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Treatment of *Helicobacter pylori* infection in mice with oral administration of egg yolk-driven anti-UreC immunoglobulin

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ABSTRACT

Background: Helicobacter pylori, the causative agent of gastritis and gastric ulcer, plays a crucial role in development of gastric carcinomas. Antibiotic therapy fails in almost 20% of cases due to development of antibiotic resistance. Development of antibodies against specific *H. pylori* targets could have significant therapeutic effect. In the present research attempts have been made to study the effect of IgY purified from egg yolk of hens immunized with recombinant UreC in treatment of mice infected with *H. pylori*.
Materials and methods: Purified IgY-HpUc was used in two forms: powdered and PBS dissolved. 10^9 bacteria in BHI were orally administered to C57BL6/j mice three times on alternate day intervals. Eight weeks after the last inoculation, the serum was assayed for infection rate by ELISA. The severity of gastritis was analyzed histopathologically. Infected mice were randomly divided into three groups. Groups one and two were treated with dietary IgY-HpUc and IgY-HpUc dissolved in PBS respectively for 28 days. The untreated group served as control.
Results: Serology and histopathology confirmed the establishment of the infection. Indirect ELISA results in the treated animals showed considerable reduction of *H. pylori* specific antibodies in their sera. Pathological examination of gastric mucosa of infected mice treated with IgY-HpUc showed considerable reduction of inflammation in the stomach tissues. The bacterial presence on mucosal layer of the stomach was considerably reduced.
Conclusions: UreC-induced IgY is specifically successful in inhibition of *H. pylori* infection and could be an alternative to antibiotic treatment.

1. Introduction

Helicobacter pylori infection is a widespread health problem and is strongly linked to gastric ulcer and cancers [1]. Although majority of the infected individuals do not show any symptoms, chronic infection often increases the risk of *H. pylori*-associated gastric diseases and even gastric carcinomas [2–4]. Successful treatment and eradication of *H. pylori* infection requires employing multiple antibiotic regimens including combination of metronidazole, amoxicillin, clarithromycin and bismuth or a proton pump inhibitor (PPI) [5–7]. Although antibiotic therapy could be successful, but it fails in 20% of cases due to the emergence of antibiotic-resistant strains [6–8]. Development of antibiotic-resistant strains will further complicate treatment of *H. pylori* infection and increase the relapse rates [9]. Thus it is important to investigate novel therapeutic approaches that lead to control or treatment of *H. pylori* infection without causing drug-resistance problems.

Use of specific antibodies has shown to be useful in control or treatment of mouse models of *H. pylori* infection [10,11]. Passive immunization with oral administration of antibodies has shown to be effective in inhibiting intestinal infection with variety of intestinal pathogens [12,13]. Oral administration of antibodies requires large amount of antibodies which is not economically affordable. Recent studies have shown that hen egg yolk is an inexpensive, alternative antibody source. The usefulness of egg yolk-driven immunoglobin Y (IgY) in the control of intestinal infection through oral administration has been demonstrated [14–19]. Passive immunization by ingestion of IgY as in fortified food products, for prevention or control of intestinal infection has been studied in variety of pathogens such as *Escherichia coli* [17], *Salmonella enterica* serovar typhimurium [20], and rotavirus [15,19,21]. These studies indicate that use of IgY against *H. pylori* antigens could be potentially beneficial. Shin et al. [2] showed that IgY produced against whole-cell lysate of *H. pylori* inhibits the growth of the bacterium and reduces gastric inflammation in *H. pylori*-infected Mongolian gerbils. However IgY produced by immunization of hens with *H. pylori* whole-cell lysates could have

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some cross-reaction with other bacteria, including human intestinal normal flora [22]. This will decrease the efficiency and specificity of produced antibody. Therefore, it is imperative to use specific key antigens of *H. pylori* for production of IgY, and obtaining efficient antibody. UreC has been reported to exhibit significant response to anti *H. pylori* IgY [23]. Antibodies against *H. pylori* antigens such as UreB (now known as UreC), CagA, VacA, NapA, HpaA, FlaA, and FlaB, have been detected in the sera of infected patients [24]. However antibody to UreB was present in all the patient sera tested [25]. In this study the effect of UreC-induced IgY on *H. pylori* colonization was evaluated. We used IgY driven from egg yolk of immunized hens with recombinant UreC subunit of urease enzyme. The IgY was orally administrated to *H. pylori*-infected C57BL/6j mice. The result of treatment, serum IgG analysis and pathological observation of mucosa in the infected and treated mice are discussed.

2. Methods

2.1. Expression and purification of UreC subunit of urease enzyme

The UreC gene was optimized by the codon bias of *E. coli* and synthesized by Shine Gene Company (china).

Transformed *E. coli* BL21DE3 bacteria containing pET28a recombinant construct was cultured in LB medium containing 70 μg/ml Kanamycin and incubated at 37 °C/200 rpm. Expression of recombinant protein was induced with isopropylthio-β-D-galactoside (IPTG). The results were studied by SDS-PAGE analysis. Recombinant protein was purified by Ni-NTA affinity chromatography. The concentration of purified recombinant UreC protein was estimated by Bradford method.

