Global Veterinaria 4 (6): 595-600, 2010
ISSN 1992-6197

The Effect of CpG ODN on Infectious Bronchitis and Newcastle Disease Vaccines Humoral Immune Response and Phagocytosis in Broilers

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Abstract: Newcastle disease (ND) virus and infectious bronchitis virus (IBV) becomes some of the most important avian infection agents. CpG oligodeoxynucleotides (ODN) are intimated to act as a danger signal to the vertebrate immune system. In 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. IB and ND vaccine specific antibody and phagocytosis were evaluated by ELISA and Phagocytosis assay methods, respectively. The comparison of antibody titer and phagocytosis percentage of CpG control and experimental groups with normal control, show significantly ($P<0.05, 0.01$) higher levels of antibody and phagocytosis in study groups. This Immunostimulating effect of CpG ODN proved to be dose dependent, with better outcome at 100 and 200 μg.

Key words: Newcastle disease vaccine · Infectious bronchitis vaccine · CpG ODN · Phagocytosis

INTRODUCTION

CpG oligodeoxynucleotides (ODN), which are short sequences containing one or more CpG motif, are intimated to act as a danger signal to the vertebrate immune system, warning of bacterial infection and activating antigen presenting cells (APCs) [1] inducing a potent Th1 type immune response [2]. These unmethylated dinucleotides are expressed approximately 20 times more frequently in bacterial than in vertebrate DNA and the vertebrate cytosine residues are more frequently methylated at position 5. These differences characterize CpG-ODN as pathogen associated molecular patterns (PAMPs) and thus enable vertebrates to protect themselves against invading pathogens [3]. They induce many effects on the host which contribute to their adjuvant activity including the upregulation of MHC class two expression on murine dendritic cells (DC), leading to enhanced antigen presentation [4], augmentation of human and murine NK cell lytic activity [5], activation of human B cells, murine B cell proliferation [6, 7] and increased chemokine expression in murine spleen and lymph node cells [3, 8]. Hemmi and Bauer, respectively, reported that CpG DNA is recognized by a member of the Toll-like receptor (TLR) family which triggers the host's innate immune system. CpG-ODN present in bacterial DNA represents one of the most recent examples of PAMPs that influence adaptive immune responses by activating the innate immune system [9]. Delivery of CpG ODN with a large variety of antigens can augment antigen-specific cell-mediated and humoral immunity [10, 11]. CpG-ODN uptake involves the binding to cell surface proteins with a non specific antigen-receptor, the immunostimulatory effects of CpG-ODN are activated by binding to an intracellular receptor, TLR9 [2]. Recently, the induction of immune response in chickens immunized with CpG ODN also has been reported [12]. 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 [12].

Newcastle disease (ND) virus and infectious bronchitis virus (IBV) becomes some of the most important avian infection agents around the world,

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because of high mortality rates. Commercial attenuated live vaccines can be used to control these diseases. Newcastle disease is a worldwide disease of poultry caused by Newcastle disease virus (NDV), a member of the genus Rubulavirus, family Paramyxoviridae [13-15]. Infectious bronchitis is an acute, highly contagious viral respiratory disease of chickens. The IBV, which belongs to the Coronaviridae family, causes the disease [16]. In the present study, the potential immunomodulatory effect of CPG-ODN on humoral immune response against ND and IB vaccines as an specific immune response index and phagocytic activity as a none specific immune response index in broilers were evaluated.

MATERIAL AND METHODS

Experimental Birds: One day old broilers of a commercial line (Ross 308) were 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 [17].

Oligonucleotides: A synthetic ODN containing unmethylated CpG dinucleotides (QIAGEN-GmbH, Hilden, Germany), ODN 2007 (TCGTCGTTGTCGTTTTGTCGTT), was selected for this study [18].

Experimental Design: 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 (50 μg CPG), 4) 50 μg CPG + vaccine, 5) 100 μg CPG + vaccine, 6) 200 μg CPG + vaccine.

Vaccination: All chicks were vaccinated by aerosol-spray on the 1st day of age with bronchitis vaccine (Intervet International B.V. Boxmeer). On the day 14 chicks were vaccinated with Infectious bronchitis vaccine administered in drinking water and Newcastle vaccine (CEVAC® VITAPEST) administered according to product prescription.

