Inhibitory Effects of Chicken Egg Yolk Antibody on Infection of *Escherichia coli* in Macrophage

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Received: FEB. 28. 2012, Revised: APR. 03. 2012, Accepted: APR. 27. 2012

ABSTRACT

The present study evaluated the potential use of immunoglobulin prepared from egg yolk of chickens immunized with *Escherichia coli* K88 (IgY-Ec) in the control of *E. coli* K88 infection in RAW 264.7 murine macrophage. The binding activity of IgY-Ec against *E. coli* K88 surface protein was more specific and increased than control IgY. In infection assay of *E. coli* in macrophage, the specific IgY-Ec to *E. coli* K88 remarkably inhibited the phagocytic activity comparing to nonspecific IgY (p<0.001). In adherence assay, bacterial adhesion on macrophage cells was definitely reduced by preincubation of IgY-Ec compared with nonspecific IgY (p<0.05). These findings suggested that IgY-Ec have the protective effects against pathogens and IgY-based diets may have potential benefits for preventing or treating various infections in domestic animals.

Key words - Egg yolk immunoglobulin (IgY), *Escherichia coli*, Infection, Prevention

I. INTRODUCTION

*Escherichia coli* is the common cause of various intestinal and extra-intestinal infections, such as diarrhea, urinary tract infection, meningitis, peritonitis, septicemia, and gram-negative bacterial pneumonia. Diarrheal disease due to enterotoxigenic *E. coli* (ETEC) is an important health problem in humans and animals. The ETEC strains that are involved in intestinal colonization followed by elaboration of diarrheagenic enterotoxin and cause severe diarrhea are the K88, K99, and 987P fimbrial adhesions (Parry & Rooke, 1985).

In many cases of infectious diarrhea, although only rehydration therapy is appropriate, antimicrobial agent therapy is designated for children and adults with acute infectious gastroenteritis in diverse circumstances, including dysentery, prolonged disease, and elimination of fecal shedding (Pickering, 2004). Among the diarrheagenic *E. coli*, there is a conventional advantage for the utilization of antibiotics for infections of ETEC, EIEC, and EAEC; these verdicts are based principally on data in traveler’s diarrhea (Cabada & White, 2008). However, the role of antibiotic therapy in children with acute diarrhea caused by a diarrheagenic *E. coli* is not fully defined. Moreover, antibiotic resistance among ETEC strains is increasing, perhaps due to indiscriminate use of antibiotics (Pickering, 2004; Estrada-Garcia et al., 2005).

Feeding colostrum from vaccinated cows has been documented to prevent diarrhea due to pathogenic *E.
coli in human infants (Hilpert et al., 1977). Distinct egg yolk immunoglobulin (IgY) can be produced in egg yolk by immunizing chickens with specific antigens. The IgY can be isolated from egg yolk in great amounts by simple techniques (Akita & Nakai, 1993). Thus, IgY has been a focus for a passive, economical and effortless generating vaccine and been distinguished to be proficient in therapy and prevention (Carlander et al., 2000). Specific IgY can control infectious disease by E. coli in piglets (Erhard et al., 1996; Jin et al., 1998), and by Salmonella in mice (Gurtler et al., 2004).

The present study was performed to evaluate its ability to inhibit E. coli-associated disease in vitro using the specific IgY against E. coli K88.

The present study, taken together, was performed to evaluate its ability to inhibit E. coli-associated disease in vitro using the specific IgY against E. coli K88, and to provide the potential advantage for controlling and preventing the diseases caused by E. coli.

II. MATERIALS AND METHODS

2.1 Bacterial culture and media:

E. coli K88 (ATCC 14028) were maintained as frozen glycerol stocks and were cultured in Luria-Bertani (LB) broth or LB broth containing 1.5% agar. Bacteria were grown at 37°C with vigorous shaking to a stationary phase in LB broth.

2.2 Cell culture

The murine macrophage cell line RAW 264.7 cells were grown at 37°C in a 5% CO₂ atmosphere in RPMI 1640 (GIBCO) containing 10% heat-inactivated fetal bovine serum (Invitrogen), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). RAW 264.7 cells seeded (1×10⁴ per well) in 96-well cell culture plates and incubated for 24 h before infection for all assays at 37°C in 5% CO₂ atmosphere.

