The nucleic acid adjuvant with high efficacy in avian vaccines and its production technique

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Unmethylated CpG motifs are capable of evoking a range of immunostimulatory effects in vertebrates and have tremendous potential to be used as therapeutic agents and adjuvants. This particular type of CpG motif has been demonstrated to be an excellent immune adjuvant mediated by Toll-like receptor 9 (TLR9) in various mammalian vaccines; however, only a few studies confirm its efficacy in avian vaccines. In the present study, immunomodulatory activities of plasmids with various copy numbers of a CpG motif were evaluated in chickens inoculated with an avian influenza vaccine. Results showed that the plasmid with 64 copies of the CpG motif (64CpG-plasmid) significantly enhanced the mRNA expressions of interferon (IFN), TLR3 and TLR7 in chicken splenocytes compared to plasmids with lesser copies of the CpG motif in vitro. Chickens inoculated with the H5N2 avian influenza inactivated vaccines (V52) coadministrated with the 64CpG-plasmid (V52-64CpG) showed significant increments of hemagglutination inhibition (HI) titers, peripheral blood mononuclear cell (PBMC) proliferation, and mRNA expressions of IFN, TLR3, TLR7 and TLR21 in splenocytes as compared to those of chickens inoculated with V52 alone, V52 adjuvanted with aluminum gel (V52-gel), or with V52-gel plus vector. Additionally, following challenge with a very virulent H5N1 strain, a higher survival rate (100%) was observed in chickens inoculated with V52-64CpG as compared to those that received V52-gel (80%) or PBS (0%). The 64CpG-plasmid significantly enhanced chicken immunity in vitro and in vivo; thus it can be a potent adjuvant in an avian influenza vaccine for chickens.

Unmethylated CpG dinucleotides (CpG motif) in either bacterial DNA or synthetic oligodeoxynucleotides (ODN) are capable of evoking a range of immunostimulatory effects in vertebrates and have tremendous potential to be used as therapeutic agents and adjuvants; they can enhance the activities of lymphocytes and antigen-presenting cells, trigger dendritic cell (DC) maturation, and drive immune systems towards the T helper 1 (Th1) immune response against specific antigens in human and other mammalian animals. Although ODN sequences with CpG motifs (CpG ODN) can be recognized as a danger signal by the innate immune system in most animals, the best contexts of CpG motifs in terms of yielding optimal immunostimulatory effects vary from species to species. CpG ODN has been demonstrated to be an excellent immune adjuvant in various mammalian vaccines; however, only a few studies confirm its efficacy in avian vaccines.

Chickens treated with the synthetic CpG ODN have shown an elevated level of serum antibodies specific for bovine serum albumin and increased survival rates against *E. coli* or *S. enteritidis* infections. Coadministration of a synthetic CpG ODN with the attenuated-infectious bursal disease (IBD) vaccine or the recombinant plasmid DNA vaccine encoding the VP2 gene of a very virulent strain of IBD virus significantly increased protection of the chickens from the IBD virus challenge as compared to vaccines administered without the CpG ODN as an adjuvant. The responses of serum antigen-specific IgG titer and the proliferation of lymphocytes in chickens that received Newcastle disease (ND) vaccine along with the synthetic CpG ODN as an adjuvant were significantly elevated compared to those receiving ND vaccine only. Relative to chickens inoculated with H5N1 AIV vaccine only, higher hemagglutination inhibition (HI) titers were observed in chickens inoculated with H5N1 AIV vaccine incorporating the synthetic CpG ODN. Moreover, intranasal immunization of the inactivated AIV H5N2 vaccine mixed with the bacterial genomic DNA extracted from *E. coli* in chickens showed the significantly increasing numbers of IgA- and IgG-secreting cells in respiratory tract. These results confirm that the adjuvant efficacy of synthetic CpG ODNs in chicken vaccines is similar to that observed in mammalian vaccines.