Sample Collection: After measurement of maternal antibody, blood samples were taken individually from all chickens in all groups, at 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 ℃ until tested. Antibodies to ND and IB virus were measured with a commercial ELISA kit (IDEXX FlockChek standard) according to the manufacturer's instructions. In each group, the geometric mean titers were calculated [19].

Cell Separation: Peripheral blood mononuclear cells (PBMCs) were isolated using standard Ficoll-Paque gradient centrifugation according to the instructions of the manufacturer Pharmacia, Freiburg, Germany. Briefly, 4 ml of Ficoll-Paque gradient was pipetted into two 15-ml centrifuge tubes. The heparinized blood was diluted 1:1 in PBS and carefully layered over the Ficoll-Paque gradient. The tubes were centrifuged for 30 min at 1500 g. The cell interface layer was carefully harvested and the cells were washed twice in PBS and resuspended in RPMI 1640 supplemented with penicillin (50 U/ml)-streptomycin (50 g/ml) [20].

Yeast Particles: Frozen stored baker's yeast (*Saccharomyces cerevisiae*) was autoclaved in PBS at 120°C for 15 min. after autoclaving, the yeast particles were washed in PBS and stored at 4°C. On the day of experiment the yeast particles were washed in Hanks balanced salt solution (HBSS) and counted using a hemacytometer, mixes with RPMI 1640 containing 10% rat fresh serum and incubated 30 min in 37°C. Yeast particles opsonized with rat fresh serum components. The yeast particles in RPMI 1640 centrifuged 10 min in 1500 g. Then the sediments were washed twice in HBSS. Sediments were solved in serum free RPMI 1640 and diluted to 107/ml [21].

Phagocytosis Assay: A sterile glass coverslip placed in each well of a multi well plate.1 ml of PBMC suspension, at 105/ml, added to each well and incubated at 37°C for 2 h. RPMI 1640 medium removed and PBMCs washed again with RPMI 1640 medium. 1 ml RPMI medium added to each well and incubated for 2 h at 37°C. 100 μl yeast suspension added to per plate and plates Incubated for 1 h at 37°C in a 5% CO2 humidified incubator. PBMCs washed twice gently with RPMI 1640 medium and 1 ml 1% w/v tannic acid solution were added. Then wash with

RPMI 1640 medium. Coverslip covered with a drop of heat-inactivated FBS and dried in air. Cells were stained with May-Grünwald freshly diluted 1: 2 with buffer, for 5 min. then coverslips rinsed in buffer. PBMCs stained in Giemsa solution, freshly diluted with buffer, for 15 min and rinse in buffer. At last coverslips inverted on microscope slides and observed at 100X magnification (Nikon microscope, Japan). On each coverslip PBMCs were counted until at least 100 PBMCs were scored. At access phagocytosis, the number of ingested yeast particles per counted PBMCs was determined [21, 21].

Statistical Analysis: Results are expressed as mean ± SEM 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

Humoral Immune Response to IB and ND Vaccine: The effect of different doses of CpG as an adjuvant on humoral immune response to IB and ND vaccines are presented in Tables 1 and 2, respectively. 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 and experimental groups (4, 5 and 6) with normal control group from day 14 to 35 show significantly ($P<0.05$, 0.01) higher levels of antibody in both IB and ND study groups. The Comparison of antibody titers of groups with different levels of CpG, from day 7 to 35, in both IB and ND study groups, show significant ($P<0.05$, 0.01) differences. The highest antibody level at the IB and ND study groups were groups 6 at the day 35 and group 6 at day 28, respectively.