2.2. Immunization of hens

200 μg (250 μl) of recombinant UreC protein with equal volume of freund's complete adjuvant (Razi institute, Iran) was injected subcutaneously into 25-week-old white Leghorn hens ($n = 6$) in the chest area. Three booster injections, with Freund's incomplete adjuvant, were given at 2-week intervals following the first injection. Each booster dose contained a total concentration of 200 μg of recombinant UreC protein with equal volume of Freund's incomplete adjuvant. Freund's complete adjuvant without protein was injected to the negative control group. Immunization of hens was confirmed by indirect Enzyme Linked Immunosorbent Assay.

96 well ELISA plates (JET BIOFILM) were coated by recombinant UreC subunit of *H. pylori* Urease Enzyme (4 μg/well) and incubated overnight at 4 °C. Blocking was performed by applying 100 μl of 5% (w/v) per well of skim milk in PBS (NaCl 0.8%, KCl 0.02%, Na_2HPO_4 0.29% and KH_2PO_4 0.02% in deionized distill water) followed by incubation at 37 °C with mild agitation for 60 min 100 μl of serum at 1:100 dilution was added to the first well and followed by a two-fold serial dilution and then incubation was performed at 37 °C with mild agitation for 45 min.

100 μl of anti-IgY antibody conjugated with horseradish peroxide enzyme (GeNet, India) at the final concentration of 1:3000 was added to each well and incubated at 37° C with mild agitation for 30 min 100 μl of substrate buffer containing 0.8% Orthophenylendiamine (OPD) (Sigma, USA) and 8% H_2O_2 in citrate buffer was added to each well and kept in dark for 15 min. The reaction was stopped by addition of 100 μl of 3 N H_2SO_4. The absorbance was measured by microplate ELISA reader (Perlong New Technology, Beijing) at 492 nm. The uncoated wells, blocked by 5% skim milk in PBS, were served as negative control. Washing was performed after each step using 200 μl of PBS-T (0.05% Tween-20 in PBS [pH 7.2]).

2.3. Isolation and purification of IgY-HpUc

Isolation of IgY-HpUc was carried out by separation of the egg yolk from the white part. The egg yolk was washed three times with distilled water, followed by addition of two volumes of phosphate buffer (sodium phosphate 0.1 M, NaCl 10 mM, pH 7). The solution was then mixed with magnetic stirrer. 3.5% (w/v) poly ethylene glycol (PEG 6000) was added to the egg yolk and mixed with magnetic stirrer to remove lipoproteins. The solution was kept at room temperature for 60 min. The mixture was centrifuged at 8000 rpm at 4° C and the supernatant was filtered through Whatman filter No. 1 to remove lipid traces. Purification of IgY was carried out by applying 12% (w/v) poly ethylene glycol (PEG 6000) to the previous filtrate and mixed by magnetic stirrer. The solution was maintained at room temperature for another 60 min, followed by centrifugation at 8000 rpm for 10 min. The sediment was then resuspended in equal volume of phosphate buffer and preserved at 4° C until further use.

2.4. SDS-PAGE analysis of IgY-HpUc

Sodium dodecyl sulfate polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out using 9% PAGE in Bio-Rad Mini Protein system. 20 μl of IgY-HpUc and 20 μl of IgY isolated and purified from non-immunized hens were separately diluted at 1:4 ratio with sample buffer (62.6 mM Tris–HCl, pH 6.8, 25% glycerol [v/v], 2% SDS[v/v] and 5% β-mercaptoethanol) and heated at 100° C for 10 min 10 μl from each sample was loaded into each well. Pre-stained protein molecular marker (Fermentas) was used as a standard molecular weight marker.

2.5. Assessment of anti–urease action for the purified IgY–HPUC antibody

For the measurement of IgY-HpUc anti-urease activity, *H. pylori* was inoculated in BHI and incubated at 37° C under microaerophilic conditions until the OD_{600} reached 0.5. Different concentrations of IgY-HpUc were added to each of these cultures, followed by six more hours of incubation under the same conditions (37 °C, Microaerophilic conditions). 25 μl urea-phenol red solution (2% urea and 0.03% phenol red) was then added to each of these cultures. The activity of IgY-HpUc was assayed by change in color and measurement of optical density at 550 nm. The amount of IgY required to inhibit 50% of urease activity (IC_{50} of IgY-HpUc) was expressed as mg/ml ($y = 8.012x + 1.932$ R^2; $= 0.996$).

2.6. Inhibition of H. pylori growth by IgY-HpUc

The colony forming units (CFU) were determined after incubation of *H. pylori* with IgY-HpUc in order to estimate adhesion potential of IgY-HpUc to the bacterium. *H. pylori* was cultured in BHI for 48 h to reach 10^9 bacteria/ml. One mg of IgY-HpUc was added to 1 ml of the bacterial suspension and incubated for 30 min at 37° C. It was then spread in 100 μl quantity on Brucella agar. BSA was used instead of IgY-HpUc to serve as negative control. The colonies grown on the agar media were comparatively counted.

2.7. H. pylori culture and development of infection in mice

The *H. pylori* used in the present study was Sydney strain SS1. The colonies were first cultured in Brucella agar medium (Difco) and then transferred to Brain Heart infusion (BHI) Broth (Merck) supplemented with 7% (v/v) fetal calf serum. The campylobacter antibiotic supplement (MERK), including antibiotic such as vancomycin, trimethoprime and polymixine B, diluted in 2 ml of sterile distilled