Age-dependent response to CpG oligodeoxynucleotides against organ invasion of Salmonella enteritidis in broiler chickens

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Bacterial infections cause significant economic losses in the broiler industry throughout the production cycle. It is known that CpG-ODN stimulated innate immune activities and enhanced the resistance to infectious pathogens in chickens. In this study, the effectiveness of CpG-ODN was evaluated in organ colonization by Salmonella enteritidis at different ages of chickens. Pre-treatment of 2-day old chickens with CpG-ODN by subcutaneous injection and subsequent challenge with S. enteritidis 3 h after treatment resulted in significant reduction of organ invasion by S. enteritidis. In a second experiment, these chickens were repeatedly treated with CpG-ODN at day 20, and 24 h later were orally infected with S. enteritidis. There was no significant reduction in organ invasion by S. enteritidis compared to PBS treated controls. In a third experiment, five-day old chickens were treated with CpG-ODN and 24 h later orally infected with S. enteritidis. There was no reduction in organ invasion compared to PBS treated control group. Our study shows that CpG-ODN increase resistance against S. enteritidis invasion in broiler chicken in early age.

Key words: CpG-ODN, Salmonella enteritidis, immunoprotection, poultry.

INTRODUCTION

Infectious diseases remain a threat to the poultry industry, and countermeasures to prevent and control them are needed (Kogut, 2009). Immunological interventions to reduce microbial pathogens in poultry would be of great value to the poultry industry and to the consumer. DNA containing six base pair motifs consisting of an unmethylated CpG dinucleotide flanked by two 5-purine and two 3-pyrimidines, are rarely present in eukaryotic but are common in prokaryotic genomes. Bacterial DNA and synthetic oligonucleotides that express these CpG motifs rapidly stimulate B cells, T cells, and macrophages to proliferate, secrete Abs, and produce a variety of Th1-associated immunomodulatory cytokines (Patel et al., 2008; Elkins et al., 1999). CpG motif (CpG-ODNs) mimicking bacterial and viral DNA has been demonstrated to retain immunostimulatory activities (He et al., 2006).

Recognition of CpG-ODNs by receptors such as Toll-like receptors, stimulates the immune system to mount a rapid innate immune response and promotes the development of Th1 protective immunity. Owing to the strength and nature of this stimulation, CpG-ODNs have been tested for immune therapeutic and protective use (Krieg, 2002; Klinman et al., 2004; Dar et al., 2009; He et al., 2006). Toll-like receptors (TLRs) are expressed in many types of immune cells and function as the key sensors of microbial infections. TLR9 recognizes bacterial DNA and its synthetic oligodeoxynucleotide containing unmethylated CpG motifs (He at al., 2007b).

An optimal immune response to an infection would be immunoresponsive. The nature of an optimal immune response is dependent upon specific conditions such as environment, nutritional status and age of birds (Kogut, 2009). Thus, according to the age of birds, modulating the innate immune response would be advantageous during the first week of life.

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Several studies have also demonstrated that immune stimulatory CpG-ODNs provide immune protection of chickens from lethal challenge of *Escherichia coli* and the parasitic infection, *Eimeria* (Dalloul et al., 2004; Gomis et al., 2003, 2004). Previously, it has been shown that intraperitoneal (ip) administered immune stimulatory CpG-ODN reduces systemic infection and mortality in neonatal chickens induced by oral and ip challenge of *Salmonella enteritidis*, respectively (He et al., 2005). The objective of this study was to determine the effect of CpG-ODN as immunostimulants at different ages in broiler chickens.

**MATERIALS AND METHODS**

**Bacteria**

A field isolate of *S. enteritidis* from a chicken with yolk sac infection was used as the source of the challenge strain. The isolate was confirmed by detection of *invA* gene by PCR. Aliquots of this field isolate were stored at -80°C in nutrient broth supplemented with 15% glycerol. The actual number of bacteria was determined by plating duplicate 10-fold serial dilutions of the culture on nutrient agar and incubating at 41°C for 24 h. The challenge inoculums were washed once in normal saline at a concentration of 10^8 cfu/ml.