Table 1: The effect of different levels of CpG on IBV 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	577 ± 64	583 ± 84^{a}	592 ± 69^{a}	578 ± 76^{a}	575 ± 69^{a}
2	574 ± 56	640 ± 92^{b}	820 ± 98^{b}	1010 ± 104^{b}	1208 ± 112^{b}
3	579 ± 68	579 ± 67^{a}	576 ± 79^{a}	587 ± 83^{a}	383 ± 79^{a}
4	581 ± 71	670 ± 86^{c}	940 ± 94^{c}	1310 ± 112^{c}	1503 ± 124^{c}
5	587 ± 63	683 ± 96^{d}	987 ± 102^{d}	1460 ± 109^{d}	1664 ± 186^{d}
6	582 ± 76	680 ± 87^{d}	985 ± 96^{d}	1510 ± 121^{e}	1753 ± 181^{e}

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

Table 2: The effect of different levels of CpG on NDV 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	374 ± 34	384 ± 67^{a}	382 ± 58^{a}	385 ± 64^{a}	383 ± 43^{a}
2	392 ± 31	1250 ± 117^{b}	1939 ± 231^{b}	2136 ± 263^{b}	2018 ± 189^{b}
3	388 ± 28	392 ± 26^{a}	391 ± 97^{a}	382 ± 57^{a}	386 ± 37^{a}
4	392 ± 44	1313 ± 241^{b}	2043 ± 248^{b}	3775 ± 311^{c}	3705 ± 223^{c}
5	396 ± 39	1655 ± 245^{c}	2338 ± 352^{c}	4968 ± 387^{d}	4405 ± 204^{d}
6	398 ± 53	1760 ± 267^{c}	3008 ± 396^{d}	5607 ± 386^{e}	5405 ± 230^{e}

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

Table 3: The effect of different levels of CpG on phagocytosis percentage 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	43 ± 8.32	43 ± 6.83	44 ± 5.78^{a}	45 ± 6.31^{a}	43 ± 6.81^{a}
2	45 ± 4.67	44 ± 7.08	46 ± 6.12^{b}	47 ± 7.13^{a}	45 ± 7.54^{a}
3	-	-	-	-	-
4	48 ± 5.09	54 ± 8.36	60 ± 8.74^{b}	64 ± 9.22^{b}	66 ± 8.84^{b}
5	47 ± 6.32	53 ± 6.83	60 ± 9.70^{b}	65 ± 8.91^{bc}	70 ± 9.80^{c}
6	48 ± 7.42	54 ± 7.14	62 ± 8.92^{b}	68 ± 9.38^{c}	72 ± 9.67^{c}

a-c values with different superscripts in each column differ significantly ($P<0.05$).

Phagocytosis Assay: The effect of different doses of CpG on phagocytosis is presented in Table 3. On day 7 of the study, there was no statistically significant difference in phagocytosis of experimental groups. The comparison of phagocytosis percentage of groups with different levels CpG (4, 5 and 6) with normal control group, from day 21 to 35, show significantly ($P<0.05$) higher percentage of phagocytosis. The Comparison of phagocytosis percentage of groups with different levels of CpG, from day 21 to 35, shows significant ($P<0.05$) differences. The highest phagocytosis percentage was groups 6 at the day 35.

DISCUSSION

The adjuvant effect of CpG ODN has been well documented by many studies. In general, immunostimulatory DNA containing CpG motifs promote Th1 humoral responses for protein-based vaccines [10, 22- 29]. The ability of CpG ODN to induce both innate and adaptive cellular immune response has made it a potential treatment or prophylactic vaccine adjuvant, because of disease requiring cellular immunity. This study, demonstrated co-administration of CpG ODN as an adjuvant with IB and ND vaccines in chicken and effect of CpG ODN on phagocytosis. The Previous studies have indicated that antibody titer, isotype, or a combination of these factors, are important in resistance to bacteria infections [30]. In the present study, the IB and ND vaccine-specific antibody titers were higher in chicken receiving IB and ND vaccine with CpG ODN when compared with those observed in PBS and CpG ODN and IB and ND vaccine alone groups. This immunostimulating effect of CpG ODN proved to be dose dependent, with better outcome at 100 and 200 µg. We observed that administration of CpG ODN in chickens did create stronger PBMCs phagocytic activity, whereas the other groups did not. Today, there is limited information concerning the biological effects of CpG ODN in chickens. In the present study, we also found that CpG ODN could also create strong humoral immune responses to IB and ND vaccine in chicken. Co-administration of the antigen and the adjuvant was essential, as injection of CpG ODN alone (without antigen) had reduced humoral immune responses 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 the Krieg's [2, 18, 31]. 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 (15) and transfection of cells with the human TLR9 gene confers the ability to respond to CpG ODN [32]. 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 [33]. 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 [34, 35]. 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. Taken together, in this study, it is found that the immunostimulatory effect of CpG ODN can be utilized to create a protective vaccine when given in combination with IB and ND vaccine and CpG ODN had the ability to augment protective immune response in chickens. Further studies would be required to evaluate the secretion of various cytokines and interferon-ã and improve our understanding of the mechanisms of the immunity induced by the constructs and action of CpG ODN in chickens.

