pylori, we performed the enzyme-linked immunosorbent assay (ELISA) by Akita et al. (2) with modification. Ninety-six-well plates were coated with H. pylori whole-cell lysate (500 ng/well). After blocking with 1% (wt/vol) bovine serum albumin, 100 μl of IgY-Hp (1 mg/ml) was added using a twofold serial dilution. The plates were then washed with phosphate-buffered saline (PBS)-Tween (0.05% Tween 20 in PBS [pH 7.2]) and incubated for 1 h after the addition of alkaline phosphatase-conjugated goat anti-chicken IgY (Promega). The plates were washed with PBS-Tween, and disodium p-nitrophenyl phosphate (Sigma) was added as substrate to each well. After incubation for 10 min, the reaction was stopped by addition of 3 M NaOH. The absorbance was measured at 405 nm using a microplate reader (Multiskan MS; Thermo Labsystems, Helsinki, Finland).

**Heat, acid, and pepsin stability of IgY-Hp.** IgY-Hp solutions were incubated at 0, 4, 10, 25, 35, 60, 70, 80, and 90°C for 10 min. The heat-treated IgY-Hp was cooled in an ice bath. For the pH stability test, the pHs of IgY-Hp solutions were adjusted to the desired pH (pH 2 to 8) with NaOH or HCl, the solutions were incubated at 37°C for 4 h, and then each IgY-Hp solution was neutralized. For the pepsin stability tests, the pHs of the IgY-Hp solutions were adjusted to pH 2, pH 4, and pH 6, respectively. IgY-Hp solution of each pH was incubated with 15 μg of pepsin (Sigma)/ml at 37°C for 0.5, 1, 2, and 4 h. After incubation, each IgY-Hp solution was neutralized to inactivate the pepsin. The remaining antibody activity was measured by ELISA, following the heat, pH, and pepsin treatments. Antibody activity was represented as a percentage of control.

**Colony counting.** H. pylori (105 CFU/ml) was incubated with IgY-Hp (1 and 10 mg/ml) for 6 h at 37°C under 10% CO2 at 50 rpm. After incubation, H. pylori was diluted with brucella broth via a 10-fold serial dilution. Each 100 μl of inoculated brucella agar containing 5% (vol/vol) bovine calf serum, and the plate contents were incubated at 37°C under 10% CO2 for 10 days. The colonies were identified as H. pylori by Gram staining and urease, oxidase, and catalase activities. The growth rate (percentage of control) was calculated by colony counting compared to results for the control.

**Urease activities.** Both IgY-Hp (1 and 10 mg/ml) and H. pylori (108 CFU/ml) were incubated at 37°C under 10% CO2 for 6 h at 50 rpm, and then 50 μl of urea base (2% urea and 0.03% phenol red) was added and allowed to react for 30 min. Urease activity was quantified by measuring the optical density at 550 nm, using a modification to the method of Fauchere and Blaser (10), and was represented as % of control.

**Urease inhibition on AGS cells.** The human gastric carcinoma cell line AGS was obtained from the Korean Cell Line Bank (Seoul, Korea). Cells (105 cells/ml) were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, N.Y.), supplemented with 10% (vol/vol) fetal bovine serum (HyClone Laboratories, Logan, Utah) and antibiotics (100 U of penicillin/ml and 100 μg of streptomycin/ml; HyClone) at 37°C under 5% CO2 for 24 h. AGS cells were checked for their integrity. The growth rate (percentage of control) was calculated by colony counting on brucella agar containing 5% (vol/vol) bovine calf serum, and the plate contents were incubated at 37°C under 10% CO2 for 10 days. The colonies were identified as H. pylori by Gram staining and urease, oxidase, and catalase activities. The growth rate (percentage of control) was calculated by colony counting compared to results for the control.

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yolk (Table 1). The egg yolk proteins obtained during purification of IgY were analyzed using SDS-PAGE. As shown in Fig. 1, the IgY finally purified, using the UF system, was pure and dissociated into heavy and light chains of 64 and 25 kDa, respectively. The electrophoretic patterns were in perfect accordance with commercially available IgY (Promega) (Fig. 1A and B, lane 2). After treating the egg yolk with κ-carrageenan, the number of proteins in the WSF were significantly decreased. These contaminating proteins were removed by salt- ing out with 19% sodium sulfate. The IgY preparation was concentrated and desalted using the UF system.