**Synthetic CpG-ODN**

The sequence of CpG-ODN was GTCGTTGTCGTTGTCGTT with phosphorothioate backbone, which was synthesized by Bioneer Company (South Korea). This CpG-ODN contains three consecutive GTCGTT motifs which have been previously shown to induce innate resistance to *S. enteritidis* infection in neonatal chickens (He et al., 2006). Synthetic CpG-ODN were diluted in sterile PBS and administered in a 0.1 ml volume by subcutaneous route.

**Animals**

Day-old broiler chicks were obtained from a local hatchery in Mashhad, Iran. Birds were placed in metal cages in a controlled environment and provided *ad libitum* access to water and a balanced un-medicated diet with nutrient rations meeting or exceeding the recommendations of the National Research Council (1994).

**Experiment design**

Each experiment consisted of three groups: group A (PBS with bacterial infection), group B (PBS without bacterial infection), and group C (CpG-ODN 50 μg/bird with bacterial infection).

**Effect of CpG-ODN treatment on Salmonella enteritidis infection in two-day old chickens**

This experiment was conducted by using 72 chickens which were randomly allocated into three groups of 24 chickens each, injected with CpG-ODN (group G) and PBS (groups A and B) subcutaneously on day 2. All the chickens in group A and C were challenged orally with *S. enteritidis* (1.5 × 10^8 cfu/ml), 3 h after CpG

**Effect of repeated CpG-ODN treatment on Salmonella enteritidis infection at 20-day old chickens**

This experiment was designed by using 45 chickens allocated into three groups of 15 chickens each as the first experiment. Fifteen chickens in group C which were already treated with CpG-ODN on day two of age, received the same dose of CpG-ODN on day 20. All the chickens in groups A and C challenged orally with *S. enteritidis* (9 × 10^8 cfu/ml), 24 h after CpG-ODN treatment.

**Effect of CpG treatment on Salmonella enteritidis infection in 5-day old chickens**

This experiment was also conducted by using 60 chickens allocated into three groups of 20 chickens each as the first experiment. All of chickens in these experiments were five-day old. All of chickens in groups A and C were challenged orally with *S. enteritidis* (1.5 × 10^8 cfu/ml), 24 h after CpG-ODN treatment.

All chickens in each experiment were euthanized with cervical dislocation 6 days after orally challenged with *S. enteritidis*. Organ invasion was determined by culturing of liver and spleen. Liver and spleen were aseptically removed from each chicken and cultured as a combined sample in an enrichment Rappaport broth overnight (18 to 24 h) at 41°C. After incubation, the broth (10 ml) was streaked on XLD agar and incubated for an additional 24 h at 41°C. The plates were then examined for the presence of *S. enteritidis*. Suspected colonies were confirmed by chemical tests (Urea agar, TSI agar, and Simmons citrate agar).

**Data analysis**

The dichotomous outcomes were considered at each experiment. Statistical differences were determined at the level of *P* < 0.05 by Chi-square test using SAS software (v. 9.2).

**RESULTS**

Effect of CpG-ODN on systemic infection of *S. enteritidis* was evaluated by organ invasion of *S. enteritidis* in experimental chickens with different age:

**Effect of CpG-ODN treatment on Salmonella enteritidis infection in two-day old chickens**

Bacterial invasions were 4.16 and 25% in group C and group A, respectively. Significantly (*P* < 0.05) fewer *S. enteritidis* positive liver and spleen were observed in group C (CpG-ODN) than group A (PBS with bacteria) at 3 h after challenged orally with *S. enteritidis* (Table 1).

**Effect of repeated CpG-ODN treatment on Salmonella enteritidis infection at 20-day old chickens**

There was not significant difference between group C(CPG-ODN) and group A (PBS with bacteria) in this
Table 1. Effect of subcutaneous injection of CpG-ODN on organ invasion of S. enteritidis on 2 and 5 days old chickens and the effect of repeated CpG-ODN on 20 days old chickens.

