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# Immunomodulatory Effect of CpG Oligodeoxynucleotide on Infectious Bursal Disease Vaccine Antibody Response and Complement Activity in Chicks

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**Abstract:** The immune stimulatory activities of bacterial DNA are attributed to the presence of a high frequency of an unmethylated CpG dinucleotide motif. The aim of the present study was to evaluate the effects of CpG ODN administration on complement activation and humoral immune response as innate and adoptive immune response indexes, respectively. In the present study Chicks were divided into 6 treatment groups; include Normal control, vaccine control, CPG control and 3 different doses of CpG with vaccine. Blood samples were collected at days 7, 14, 21, 28 and 35. IBD vaccine specific antibody and complement activity were evaluated by ELISA and hemolytic technique, respectively. The comparison of antibody titer and complement activity of CpG control and experimental groups with normal control, show significantly ($P<0.05$, 0.01) higher levels of antibody and complement activity in study groups. According to the present study, it is found that the immunostimulatory effect of CpG ODN can be utilized to create a protective vaccine when given in combination with IBD vaccine plus complement activity. CpG ODN had the ability to augment innate and adoptive immune response in chickens.

**Key words:** CpG Oligodeoxynucleotide · Infectious Bursal Disease Vaccine · Antibody response · Complement activity · Chicks

## INTRODUCTION

DNA from bacteria, but not vertebrates, is a powerful stimulator of the innate immune system. The immune stimulatory activities of bacterial DNA are attributed to the presence of a high frequency of an unmethylated CpG dinucleotide motif, which is an unmethylated cytosine followed by guanosine (CpG) dinucleotide flanked by certain nucleotides [1]. It has been well documented that bacterial DNA and synthetic oligodeoxynucleotide (ODN) containing unmethylated CpG-dinucleotides (CpG-ODN) activate B-lymphocyte and innate immune cells (macrophage, dendritic cells and natural killer cells) to secret cytokines [interleukin-1b (IL-1b), IL-6, IL-12, IL-18, tumor necrosis factor-a (TNF-a), interferon-a (IFN-a) and IFN-g] and to promote adaptive immune responses [10]. Recognition of CpG-ODN and signaling of cell activation are mediated by the Toll-like receptor 9 (TLR9) [2,3]. Interaction of CpG-ODN with TLR9 occurs exclusively in the intracellular compartment [4]. Internalization by endocytosis and subsequent endosomal maturation is therefore the prerequisite for CpG-ODN activity [5].

Recently, the induction of immune response in chickens immunized with CpG ODN also has been reported [6]. The birds were immunized with bovine serum albumin (BSA) and the serum antibody response was followed. A significantly higher BSA-specific response was observed in the CpG-treated group. Moreover, immunostimulatory DNA resulted in more persistent responses to immunization [6].

The avian immune system has a variety of tools at its disposal to combat virus infections, including the complement system that, as an innate immune component, is immediately ready to target and eliminate virus particles and to interact with the surface of virus-infected cells [7,8]. Complement activation is a crucial component of both innate immunity, in the forms of alternative (APW) and mannanbinding lectin (MBL) activation pathways [9-11] and adaptive immunity (classical, antibody-dependent complement activation, CPW) [12] acquired over time following virus infection or vaccination [13]. The purpose of vaccination of chickens, as of other species, is usually to induce enhanced specific and protective immune

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responses such as specific antibodies or memory T cells [14] against important viral diseases at an appropriate time.

The aim of the present study was to evaluate the effects of CpG ODN administration on complement activation and humoral immune response as innate and adoptive immune response indexes, respectively.

## MATERIALS AND METHODS

**Experimental Birds:** One day old broiler chicks (Ross 308) kept in open floor pens at a maximum initial density of 10 birds per $m^2$. Wood shavings were used as litter. The birds had access to food and water *ad libitum*. Ambient temperature and ventilation were regulated in keeping with standard breeding practices. A series of commercial pelleted feed mixes for broiler rearing formulated to meet or exceed the nutritional requirements of broilers as recommended by the NRC [15]. A completely randomized experimental design was used and chicks were divided into 6 treatment groups, with 3 replicates per treatment and 10 chicks per replicate. Treatments were 1) Normal control (phosphate-buffered saline (PBS)), 2) vaccine control, 3) CpG control, 4) 50 µg CpG + vaccine, 5) 100 µg CpG + vaccine, 6) 200 µg CpG + vaccine.

**Oligonucleotides:** A synthetic ODN containing unmethylated CpG dinucleotides (QIAGEN-GmbH, Hilden, Germany), ODN 2007 (TCGTCGTTGTCGTTTTGTCGTT), was selected for this study [16].

**Vaccination:** All Chicks in groups were vaccinated twice at days 18 and 24 with attenuated live IBD vaccine (Nobilis® Gumboro D78, Intervet International B.V. Boxmeer, Holland).