The IgY and IgY-Hp obtained from hens without and with \textit{H. pylori} immunization, respectively, were examined for immunological properties by ELISA. The ELISA titers were 640 and 10,240 for IgY and IgY-Hp, respectively. These results indicate that IgY-Hp is highly specific to \textit{H. pylori}.

The effects of IgY-Hp on growth and urease activity of \textit{H. pylori} in vitro. To determine the effect of IgY-Hp on the growth and urease activity of \textit{H. pylori}, we compared growth rates and urease activity with those found for the control, after incubation with 1 mg and 10 mg of IgY-Hp/ml, respectively (Fig. 4). When \textit{H. pylori} (10⁶ CFU/ml) was incubated with IgY-Hp (1 and 10 mg/ml) for 6 h, the urease activity of \textit{H. pylori} was 75.4% ± 8.5% and 15.5% ± 7.4% compared with that of the control (Fig. 4).

FIG. 2. The reaction between IgY-Hp and \textit{H. pylori}. One hundred microliters of IgY-Hp (1 mg/ml) was added with a twofold serial dilution in 96-well plates coated with \textit{H. pylori} whole-cell lysate (500 ng/well), and the titers were measured using ELISA. IgY was isolated from the egg yolk of nonimmunized hens, and IgY-Hp was obtained from \textit{H. pylori}-immunized hens.

FIG. 3. Heat, pH, and pepsin stability of IgY-Hp. IgY-Hp was treated at various temperatures for 10 min (A), at various pHs for 4 h (B), and with pepsin (15 μl/ml) (C) at pH 2, 4, and 6 for 0.5, 1, 2, and 4 h. Remaining activities after the treatments were measured using ELISA and are expressed as a percentage of the initial activity.

FIG. 4. Effects of IgY-Hp on growth and urease activity of \textit{H. pylori} in vitro. When \textit{H. pylori} (10⁶ CFU/ml) was incubated with IgY-Hp (1 and 10 mg/ml) for 6 h, the urease activity of \textit{H. pylori} was 75.4% ± 8.5% and 15.5% ± 7.4% compared with that of the control (Fig. 4).

Effect of IgY-Hp on \textit{H. pylori}-induced gastritis in the Mongolian gerbils model. Mongolian gerbils were inoculated with \textit{H. pylori}. Two weeks after the inoculation, the status of infec-

The effects of IgY-Hp were evaluated by measuring the serum antibody (IgG) against \textit{H. pylori}. The \textit{H. pylori}-infected gerbils were determined by high IgG titer (data not shown).

Oral administration of 10 mg of IgY-Hp/ml for 4 weeks to the \textit{H. pylori}-infected gerbils improved lymphocyte infiltration compared to that found for the \textit{H. pylori} infection group with no IgY-Hp (1.2 ± 0.8 versus 2.5 ± 0.5; \( P < 0.01 \)) (Fig. 5). However, the group that was administered 1 mg of IgY-Hp/ml showed no statistically significant difference with the \textit{H. pylori} infection group (2.4 ± 1.1 versus 2.5 ± 0.5) (Fig. 5). The group treated with 10 mg of IgY/ml significantly improved neutrophil infiltration (0.9 ± 0.7 versus 2.3 ± 0.8; \( P < 0.01 \)) (Fig. 5). There was no statistically significant difference between the group treated with 1 mg of IgY-Hp/ml and the \textit{H. pylori} infection group (2.2 ± 0.5 versus 2.3 ± 0.8) (Fig. 5).

A high dose of IgY-Hp treatment decreased \textit{H. pylori}-induced lymphocyte and neutrophil infiltration in gastric mucosa. However, a low dose of IgY failed to protect gerbils from \textit{H. pylori}-induced gastric mucosal injury.

Effect of IgY-Hp on \textit{H. pylori} attachment on AGS cells.
from the values for the control (treatment of H. pylori) the emergence of antibiotic-resistant strains (5, 22). Moreover, antimicrobial therapeutic cures of H. pylori infections do not lead to immunity from reinfection (28), and the emergence of antibiotic-resistant strains can increase failure-of-therapy and relapse rates (26). Consequently, there have been tremendous efforts to seek alternatives to antibiotic-based therapies for a more widely available means of treating, suppressing, or preventing H. pylori infections without drug resistance problems.