<table>
<thead>
<tr>
<th>CpG-ODN</th>
<th>No of chickens with bacterial invasion/total chickens (%) in treatment group</th>
<th>No of chickens with bacterial invasion/total chickens (%) in control group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG-ODN inoculated at 2 days old chickens</td>
<td>1/24 (4.16%)</td>
<td>6/24 (25%)</td>
<td>0.044</td>
</tr>
<tr>
<td>CpG-ODN inoculated at 2 days old chickens and repeated at 20 days</td>
<td>0/15 (0%)</td>
<td>4/15 (27%)</td>
<td>0.09</td>
</tr>
<tr>
<td>CpG-ODN inoculated at 5 days old Chickens</td>
<td>3/20 (15%)</td>
<td>3/20 (15%)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Organ (liver and spleen) invasion by S. enteritidis was evaluated in experimental chickens that were treated with CpG-ODN and control PBS followed by oral inoculation of S. enteritidis 3 h later. Liver and spleen were removed 6 days after oral inoculation and cultured for the presence of S. enteritidis. Data represent the number and percentage of liver/spleen S. enteritidis positive outcome in the experimental groups.

experiment (P = 0.09) (Table 1).

Effect of CpG treatment on Salmonella enteritidis infection in 5-day old chickens

Bacterial invasion was 15% in group C and group A (P = 1.0). Thus, there was not significant difference between these two groups (Table 1).

DISCUSSION

It is generally accepted that disease prevention by immune stimulation is preferred to the use of therapeutic agents in the food animal industry. This has become especially important due to a number of food safety and human health issues (Gomis et al., 2003). Synthetic CpG-ODN has been shown to be effective stimulators of immune system in number of species including mice, primates, human, and poultry (Gomis et al., 2003). Newly hatched chickens, which have severe systemic diseases, experience high morbidity and mortality. Among those diseases that target newly hatched chicks, salmonellosis due to S. enteritidis and colibacillosis due to E. coli are the most economically important (Barnes, 2001). It has been demonstrated that CpG-ODN has an immunoprotective effect against E. coli that often infects neonatal broiler chickens (Gomis et al., 2004). However, some studies were conducted on the effect of CpG-ODN against S. enteritidis in neonatal chickens (He et al., 2005; He et al., 2007a; Mackinnon et al., 2009; Taghavi, 2008). In present study, organ invasion of S. enteritidis was significantly reduced in CpG-ODN group in two-day old chickens compared to control group.

The different routes of treatment and age of the birds when treated with CpG-ODN may account for the differences observed between studies (MacKinnon et al., 2009). In our study, three experiments were conducted with chickens in different ages. In all of these experiments, CpG-ODN was injected subcutaneously. Gomis et al. (2003) were found that the subcutaneous administration of CpG-ODN was most effective in protection of chicken against E. coli infection.

Our results in the second experiment showed that there was no significant difference in organ invasion of S. enteritidis between chickens in treatment group which received repeated dose of CpG-ODN at day 20, and the control group. However, a tendency to be significantly effective was seen in repeated treatment of CpG-ODN. The low number of animals in this experiment might be a limited factor. On the other hand, CpG-mediated protection appear to last only a few days. Frequent administration of CpG at short intervals may prolong protection as previously reported in mice. In our study, only one repeated administration of CpG-ODN with a period of 18 days interval had any effect on organ invasion of salmonella compared to control groups.

Our results showed that there was no significant difference in organ invasion of salmonella in third experiment between control group and treatment chickens which received CpG-ODN at day 5. In conclusion, CpG-ODN was an effective immunoprotective agent in chickens only in the first few days of life. Further research on the age-dependent CpG-ODN protection and particularly repeated treatment effect of CpG-ODN is required against Salmonella invasion to the organs in poultry.

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