REFERENCES

1. Pisetsky, D.S., 1996. Immune activation by bacterial DNA: a new genetic code. Immunity, 5: 303-310.
2. Krieg, A.M., 2002. CpG motifs in bacterial DNA and their Immune effects. Annu. Rev. Immunol., 20: 709-760.
3. Klinman, D.M., K.M. Barnhart and J. Conover, 1999. CpG motifs as immune adjuvants. Vaccine, 17: 19-25.
4. Sparwasser, T., E.V. Koch, R.M. Vabulas, K. Heeg, G.B. Lipford, J.W. Ellwart and H. Wagner, 1998. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. Eur. J. Immunol., 28: 2045-2054.
5. Ballas, Z.K., W.L. Rasmussen and A.M. Krieg, 1996. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. J. Immunol., 157: 1840-1845.
6. Liang, H., Y. Nishioka, C.F. Reich, D.S. Pisetsky and P.E. Lipsky, 1996. Activation of human B cells by phosphorothioate oligodeoxynucleotides. J. Clin. Invest., 98: 1119-1129.

7. Messina, J.P. G.S. Gilkeson and D.S. Pisetsky, 1991. Stimulation of in vitro murine lymphocyte proliferation by bacterial DNA. J. Immunol., 147: 1759-1764.
8. Takeshita, S., F. Takeshita. D.F. Haddad. K.J. Ishii and D.M. Klinman, 2000. CpG oligodeoxynucleotides induce murine macrophages to up-regulate chemokine mRNA expression. Cell. Immunol., 206: 101-106.
9. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda and S. Akira, 2000. A Toll-like receptor recognizes bacterial DNA. Nature, 408(6813): 740-745.
10. Chu, R.S., O.S. Targoni, A.M. Krieg, P.V. Lehmann and C.V. Harding, 1997. CpG oligodeoxynucleotides act as adjuvants that switch on T Helper 1 (Th1) immunity. J. Exp. Med., 186(10): 1623-1631.
11. Roman, M., E. Martin-Orozco, J.S. Goodman, M.D. Nguyen, Y. Sato, A. Ronaghy, R.S. Kornbluth, D.D. Richman, D.A. Carson and E. Raz, 1997. Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants. Nature, 3(8): 849-854.
12. Vleugels, B., C. Ververken and B.M. Goddeeris, 2002. Stimulatory effect of CpG sequences on humoral response in chickens. Poult. Sci., 81: 1317-1321.
13. Awan, M.A., M.J. Otte and D. James, 1994. The epidemiology of Newcastle disease in rural poultry: A review. Avian Pathol., 23: 405-423.
14. Brown, C., D.J. King and B.S. Seal, 1999. Pathogenesis of Newcastle disease in chickens experimentally infected with viruses of different virulence. Vet. Pathol., 36: 125-132.
15. Swayne, D.E. and D.J. King, 2003. Avian influenza and Newcastle disease. J. Am. Vet. Med. Assoc., 222: 1534-1540.
16. Davies, H.A. and M.R. Macnaughton, 1979. Comparison of the morphology of three corona viruses. Arch. Virol., 59: 25-33.
17. National Research Council: Nutrient Requirements of Poultry. 1994. 9th rev. edn. National Academy Press. Washington, D.C.,
18. Rankin, R., R. Pontarollo, X. Ioannou, A.M. Krieg, R. Hecker, L.A. Babiuk and S. Van Drunen Littel-van den Hurk. 2001. CpG motif identification for veterinary and laboratory species demonstrates that sequence recognition is highly conserved. Antisense Nucleic. Acid. Drug. Dev., 11(5): 333-40.
19. Snyder, D.B., W.W. Marquardt. E.T. Mallinson. P.K. Savage and D.C. Allen, 1984. Rapid serological profiling by enzyme-linked immunosorbent assay. III. Simultaneous measurements of antibody titers to infectious bronchitis virus, infectious bursal disease and Newcastle disease viruses in a single serum dilution. Avian. Dis., 28: 12-24.
20. Hay, F.C. and O.M.R Westwood, 2002. Practical Immunology, Black Well Science, Ltd, Berlin, Germany, 4th Edn., pp: 203- 210.
21. Bos, H. and W. De Souza, 2000. Phagocytosis of yeast: a method for concurrent quantification of binding and internalization using differential interference contrast microscopy. Journal of Immunological Methods, 238: 29-43.
22. Wai, M.L., B.B. Marcel. M. Paule and S. Redelmeier, 2002. Enhanced immune response to T-independent antigen by using CpG oligodeoxynucleotides encapsulated in liposomes. Vaccine, 20: 148-158.
23. Brazolot Millan, C.L., R. Weeratna, A.M. Krieg, C.A. Siegrist and H.L. Davis, 1998. CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. Proc. Natl. Acad. Sci. U.S.A., 95: 15553-15558.
24. Davis, H.L., I.I. Suparto, R.R. Weeratna, D.D. Iskandriati, S.S. Chamzah, A.A. Ma'ruf, C.C. Nente, D.D. Pawitri, A.M. Krieg, S.W. Heriyanto and D.D. Sajuthi, 2000. CpG DNA overcomes hyporesponsiveness to hepatitis B vaccine in orangutans. Vaccine. 18(18): 1920-1924.
25. Sun, S., X. Zhang, D.F. Tough and J. Sprent, 1998. Type 1 interferon mediated stimulation of T cells by CpG DNA. J. Exp. Med., 188(12): 2335-2342.
26. Threadgill, D.S., L.L. McCormick. T.L. McCool. N.S. Greenspan and J.R. Schreiber, 1998. Mitogenic synthetic polynucleotides suppress the antibody response to a bacterial polysaccharide. Vaccine, 16: 76-82.
27. Carpentier, A.F., L. Chen, F. Maltonti and J.Y. Delattre, 1999. Oligodeoxynucleotides containing CpG motifs can induce rejection of a neuroblastoma in mic. Cancer. Res., 59: 5429-5432.
28. Ioannou, X.P., S.M. Gomis, B. Karvonen, R. Hecker, L.A. Babiuk and S. Van Drunen Little-van den Hurk. 2002. CpG-containing oligodeoxynucleotides, in combination with conventional adjuvants, enhance the magnitude and change the bias of the immune responses to a herpesvirus glycoprotein. Vaccine, 21: 127-137.

