

## Effects of DDA, CpG-ODN, and plasmid-encoded chicken IFN- $\gamma$ on protective immunity by a DNA vaccine against IBDV in chickens

Ha Jung Roh, Haan Woo Sung, Hyuk Moo Kwon\*

School of Veterinary Medicine and Institute of Veterinary Science, Kangwon National University, Chuncheon 200-701, Korea

This study examined the adjuvant effects of dimethyl dioctadecyl ammonium bromide (DDA), CpG oligodeoxynucleotides (CpG-ODN), and chicken interferon- $\gamma$  (ChIFN- $\gamma$ ) on a DNA vaccine (pcDNA-VP243) against the infectious bursal disease virus (IBDV). A plasmid encoding chicken IFN- $\alpha$  was constructed. Twice at 2-week intervals, two-week-old chickens were injected intramuscularly and intraperitoneally with either a DNA vaccine alone or a DNA vaccine together with the respective adjuvants. On week 2 after the second immunization, the chickens were orally challenged with the highly virulent IBDV. The groups that received the DNA vaccines plus either DDA or CpG-ODN showed significantly lower survival rates than the group that received the DNA vaccine alone. However, the survival rates for the DNA vaccine alone and for the DNA vaccine plus ChIFN- $\gamma$  were similar. The chickens had no detectable antibodies to the IBDV before the challenge but all the surviving chickens in all groups except for the normal control group showed the induction of antibodies to the IBDV at day 10 after the challenge. As judged by the lymphocyte proliferation assays using the a WST-8 solution performed on the peripheral blood and splenic lymphocytes, the stimulation indices (SI) of the peripheral blood lymphocytes in all groups except for the normal control group were similar immediately before the challenge. At 10 days post-challenge, the SI for DNA vaccine plus either CpG-ODN or ChIFN- $\gamma$  was similar to that of the DNA vaccine control group. For splenic lymphocytes, the SI in the DNA vaccine plus CpG-ODN and DNA vaccine plus ChIFN- $\gamma$  groups were higher than for the DNA vaccine control. These results suggest that DDA actually compromises the protection against the IBDV by DNA vaccine, and CpG-ODN and IFN- $\gamma$  had no significant effect.

**Key words:** chicken IFN- $\gamma$ , CpG-ODN, DDA, DNA vaccine, infectious bursal disease virus

### Introduction

New types of vaccines such as recombinant vaccines or DNA vaccines in some diseases have largely supplanted the traditional methods of vaccination, which utilize live or dead whole microorganisms [9,30,38]. Although DNA vaccines have many advantages over conventional vaccines, some new generations of vaccines have the drawback that DNA vaccines require repeated doses or mixing with strong adjuvants to achieve an effective immune response [13,25,28]. Therefore, recent vaccine studies have focused on discovering efficient adjuvants.

Adjuvants with DNA vaccines are essential for achieving sufficient levels of protection and long-term immunity. Suitable adjuvants must be able to increase the immune response without having any undesirable side effects. Conventional oil-based adjuvants deteriorate the meat quality at the injection sites. Hence, either naturally occurring adjuvants such as cytokines or other types of compatible adjuvants are needed [25].

Interferon- $\gamma$  (IFN- $\gamma$ ) is a cytokine produced by stimulated T cells and has important effects in immunomodulation [8]. Its properties as an adjuvant have been examined in a large number of animal studies [1,5,11,25,40,46]. Dimethyl dioctadecyl ammonium bromide (DDA) has been recognized as an adjuvant for enhancing both humoral and cell-mediated immune responses [18]. Unmethylated cytosine-phosphate-guanosine (CpG) dinucleotides are sequences that are found in the bacterial DNA that have adjuvant properties in the mammalian immune system by activating antigen-presenting cells (APCs) [20,24]. In addition, CpG-containing oligodeoxynucleotides (CpG-ODNs) induce the secretion of various cytokines, including interleukin-6 (IL-6) and type II interferon (IFN- $\gamma$ ) [47,48].

