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Preventive effect of anti-VacA egg yolk immunoglobulin (IgY) on *Helicobacter pylori*-infected mice

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ABSTRACT

Background: *Helicobacter pylori*, a gram-negative bacterium, is the causative agent of gastric disorders and gastric cancer in the human stomach. Vacuolating cytotoxin A (VacA) is among the multi-effect protein toxins released by *H. pylori* that enables its persistence in the human stomach.
Methods: To evaluate the effect of anti-VacA egg yolk immunoglobulin (anti-VacA IgY) on *H. pylori* infection, a highly specific anti-VacA IgY was produced from egg yolks of hens immunized with a mixture of two purified recombinant VacAs. Female C57BL/6 mice were supplemented anti-VacA IgY daily with drinking water for 2 weeks before and 4 weeks after *H. pylori* ATCC 43504 inoculation. Anti-VacA IgY recognized both native and denatured structures of VacA by enzyme-linked immunosorbent assay and immunoblotting analyses, respectively.
Results: Oral administration of anti-VacA IgYs significantly ($p < .05$) reduced the serum levels of anti-*H. pylori* antibodies compared to those in the *H. pylori*-infected, untreated group. The reduction in the immune response was accompanied by a significant ($p < .05$) decrease in eosinophilic infiltration of the stomach in anti-VacA IgY treated group compared to other groups. Concomitantly, *H. pylori*–induced histological changes and *H. pylori* antigen-positivity in gastric tissues were decreased significantly ($p < .05$) in anti-VacA IgY treated group similar to the control group.
Conclusions: Oral administration of anti-VacA IgY is correlated with a protective effect against *H. pylori* colonization and induced histological changes in gastric tissues. Our experimental study has proved that it is expected to be a new drug candidate of Hp infection by further study.

1. Introduction

There are many possible factors and pathogenic determinants of *H. pylori* that induce most gastroduodenal diseases [1]. Among the *H. pylori* virulence factors, CagA protein plays a key role in *H. pylori*-associated gastric cancer by activating oncogenic signaling pathways and inactivating tumor suppressor pathways [2]; vacuolating toxin (VacA) is an extracellular protein that induces formation of cytoplasmic vacuoles and leads to apoptosis, causing *H. pylori*–induced epithelial cell damage in the host [3,4]. VacA may also play a critical role in the chronicity of *H. pylori* infection by suppressing T cell proliferation [5,6]. Generally, there are two variable regions within the *vacA* gene of clinical isolates of H. *pylori* strains. One is the s-region within the signal peptide, and this region has two types (s1 and s2) at the 5′ end of *vacA*, while the other is the m-region, which contains two additional families (m1 and m2) in the mid-regions of *vacA* [7]. Strains encoding s1/m1 *vacA* typically produce m1VacA with vacuolating activity in AGS and HeLa cells, whereas m2VacA, produced by strains containing s2/m2 *vacA*, cannot induce vacuoles in cultured cells [4]. In addition, colonization with *vacA* s1/*cagA*-positive *H. pylori* strains is associated with inflammation and epithelial degeneration in the gastric mucosa and an increased risk of peptic ulcer disease, whereas colonization with s2/m2 *vacA*/*cagA*-negative strains is associated with mild gastric histopathology and not associated with a significant risk of

Abbreviations: *H. pylori*, *Helicobacter pylori*; VacA, Vacuolating cytotoxin A; anti-VacA IgY, anti-VacA egg yolk immunoglobulin; IgY, egg yolk immunoglobulin; HRP, horseradish peroxidase; Hp group, *H. pylori* infected group; ABC, avidinbiotin-peroxidase; CC, chief cells; PC, parietal cells.

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peptic ulcer disease [8,9]. VacA may be a useful diagnostic tool and promising candidate for vaccine development.

Although the prevalence of *H. pylori* infection in Korea has shown a decreasing trend, primary antibiotic resistance rates are increasing at higher rates such as higher resistance to clarithromycin compared to in other developed countries. Thus, strategies for eradicating *H. pylori* have been changed from treatment to prevention [10]. Therefore, the development of a vaccine to eradicate and prevent reinfection of *H. pylori* is very important [11–13].

New treatments using natural resources such as probiotics and natural plant extracts have been investigated [14]. Among these alternatives, the potential therapeutic effects of anti-*H. pylori* immunoglobulin derived from egg yolk (IgY) have been reported [15–17]. Egg yolk antibodies (IgYs) typically do not interfere with mammalian immunoglobulin G (IgG) or activate the mammalian complement; therefore, egg IgYs do not mediate inflammatory responses in the gastrointestinal tract [18]. In addition, the simple and non-invasive preparation of immunoglobulin from egg yolks makes this antibody an excellent tool for passive immunization [19]. Anti-*H. pylori* IgY can be produced by hens immunized with *H. pylori* antigens. As mothers give their children immunity through the placenta or mother's milk, a hen gives a chick immunity against *H. pylori* through its eggs. Anti-urease IgY decreases the activity of urease from *H. pylori* [20].

In this study, we investigated the potential of anti-VacA IgY produced from egg yolks of hens immunized with recombinant VacA proteins to protect against *H. pylori* infection in a mouse model with *H. pylori* [21]. The effects of daily administration of anti-VacA IgYs on *H. pylori* infection was assessed by examining histological changes in gastric tissue and through serological tests.