2.3 Production of specific egg yolk immunoglobulin (IgY)

The preparation of immunoglobulin from egg yolk of chickens immunized with E. coli K88 was performed as described in a previous study (Park et al., 2011). Briefly, the specific antigen was collected from E. coli K88, and was then mixed with an oil adjuvant to produce a combined vaccine. This combined vaccine was inoculated into layer chickens three times at three week intervals. The eggs were selected two weeks following the last inoculation, washed with sodium hypochlorite and dried. The yolk sac in dried eggs was separated from the egg white and prepared as dried egg yolk sac powder, which was tested for the antibody titer.

2.4 Cytotoxic assay

To determine the cytotoxicity of IgY, RAW 264.7 cells were grown in the presence of different concentrations of IgY (0, 50, 100, 200, and 400 μg/mL) in a 96-well cell culture plate for 48 h. Cell viability was measured by 3-(4,5-dimethylthiazol-2-y1)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) cleavage assay.

2.5 Isolation of bacterial surface proteins and western blot

To extract the bacterial surface proteins, a 10 g (wet weight) sample of E. coli was washed twice with cold EDTA/NaCl (25 mL, 0.12 M-EDTA, 0.77 M-NaCl, pH 7.2). The combined extracts obtained after centrifugation (20,000 × g, 30 min) were concentrated by negative pressure dialysis against 0.01 M PBS and
again centrifuged for 15 min. The resulting supernate portion was lyophilized and resuspended with PBS. Sample (40 μg) was mixed with sodium dodecyl sulfate sample buffer, incubated at 100°C for 5 min before loading, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were electrotransferred to nitrocellulose membranes and were incubated with IgY-Ec or non-immunized IgY (control IgY). The binding of primary antibody was visualized using HRP-conjugated secondary chicken Ig. The blot was developed with enhanced ECL according to the manufacturer's instructions (iNtRON Biotechnology).

2.6 Bacterial adherence assay

RAW 264.7 cells were cultured in 12-well plates with 18 mm-diameter glass coverslips at 10^5 cells/well 1 day before the infection. The cells were pre-treated with IgY-Ec (200 μg/mL) or control IgY for 4 h.

During the last 40 min of pre-treatment, MEM that contained cytochalasin D (500 μg/mL) was added to the cells, which were subsequently infected by E. coli at an MOI of 10 for 30 min. The infected cells were fixed in 4% (w/v) periodate-lysine-paraformaldehyde-sucrose for 1 h at 37°C. To monitor adherent bacteria on the cell surface within of infection, the preparations were stained with anti-E. coli K88 polyclonal pig serum (1:500) in blocking buffer for 1 h at 37°C and subsequently stained with FITC-conjugated goat anti-pig IgG (1:1000) in blocking buffer for 1 h at 37°C. Finally, the preparations were mounted with DakoCytomation fluorescent mounting medium. Immunofluorescence images were collected with a microscope (DMIREE2; Leica) equipped with a camera (DC350F; Leica) and IM50 imaging software (Leica). One hundred macrophages were selected randomly, and bacteria adhered to these cells were counted.

2.7 Determination bacterial uptake

For analysis of bacterial uptake efficiency, overnight-cultured cells were pre-treated with IgY-Ec (200 μg/mL) or control IgY for 4 h prior to infection. Following treatment, E. coli was deposited onto cells at MOIs of 10, centrifuged at 500 × g for 10 min at room temperature and incubated at 37°C in 5% CO2 for 0, 30, and 60 min. Cells were washed once with media and then incubated with RPMI 1640 media with gentamicin (30 μg/mL) for 30 min to kill any remaining extracellular bacteria. To evaluate the number of viable bacteria at different periods of time, the infected cells were washed three times with PBS and then lysed with distilled water. The number of viable bacteria was determined by counting the cfu. All assays were conducted in triplicate and repeated at least three times on different days.