Infectious bursal disease (IBD) or Gumboro disease, which is caused by the infectious bursal disease virus (IBDV), is an acute and highly contagious disease in chickens 3 weeks of age and older. The disease has an important economic impact on the poultry industry worldwide because it is associated with a high mortality and immunosuppression in recovered chickens, which leads

\*Corresponding author  
Tel: +82-33-250-8652; Fax: +82-33-244-2367  
E-mail: kwonhm@kangwon.ac.kr

both to a variety of secondary infections and a decreased response to vaccinations [26]. After the emergence of highly virulent IBDV strains that cause high mortality in Europe and the antigenic variants of IBDV in USA, these IBDVs have spread rapidly despite conventional vaccination programs to protect chickens against IBDV infections [1,4,34].

This study examined the adjuvant effects of a plasmid encoding chicken IFN- $\gamma$ , of DDA, and of CpG-ODN on a plasmid DNA vaccine (pcDNA-VP243) against the highly virulent IBDV SH/92 strain, which was developed in our laboratory [17], in chickens. The results suggest that CpG-ODN and IFN- $\gamma$  failed to enhance the DNA vaccine efficacy, and that DDA actually had an adverse effect on the level of protection against the IBDV.

## Materials and Methods

### Chickens

The fertilized eggs of specific pathogen free (SPF) White Leghorn were obtained (Sunrise Farm, USA) and hatched. The hatched chicks were placed into plastic isolators operated under a positive air pressure and provided with food and water *ad libitum* during the experimental period.

### Construction and preparation of plasmids

The DNA vaccine, pcDNA-VP243, encoding the VP2, VP4, and VP3 proteins of the highly virulent IBDV SH/92 strain, is described elsewhere [17]. The plasmid DNA was purified from the transformed *Escherichia coli* using the Endofree Plasmid Giga Kit (Qiagen, USA).

For cloning of the IFN- $\gamma$  gene, the spleens were obtained aseptically from 8-week old SPF chickens. After the spleens had been passed through a plastic cell strainer (Becton Dickinson Labware, USA), the spleen lymphocytes were separated by Histopaque-1077 (Sigma, USA). The prepared splenocytes were rinsed three times in Hanks balanced salt solution (HBSS) and incubated at  $1 \times 10^7$  cells/mL for 6 h in RPMI-1640 medium containing 10% fetal bovine serum (FBS) supplemented with 12.5  $\mu$ g/mL Concanavalin A (ConA, Sigma, USA), at 40°C and 5% CO<sub>2</sub>. The total RNA was isolated and purified from harvested splenocytes using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's recommendation, and the cDNA was synthesized using random primers (Invitrogen, USA). The PCR fragments were synthesized from cDNA using the primers, CIG-F (5' GCCGCCGCCATGACTTGCCAGAC TTACAAC 3') and CIG-R (5' TTAGCAATTGCATCTCCT CTG 3'), which were synthesized according to the published sequence of chicken IFN- $\gamma$  [6]. PCR was performed with 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. The final extension step was performed at 72°C for 10 min. The PCR products were analyzed on a 1.0 % agarose gel.

The PCR products were purified utilizing a GENECLAN

Turbo kit (Q-biogene, USA) according to the manufacturer's instructions. The purified PCR products were cloned into the pcDNA 3.1/V5/His-TOPO vector (Invitrogen, USA) and transformed into competent *Escherichia coli* (TOP 10) cells (Invitrogen, USA). The plasmid DNA was isolated using the E.N.Z.A plasmid miniprep kit I (Omega Bio-tech, USA). The nucleotide sequence and the orientation of the plasmid construct were confirmed by DNA sequencing. The verified plasmid construct was named pcDNA-ChIFN- $\gamma$ , and large quantities of the plasmid were prepared using a Endofree Plasmid Giga Kit (Qiagen, USA).

### Synthetic CpG-ODN and preparation of DDA solution

The CpG-ODN (2007) sequence [32] is TCGTCGTTGT CGTTTTGTCGTT (underlining indicates CpG dinucleotides), which was produced with a phosphorothioate backbone (Bioneer, Korea). Synthetic CpG-ODN (10  $\mu$ g/bird) along with the DNA vaccine was injected. A DDA (Sigma, USA) solution (2 mg/bird) was prepared as described previously [16] and also injected along with the DNA vaccine.