Recent work in several laboratories, using several animal models, has shown that immunization with defined native and recombinant antigens of H. pylori can protect against H. pylori infections (7, 11, 16). The mechanisms of protection are still poorly understood. Although prophylactic and therapeutic immunization has been successful in animal models, efficacy data for humans are still lacking.

On the other hand, treatment of H. pylori infection by oral administration of active antibodies specific to H. pylori may have merit, due to the antibody being recognized by H. pylori, thus inhibiting adhesion of the bacterium to human epithelial cells more efficiently. Many studies have shown that egg yolk from an immunized hen has an antibody capable of specific recognition in an abundant quantity and is therefore economical (15, 19, 27). We estimated that 1 ml of the egg yolk (15 ml per egg) contains about 9.4 mg of IgY. A hen lays about 250 eggs (about 4,000 ml of egg yolk) in a year; thus, the eggs laid by an immunized hen in a year would yield 40 g of IgY. Oral administration of IgY from chicken egg yolk has been used successfully by many researchers in preventing many intestinal diseases, such as those caused by enterotoxigenic E. coli (20) and human rotavirus (9, 23). Sugita-Konishi et al. (24) reported that IgY, obtained from hens immunized with a mixture of formalin-treated pathogenic bacteria, inhibited the growth of Pseudomonas aeruginosa. The production of Staphylococcus aureus enterotoxin A and adhesion of Salmonella typhi-murium serovar Enteritidis to cultured human intestinal cells were also inhibited. IgY antibodies inhibited the colonization of teeth by Streptococcus mutans, thus preventing plaque formation in humans (13). However, there has been no report so far on the use of IgY in the prevention and treatment of H. pylori infections.

In this study, IgY-Hp, obtained from hens immunized with H. pylori whole-cell lysate, dramatically inhibited the growth of H. pylori in vitro. If the antibody IgY had no specific effect, no inhibition of bacterial growth would occur. It seems clear from our observations, using gerbil models, that IgY-Hp decreased H. pylori-induced gastric mucosal injury, as determined by the degree of lymphocyte and neutrophil infiltration. Therefore, the therapeutic value of orally administered IgY-Hp, against the experimental model in gerbils, lies in its ability to inhibit the bacterial organism. More convincing evidence in support of the specificity of these antibodies comes from inhibition of H. pylori attachment to AGS cells, as confirmed by SEM. Although the mechanisms by which IgY-Hp prevented H. pylori colonization are yet not elucidated, our results suggest that IgY-Hp would inhibit H. pylori adherence properties. Of interest, the IgY-Hp used in this study significantly inhibited urease activity. The relationship between inhibitions of urease activity and adhesion properties needs to be clarified. In this study, IgY-Hp seems to have two properties: inhibiting adhesion of
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ACKNOWLEDGMENTS

This work was supported by grant no. 2000-1-20800-005-3 from the Basic Research Program of the Korea Science & Engineering Foundation.

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FIG. 6. SEM findings for H. pylori-infected AGS cells in the presence or absence of IgY-Hp. (A) AGS cells. (B) AGS cells infected with H. pylori (10^6 CFU/ml). (C) AGS cells infected with H. pylori (10^6 CFU/ml) pretreated with IgY-Hp (10 mg/ml).

the bacterium to gastric epithelial cells and demonstrating powerful urease-inhibiting activity.

For practical application of IgY-Hp together with food or pharmaceutical materials to treat H. pylori infection, the stability of IgY-Hp toward heat, acid, or pepsin was studied by measuring the remaining activity by ELISA. Immunologically, IgY-Hp stayed active at 60°C for 10 min, suggesting that pasteurization can be applied to sterilize the product. The fortification of food products with this immunoglobulin, together with its higher productivity and effectiveness, would significantly decrease the activities of H. pylori infections.

In conclusion, the encouraging results of this study indicate that the IgY-Hp obtained from hens immunized by H. pylori may provide a novel approach to the management of H. pylori infections in humans. However, many problems remain unsolved for the clinical application, such as the effect of IgY-Hp in humans, the persistence of the effect of IgY-Hp after cessation of the application, and the potential for eradicating an established infection.
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