2.8 Statistical analysis

The results obtained were expressed as the mean ±S.D. for the replicate experiments. Student’s t-test was used to make a statistical comparison between the groups. Results with p<0.05 were considered statistically significant.

III. RESULTS AND DISCUSSIONS

3.1 Cytotoxic effect and morphologic change of macrophage

To evaluate the cytotoxicity of IgY, RAW 264.7 cells were grown in the presence of different concentrations of IgY (0, 50, 100, 200, and 400 μg/mL) in a 96-well cell culture plate for 48 h. Cell viability was assessed by the MTT assay and correlated with OD values. As a result, there was no detectable cytotoxic effect with any of IgY at concentrations
between 0 and 200 μg/mL, showing the survival rates of more than 98% in IgY-treated cells comparing to untreated cells, which was set at 100%. In light of these findings, IgY was used at concentration less than 200 μg/mL in all of the experiments performed in this study.

3.2 Specific binding activity of IgY-Ec on bacterial surface proteins
To determine the binding capacity and pattern of IgY-Ec on E. coli K88 surface proteins, the extract of E. coli K88 surface proteins was subjected by immunoblotting using an anti-E. coli K88 IgY (IgY-Ec) or an non-immunized IgY (control IgY). As shown in Fig. 1, the IgY from immunized chicken to E. coli K88 binds to E. coli K88 surface antigens with the more higher binding site than control IgY, demonstrating the specific binding ability of IgY against bacteria. Moreover, IgY-Ec displayed some different binding patterns to E. coli K88 surface antigens comparing with control IgY. Thus, this finding suggests that E. coli K88-specific IgY possesses specific binding ability against E. coli K88.

3.3 Effects of IgY-Ec on bacterial adherence on macrophage
To evaluate the bacterial adherence, macrophage cells incubated in the presence of IgY-Ec or control IgY for 4h were subsequently infected with E. coli K88 for 30 min. One hundred macrophage cells were collected randomly, and bacteria that adhered to the cells were counted. The results revealed that the number of adherent bacteria to cells pretreated with IgY-Ec (34.01 ±3.77) was significantly lower than that of cells pretreated with control IgY (46.25 ± 2.52), showing the reduction rate of 26.48 ± 1.36 % (p<0.05) (Fig. 2). This result precisely elucidates that IgY-Ec disturbs with the adherence of E. coli K88 to murine macrophage cell surfaces.

When we determined the binding capacity and pattern of IgY-Ec on E. coli K88 surface proteins, the IgY from immunized chicken to E. coli K88 binds to E. coli K88 surface antigens with the more higher binding site than control IgY. Moreover, IgY-Ec displayed some different binding patterns to E. coli K88 surface antigens, indicating the modification of E. coli surface proteins caused by binding with specific IgY-Ec was observed comparing with control IgY. Similarly, there has been described that specific IgY could attach to constructs exposed on the surface of S. typhimurium, leading to structural alterations(Lee et al., 2002). Thus, this finding suggests that E. coli K88-specific IgY possesses specific binding ability...
against *E. coli* K88.

IgY could be an alternative source of immunoglobulins for the prevention of enterotoxigenic *E. coli* ETEC infection, because it has been demonstrated to inhibit the binding of *E. coli* to the intestinal mucosa (Jin et al., 1998). IgY raised against ETEC antigen has been treated orally to piglets and recommends a prospective prophylactic and therapeutic approach for management of ETEC-involved diarrhea (Marquardt et al., 1999). Some investigator studied the passive protective effect of IgY against ETEC infection in neonatal piglets (Yokoyama et al., 1992). The passive protective effect of anti-ETEC IgY against fatal enteric colibacillosis in neonatal calves has also been studied (Ikemori et al., 1992). Calves fed milk containing IgY had temporary diarrhea, perfect survival, and exceptional body weight gain.

3.4 Effect of IgY-Ec on bacterial phagocytic activity in macrophage

To evaluate the effects of IgY-Ec on the phagocytosis of *E. coli* K88, RAW 264.7 macrophages were pre-treated with IgY-Ec (200 μg/mL) or non-immunized IgY (control IgY) for 4 h. And then infected with *E. coli* K88, and bacteria attached to cells were scored by immunofluorescence microscopy. One hundred cells were examined per coverslip. Data are the averages of triplicate samples from three identical experiments, and error bars represent the standard deviations. Statistically significant differences between bacterial adherence of control IgY and that of IgY-Ec are indicated by an asterisk (*, P < 0.05).