29. Jakob, T., P.S. Walker, A.M. Krieg, M.C. Udey and J.C. Vogel, 1998. Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. J. Immunol., 16: .3042-3049.
30. Ricardo, S.C and B.P. Patricia, 2001. CpG DNA as a Th1-promoting adjuvant in immunization against Trypanosoma cruzi. Vaccine, 19: 242-234.
31. Heeg, L and S. Zimmermann. 2000. CpG DNA as a Th1 trigger. Int. Arch. Allergy Immunol., 121: 87-97.
32. Takeshita, F., C.A. Leifer, I. Gursel, K.J. Ishii, S. Takeshita, M. Gursel and D.M. Klinman, 2001. Cutting edge: role of Toll-like receptor 9 in CpGDNA-induced activation of human cells. J. Immunol., 167(7): 3555-3558.
33. Kerkmann, M., S. Rothenfusser, V. Hornung, A. Towarowski, M. Wagner, A. Sarris, T. Giese, S. Endres and G. Hartmann, 2003. Activation with CpG-A and CpG-B oligonucleotides reveals two distinct regulatory pathways of type I IFN synthesis in human plasmacytoid dendritic cells. J. Immunol., 170(9): 4465-4474.
34. Hornung, V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdorfer, T. Giese, S. Endres and G. Hartmann, 2002. Quantitative expression of Toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J. Immunol., 168(9): 4531-4537.
35. Rothenfusser, S., V. Hornung, A. Krug, A. Towarowski, A.M. Krieg, S. Endres and G. Hartmann, 2001. Distinct CpG oligonucleotide sequences activate human gamma delta T cells via interferonalpha/-beta. Eur. J. Immunol., 31(12): 3525-3534.