The response to CpG motifs is known to be mediated by TLR9 in mammals; TLR9-deficient mice did not show any inflammatory cytokine production by macrophages, proliferation of B cells, or maturation of DC in response to CpG motifs. However, TLR9 is not found in the two most likely locations of this gene in chickens, and thus it has been proposed that chickens may lack the TLR9 orthologue. Substantial evidence has demonstrated the adjuvant efficacy of synthetic CpG ODNs in chicken vaccines. Thus, further investigation into the mechanism of action of the immunomodulatory effects observed from CpG motifs on chicken TLR family members is warranted. Unfortunately, the high cost of synthetic CpG ODN production is one of the critical issues preventing its widespread application to animal or avian vaccines at this time. The objectives of this study are to construct plasmids with various copies of a designed CpG motif in order to examine the immunomodulatory activities of these plasmids in vitro, as well as the adjuvant efficacy of plasmids with high copy numbers of the designed CpG motif when coadministrated with an inactivated AIV H5N2 vaccine to chickens.

The immunostimulatory effects of synthetic CpG ODNs have been well documented in large mammalian animals as well as in rodents. Results of this study confirmed that plasmids with multiple copies of CpG ODN effectively enhanced immune responses against viral infection in chickens by increasing the mRNA expressions of IFN, TLR3 and TLR7 in vitro, elevating PBMC proliferation, ratios of CD8+/CD4+ and the mRNA expressions of IFN, TLR3, TLR7 and TLR21 in vivo, and by protecting animals from the challenge of very virulent AIV H5N1 strain. Huge amounts of evidence show that the mode of action of CpG ODNs on enhancing immune responses is primarily via TLR9 in mammals. TLRs are one of the most important pathogen recognition receptor families capable of recognizing several classes of pathogen-association molecular patterns and orchestrating appropriate innate and adaptive immune responses. TLR3, TLR7, TLR8 and TLR9 are four known toll-like receptors for recognizing foreign nucleic acids as a danger signal in mammalians cells, and these nucleic acid-recognizing TLRs regulate the induction of type I interferon and other antiviral gene functions. The mammalian receptor for CpG ODNs has been identified as TLR9 based on studies with knockout mice and other in vitro studies implementing recombinant mouse and human TLR9. However, TLR9 is not found in the two most likely locations for the chicken TLR9 gene, and thus it has been proposed that chickens may be lacking the TLR9 orthologue. The closest homologue of the TLR9 LRR in chickens is the TLR7 gene, which may fulfill the roles of both receptors in this family. Alternatively, chicken TLR21, absent in humans and other mammals, may act as a functional homologue to mammalian TLR9 in the recognition of CpG. motifs; expression of chicken TLR21 in HEK293 cells resulted in activation of NF-kappaB in response to CpG motif stimulation, typically recognized by mammalian TLR9 and its silencing in chicken macrophages inhibits the response to CpG motif, indicating its similar function as mammalian TLR9. Additionally, in the chicken genome, TLR8 lies in tandem with TLR7, and the draft chicken TLR8 locus has a gap where the Toll/interleukin I resistance (TIR) domain should lie; that is, it is possibly a pseudogene. The absence of TLR8 and TLR9 in the chicken shows the evolutional differences of TLR family members in the immune system between avian and mammals. Therefore, the mechanism of immunomodulation of intracellular signals via TLR9 activated by CpG motifs cannot operate in chickens lacking functional TLR8 and TLR9. In another of the view, TLR3 and TLR7 expressions contribute to the immune response and appear to play a central role in mediating both the antiviral and inflammatory responses of innate immunity; thus, the actions of CpG motifs on their expressions are critical for the protection of chickens against viral infections. Results of the present study showed that the 64CpG-plasmid significantly increased the mRNA expressions of IFN, TLR3, TLR7 and TLR21, suggesting that the plasmid with multiple copies of this typically designed CpG motif can be recognized as a danger signal and can thus evoke the anti-viral activities and the consequently associated receptors. Previous research has demonstrated that the synthesized CpG ODN can induce IFN synthesis in vitro and induce proliferation of PBMC in chickens. Evidence from the present study can serve

as the first report on the induction of TLR3, TLR7 and TLR21 gene expressions by administration of plasmids with multiple copies of CpG ODN as an AIV vaccine adjuvant in chickens. A similar result on the enhancement of mRNA expressions of TLR3 and TLR7 by CpG ODN has also been observed in porcine antigen-presenting cells. Therefore, the resulting immunomodulatory activities of CpG ODN in chickens are likely the same as those found in mammals despite the fact that a functional TLR9 is known to be absent in aves.