2. Materials and methods

2.1. Helicobacter pylori strains and culture conditions

Helicobacter pylori ATCC 49503 (strain 60,190) and ATCC 43504, wild-type, cytotoxic, *cagA*$^+$ strains with the *vacA* genotype s1/m1; ATCC 51932 (Tx30a), a wild-type, *cagA*$^-$ with the *vacA* genotype s2/m2 were purchased from American Type Culture Collection (Manassas, VA, USA). Sydney strain (SS1), a mouse-adapted strain of *H. pylori* [22], was also used. *H. pylori* were grown on Mueller-Hinton (MH) agar plates supplemented with 5% horse serum. The plates were incubated at 37 °C for 2–3 days under microaerophilic conditions in an incubator with 5% CO_2.

2.2. Immunization

Brown Leghorn hens (n = 12) were immunized intramuscularly by injection with 100 μg/mL mixtures of purified m1 and m2 VacA (Supplementary information and Fig. S1) emulsified with an equal volume of Freund's complete adjuvant. The leg muscle of each hen was injected at three different sites. Three booster injections with 50 μg/mL of mixtures of purified m1 and m2 VacA were given at 2-week intervals following the first injection. Two months after immunization, eggs were collected, first weekly for 1 month and then daily. The egg-yolk was stored after separation from the egg white, washed with distilled water, pooled, and freeze-dried.

2.3. Isolation and purification of anti-VacA IgY

IgY was isolated and purified as previously described [23]. Egg yolk was separated from the egg white, and the yolk preparation was mixed with a 5-fold volume of distilled water. After adding an equal volume of chloroform and centrifugation at 4000*g* for 30 min at 4 °C, the water-soluble fraction was collected and filtered through a Whatman No. 3 filter paper to remove solid lipid materials.

2.4. Determination of anti-VacA IgY antibody

An enzyme-linked immunosorbent assay (ELISA) was used to assess the maintenance of VacA antibodies in the collected egg yolks. The 96-well plates were coated overnight at 4 °C with 100 μL of varying concentrations of recombinant m1 VacA or m2 VacA antigen prepared by two-fold serial dilution of 10 μg of proteins with 0.2 M sodium carbonate buffer. After blocking with 1% skim milk, anti-VacA IgY that had been diluted 1:100 in PBS was added. The plates were then washed with PBS containing 0.05% Tween 20. For IgY affinity titration, 100-μL aliquots containing varying concentrations of anti-VacA IgY prepared by two-fold serial dilution of 10 μL of egg yolk extract with 0.2 M sodium carbonate buffer were reacted with a fixed concentration of various strains of *H. pylori* antigen (100 ng/mL *H. pylori* lysates). A secondary antibody goat anti-chicken horseradish peroxidase (HRP)-conjugated antibody (Santa Cruz Biotechnology, CA, USA) diluted 1:5000 in 1% skim milk was used. After washing with PBS/T, 1-Step™ Ultra TMB-ELISA (Pierce, Rockford, IL, USA) was added to each well to amplify the HRP-mediated signal. After incubation for 10 min, the reaction was stopped by adding 2 M sulfuric acid. The optical density at 450 nm was measured using an ELISA reader (Tecan, Männedorf, Switzerland).

2.5. Immunoblot analysis to determine cross-reactivity of anti-VacA IgY

For immunoblotting, 15 μg of lysate and 2 μg of recombinant VacA protein/lane were separated by 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Schleicher & Schuell, Dassel, Germany). The membranes were then blocked with 3% bovine serum albumin in Tris-buffered saline (TBS) containing 0.2% Tween 20 (TBST) for 1 h at room temperature. The primary antibody was a dilution of 1:200 of 10 μg/mL purified anti-VacA IgY in blocking solution. The membranes were incubated overnight at 4 °C. After washing, the membranes were incubated at 25 °C for 2 h in HRP-conjugated goat anti-chicken IgY (Santa Cruz Biotechnology) diluted 1:5000 in 5% skim milk, followed by washing. The color reaction was developed using a commercially enhanced chemiluminescence system (Pierce) and exposed to a medical X-ray film (Kodak, Tokyo, Japan).

2.6. Neutralization of the native VacA toxin activity by anti-VacA IgY

Purified anti-VacA IgY antibodies were tested for their ability to neutralize VacA vacuolating activity and cytotoxic activity. Briefly, prior to adding AGS human gastric epithelial cells (KCLB 21739) monolayer, ATCC 43504 cell free supernatant (CFS) was pretreated with the purified anti-VacA IgY for 30 min at 4 °C. The neutralizing activity of immunoglobulins was assessed in an *in vitro* neutral red uptake assay (550 nm) and MTT assay (595 nm) as described supplementary information and previous reports [4,24].

2.7. Animals

Eight-week old specific pathogen-free female C57BL/6 mice were used. The animals were kept in isolated cages with a 12-h light-dark schedule and fed with standard food pellets and water *ad libitum*. Animal procedures were performed in accordance with NIH guidelines and all animal experiments were conducted in accordance with the rules and regulations of the Institutional Animal Use and Care Committee at Kyungpook National University, Daegu City, Republic of Korea.

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