**Sample Collection:** After measurement of maternal antibody, blood samples were taken individually from all chickens in all groups, on days 7, 14, 21, 28 and 35 via heart and wing vein puncture. Serum was centrifuged at 1000 rpm for 15 min, stored at -20°C until tested.

**Complement Assays:** Complement activity was determined with a hemolytic technique [17] using an adapted light-scattering method. In brief, sera were diluted serially in appropriate ($Ca^{2+}$ containing) buffers in flat-bottomed 96 well microtitre plates and incubated with sensitized (haemolysin, Biomerieux, ref. no. 72202) sheep erythrocytes for measurement of Classic pathway. Plates were shaken every 30 min during the period of incubation. The results (the amount of light scattering by erythrocytes upon lysis) were read at 655 nm in a microtitre reader. Readings were log-log transformed and the hemolytic titre was expressed as the titre that lysed 50% of erythrocytes ($CH_{50}$ U/ml).

**Antibody Titres:** Antibodies of IBD were measured with a commercial ELISA kit (IDEXX Corb, Portland, USA) according to the manufacturer's instructions. In each group, the geometric mean titers were calculated.

**Statistical Analysis:** Results are presented as mean ± S.E.M. Multiple comparisons were performed by ANOVA and followed by the Tukey honestly significant difference (HSD) test. In all analyses, the level of significance was set to ($P<0.05$ or 0.01).

## RESULTS

**Hemolytic Complement Activity:** All CpG administrated birds responded to complement activity test by a significant increase ($P<0.05$) in activity of classical complement pathway. The peak of the complement activities was observed at 21 days post administration, in the CPW (Figure 1) pathway. After a peak, complement levels decreased to levels similar to initial levels. Vaccination with IBDV induced significantly higher CPW activities in comparison to control group, too.

**Humoral Immune Response to IBD Vaccine:** The effect of different doses of CpG as an adjuvant on humoral immune response to IBD vaccines are presented in Table 2. On day 7 of the study, there was no statistically significant change in the antibody titers of experimental groups. The comparison of antibody titer of CpG control group and experimental groups (4, 5 and 6) with normal control group from day 14 to 35 show significantly ($P\leq0.05$, 0.01) higher levels of antibody in study groups.

The Comparison of antibody titer of groups with different levels of CpG, from day 7 to 35, in study groups, show significant ($P\leq0.05$, 0.01) differences. The highest antibody level at study groups was in group 6 at the day 35.

Table 1: The effect of different levels of CpG on Calcium-dependent (classical) complement response from day 7 to day 35. The values are presented as mean ± SEM

| | Day | | |
|---|---|---|---|
| Group | Day 7 | Day 21 | Day 35 |
| 1 | 121 ± 34[a] | 124 ± 58[a] | 127 ± 43[a] |
| 2 | 247 ± 31[c] | 404 ± 31[c] | 342 ± 89[c] |
| 3 | 214 ± 328[b] | 342 ± 97[b] | 322± 37[b] |
| 4 | 289 ± 44[d] | 451 ± 248[d] | 362 ± 23[d] |
| 5 | 267 ± 39[cd] | 467 ± 352[d] | 374 ± 04[d] |
| 6 | 274 ± 53[d] | 472 ± 396[d] | 381 ± 30[d] |

a-d values with different superscripts in each column differ significantly (P<0.05, 0.01).

Table 2: the effect of different levels of CpG on IBDV antibody titer from day 7 to day 35. The values are presented as mean ± SEM

| | Day | | | | |
|---|---|---|---|---|---|
| Group | Day 7 | Day 14 | Day 21 | Day 28 | Day 35 |
| 1 | 1632 ± 134 | 598 ± 67[a] | 585 ± 58[a] | 588 ± 64[a] | 591 ± 43[a] |
| 2 | 1642± 231 | 593 ± 17[a] | 582 ± 31[a] | 1183 ± 163[b] | 1637 ± 189[b] |
| 3 | 1634 ± 328 | 613 ± 26[a] | 593 ± 97[a] | 582 ± 57[a] | 588 ± 37[a] |
| 4 | 1640 ± 244 | 589 ± 41[a] | 595 ± 48[a] | 1448 ± 211[c] | 2241 ± 223[c] |
| 5 | 1653 ±139 | 571 ± 45[a] | 582 ± 52[a] | 1486 ± 287[d] | 2412 ± 204[d] |
| 6 | 1636 ± 253 | 607 ± 67[a] | 589 ± 96[a] | 1508 ± 286[d] | 2434 ± 230[d] |

a-d values with different superscripts in each column differ significantly (P<0.05, 0.01)