It has been reported that aluminum gel adjuvant primarily enhances humoral immunity rather than cell-mediated immunity, whereas CpG motifs can have a strong adjuvant effect on both cellular and humoral immune responses. Consistent results were also observed in the present study; the aluminum gel significantly increased the HI titer in the vaccinated animals as compared with those inoculated with V52 only; however, it had no significant effects on stimulating PBMC proliferation, ratios of CD8+ to CD4+, or mRNA expressions of IFN, TLR3, TLR7 and TLR21.

The application of CpG motifs as an adjuvant in animal vaccines is limited due to the cost of production. In order to reduce the cost of production, construction of multiple copies of an effective CpG motif in one plasmid can be successfully performed, and thus the cost-effective production of CpG motif on a large scale can be easily achieved. The final product of one plasmid containing 64 copies of a CpG motif has been successfully produced and harvested in large-scale. The plasmid containing 128 copies of CpG motif has also been cloned; however, only a few amounts of plasmids have been successfully harvested to date. The low production of this 128CpG-plasmid may result from its large size, over 6kb. In addition, the 128CpG-plasmid cannot stimulate activities of immune cells as high as the 64CpG-plasmid (unpublished data), and thus the 128CpG-plasmid was not used in the present study.

Results of this in vitro study showed that the minimal effective copy number of CpG ODN in one plasmid to stimulate IFN gene expression was 32, whereas the effective copy numbers in plasmids shown to enhance TLR3 and TLR7 expressions are at least 4 and 8 copies of the CpG motif, respectively. However, dramatic increments of IFN, TLR3 and TLR7 expressions were observed in 64CpG-plasmid stimulated cells. These results suggest that the immunomodulatory activities of these plasmids are not be linearly correlated with the CpG motif copy number in one plasmid. Previous research has demonstrated that plasmids with 10 copies of a CpG motif can slightly enhance MHC II, CD40 and CD86 expressions on murine bone marrow-derived DC in vitro as compared to those with 0 or 5 copies of the CpG motif; however, no significant changes in these cell surface markers following treatment with plasmids containing 5 copies of the CpG motif versus the empty vector. Thus, only plasmids with more than 15 copies of CpG ODN showed significantly greater adjuvant activities in enhancing the efficacy of an HIV-1 DNA vaccine in vivo. Plasmids possessing 16 CpG motif repeats have also been found to enhance ovalbumin (OVA)-specific T cell proliferation and cytotoxic T cell activity in mice. Thus, the copy numbers of CpG motifs in one plasmid required for these plasmids to induce a strong enough signal transduction may vary in different animals. Whether or not there is a threshold to reach different levels of CpG motif-dependent signal transduction is still unknown.

In conclusion, the 64CpG-plasmids significantly increased the mRNA expressions of anti-viral cytokines and associated toll-like receptors in vitro and in vivo, and thus acted as an effective adjuvant in stimulating humoral and cell-mediated immunities by increasing HI titers, PBMC proliferation, ratios of CD8+ to CD4+ in splenocytes, and survival rate following challenge with a very virulent H5N1 AIV strain when coadministrated with inactivated AIV H5N2 vaccine in chickens. Although the underlying mechanism involved in the activation by CpG ODNs and the relationship between CpG ODNs and other TLR family members are not well understood, results from this study confirmed that plasmids with multiple copies of a CpG motif can be a potent adjuvant in enhancing chicken immunization against bacterial and viral infections.