![Graph showing bacterial adherence to macrophages](image)

Fig. 2. Effects of IgY-Ec on bacterial adherence to macrophage surfaces. Before bacterial infection, RAW264.7 cells were pre-treated with *E. coli* K88 IgY (IgY-Ec, 200 μg/mL) or non-immunized IgY (control IgY) for 4 h. And then infected with *E. coli* K88, and bacteria attached to cells were scored by immunofluorescence microscopy. One hundred cells were examined per coverslip. Data are the averages of triplicate samples from three identical experiments, and error bars represent the standard deviations. Statistically significant differences between bacterial adherence of control IgY and that of IgY-Ec are indicated by an asterisk (*, P < 0.05).

Interestingly, the surface alterations of *S. typhimurium* by binding of specific IgY may hinder the adhesion of bacteria to target cell surfaces, that is essential for establishing infection in epithelial cells (Lee et al., 2002). And specific IgY blocked the *Salmonella* spp. adhesion to a greater extent than nonspecific IgY, correlating to growth inhibitory effect. *E. coli* K88, K99, and 987P strains adhered equally to porcine duodenal and ileal epithelial cells but failed to so in the presence of homologous anti-fimbrial IgY (Yokoyama et al., 1992; Ikemori et al., 1993). Some study revealed that the specificity of IgY against *Helicobacter pylori* comes from inhibition of *H. pylori* attachment to AGS cells (Shin et al., 2002). In line
with the evidence, IgY-Ec interferes with the adherence of *E. coli* K88 to macrophage cell surfaces, suggesting that IgY-Ec could negatively affect the adherence of *E. coli* K88 to macrophage surface membrane for invasion of bacteria.

![Graph showing the effect of IgY-Ec on bacterial phagocytic activity in macrophage](image)

**Fig. 3.** Effect of IgY-Ec on bacterial phagocytic activity in macrophage. RAW 264.7 macrophages were pre-treated with IgY-Ec (200 μg/mL) or control IgY for 4 h prior to infection, and then *E. coli* K88 were deposited onto cells. Bacterial internalization efficiency was calculated by measuring the protection of internalized bacteria from gentamicin killing. Data are the averages of triplicate samples from three identical experiments, and error bars represent the standard deviations. Statistically significant differences relative to the untreated control sample are indicated by asterisks (*, P < 0.05; ***, P < 0.001).

*E. coli* is a commensal bacterium in the intestine of poultry, cattle, and pigs that are utilized for food products, and food originated by animal can be contaminated with *E. coli*, which has mostly been associated with intestinal pathogenic *E. coli* (Nataro & Kaper, 1998). There is a large range of opinion on high resistance levels to antibiotics against diarrheagenic *E. coli*. Recurrent antibiotic use, mainly one month before exposure, is a possibility of risk for progressing infection or colonization with resistant bacterial pathogens (Magee *et al.*, 1999; Vanden Eng *et al.*, 2003). Therefore, alternative therapies for *E. coli*-associated diarrhea are urgently required. Several studies concerning to the relation of IgY with the infection of pathogen evidenced that IgY against *H. pylori* would significantly decrease the *H. pylori* infection in AGS cells (Shin *et al.*, 2002). Moreover, there has been reported that IgY improved the phagocytosis of *S. aureus* by neutrophils (Nie *et al.*, 2004). In this study, the entry of *E. coli* K88 in IgY-Ec-treated cells were significantly reduced compared to control IgY, suggesting that IgY-Ec has an inhibitory effect on phagocytosis of *E. coli* K88 into macrophages. Taken together, the IgY from immunized chicken to *E. coli* K88 contribute to the suppression of bacterial infection in macrophages.

IV. Acknowledgements
This study was supported by Technology Development Program for (‘Agriculture and Forestry’), Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea (No. 20080439)

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