## DISCUSSION

Bacterial DNA represents a pathogen-associated molecular pattern (PAMP), which is recognized as a 'danger signal' by the pathogen-recognition receptors (PRRs) of vertebrate immune systems. The non-self recognition that takes place between the PAMP and the PRRs leads to a coordinated set of immune responses, including innate and acquired immunities. This immunostimulatory DNA sequence carries species-specific structural characteristics for initiating the signal pathways of immune cells. Thus, synthetic oligodeoxynucleotides containing unmethylated CpG motifs may act as immune adjuvants. CpG ODNs provoke a rapid, concerted activation of the innate immune system, by directly triggering nonspecific responses. Their adjuvant effects include (a) secreting cytokines (IFN-α/β and IFN-γ) to facilitate the anti-viral response; (b) increasing the cytotoxic activity of NK cells and macrophages; (c) enhancing the production of antibodies; and (d) inducing antigen-specific Th1-responses by elevating the actions of IFN-α/β, IFN-γ, IL-12 and IL-18. As a vaccine adjuvant, CpG ODN is as potent as the gold standard, complete Freund's adjuvant (CFA), with respect to its immunostimulatory effects on B and T cells. Moreover, it is less toxic and induces a higher level of Th1 activity. As numerous reports have provided evidence of the advantages of CpG ODN immunostimulation and also of the beneficial effects of these substances in most vaccination applications, more potent CpG ODNs have been used in new strategies for developing various vaccination programs [18,19].

In this study, we demonstrated the effects of co-administration of CpG ODN as an adjuvant with IBD vaccine in chicken on humoral immune response and complement activation.

When considering antisense and CpG ODN therapy, it is important to understand how phosphorothioated oligos interact with complement. CpG ODNs are often injected locally and systemic toxicity is thereby limited. However, local tissue concentrations may well exceed the threshold for complement activation that can influence therapeutic outcome since C3a and C5a release may affect the local immune response [20-23, 24, 25, 26]. Several groups have reported that C5a may have a negative impact on Th1 responses, mostly using LPS-induced TLR4 stimulation and simultaneous complement activation [24, 26, 27]. P-S oligos prolong coagulation and activate complement when studied in the context of antisense DNA and human blood components [28-30].

Both C3a and C5a are known chemoattractants and their receptors, expressed on monocytes/macrophages [31,32], granulocytes [31] and DCs [33,34], as well as lymphocytes [35,36], facilitate cell migration to the site of inflammation. CpG ODNs target pDCs specifically due to their strong TLR9 expression and CpG 2006 can elicit high levels of C3a and C5a. This favors a model whereby CpG acts not only on TLR9 but also activates complement on site to attract immature APCs. The immature APCs will then respond to TLR9 stimulation as well as complement split products and subsequently mature to functional APCs. Herein, we demonstrate that CpG 2006, at a concentration of 20 μg/ml, activates the complement cascade, resulting in elevated C3a levels. At a concentration of 60 μg/ml, CpG 2006 strongly initiates activation of the complement cascade with resulting high levels of C3a and C5a. This is an unrealistic i.v. dose, but we argue that this model system mimics the complement activity taking place in the extracellular fluids where in situ injections of CpG result in much higher local concentrations.

In the present study, the IBD vaccine-specific antibody titers were higher in chicken receiving IBD vaccine with CpG ODN when compared with those observed in PBS and GpC ODN and IBD vaccine alone groups ($P<0.05$, 0.01). This Immunostimulating effect of CpG ODN proved to be dose dependent, with better outcome at 100 and 200 μg. We observed that administration of vaccine and CpG ODN together and alone, in chickens did create stronger complement activity, whereas the other groups did not. However CpG plus vaccine gives better results. Today, there is limited information concerning the biological effects of CpG ODN in chickens. Co-administration of the antigen and the adjuvant was essential, as injection of CpG ODN alone (without antigen) did not have humoral immune responses increasing ability. As the previous studies, we also found that the adjuvanticity was clearly attributed to the activity of CpG motifs, since control ODN in which the CpG dinucleotides was inverted to GpC provided no immunomodulation. This result was accordant with previous studies [37-39]. Evidence from mice and humans indicates that Toll like receptor 9 (TLR9) specifically recognizes CpG DNA. TLR9-deficient mice fail to respond to CpG ODN [40] and transfection of cells with the human TLR9 gene confers the ability to respond to CpG ODN [41]. While TLR-9 is the only known CpG ODN receptor to date, a recent report indicating that different classes of ODN utilize different signaling pathways suggests that other receptors or co-receptors may be involved [42]. In addition, while CpG ODN strongly activates cells that express TLR-9 and has essentially no direct effect on cells that do not express TLR-9, ODN can increase activity of these latter cells indirectly via PDC-derived cytokines such as IFN a and b [43,44] In chicken, TLR9 is absent in the chicken genome. Therefore, it is speculated that CpG ODN maybe works through other similar receptors, or indirectly stimulate avian immune system by a similar signaling mechanism.

In conclusion, according to present study, it is found that the immunostimulatory effect of CpG ODN can be utilized to create a protective vaccine when given in combination with IBD vaccine and CpG ODN had the ability to augment protective immune response in chickens. Also, CpG ODN administration can augment complement activation.

In conclusion, further studies would be required to evaluate the secretion of various cytokines and interferon-γ and improve our understanding of the mechanisms of the immunity induced by the constructs and action of CpG ODN in chickens.

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