### In vitro transcription and translation

The in vitro expression of pcDNA-ChIFN- $\gamma$  was performed using the TNT Quick Coupled Transcription/Translation System (Promega, USA) and visualized using the Transcend Colorimetric Translation Detection System (Promega, USA) according to the manufacturer's recommendations. The samples were electrophoresed on a 12% discontinuous SDS-PAGE gel and transferred onto nitrocellulose membranes for visualization. The membranes were washed with Tris-buffered saline (TBS) and incubated in a blocking buffer (TBS containing 0.5% Tween 20). For visualization, streptavidin alkaline phosphatase was added to the membranes, which were rocked gently for 60 min and visualized by adding Western Blue Stabilized Substrate (Promega, USA).

### Immunization protocols

Two-week-old SPF chickens were randomly divided into 6 experimental groups (9 birds/group), which included the normal control (without vaccination and challenge), challenge control (without vaccine but with challenge), DNA vaccine alone (with pcDNA-VP243 DNA vaccine and challenge), DNA vaccine plus DDA (with pcDNA-VP243 DNA vaccine plus DDA and challenge), DNA vaccine plus CpG-ODN (with pcDNA-VP243 DNA vaccine plus CpG-ODN and challenge), DNA vaccine plus ChIFN- $\gamma$  (with pcDNA-VP243 DNA vaccine plus ChIFN- $\gamma$  plasmid and challenge). Twice at a 2-week interval, 2-week-old chickens were injected intramuscularly (100  $\mu$ g) and intraperitoneally (100  $\mu$ g) with the DNA vaccines. For the DNA vaccine plus ChIFN- $\gamma$  group, 125  $\mu$ g of the pcDNA-ChIFN- $\gamma$  plasmid was injected intramuscularly into separate sites.

Two weeks later, all the groups except for the normal control group were re-injected with the same dose and by

the same route as used for the primary immunization. Two weeks after the second immunization, all the groups except for the normal control were challenged orally with a  $1 \times 10^{4.8}$  50% egg lethal dose (ELD<sub>50</sub>) of the highly virulent IBDV SH/92 strain [21]. The chickens were then monitored daily for any clinical signs over a 10 day period. At 10 days post-challenge, all the remaining chickens in each group were euthanized, and the spleens and the bursa of Fabricius were collected aseptically. The bursa/body weight (B/B) ratios were calculated by (bursa weight)/(body weight)  $\times 1,000$ .

### Peripheral blood and splenic lymphocyte proliferation assay

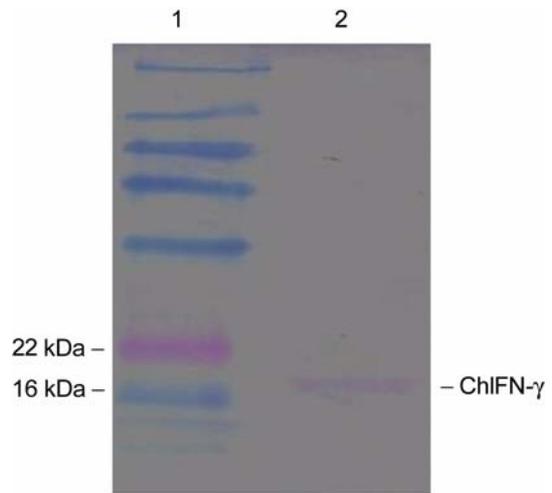
The lymphocyte proliferation assay was performed as described previously [17]. Briefly, the whole blood and spleens were collected aseptically from the chickens before the challenge as well as at 10 days after the challenge, and the cell suspensions were obtained. The lymphocytes were separated using Histopaque-1077 (Sigma, USA), washed three times, and then resuspended in RPMI-1640 medium supplemented with 10% FBS. The cells (PBL at  $1.25 \times 10^6$  cells/well and spleen at  $2.5 \times 10^6$  cells/well) were placed in 96-well flat-bottomed tissue culture plates. ConA (12.5  $\mu\text{g}/\text{mL}$ ) was added to each well except for the negative control well. The plates were incubated for 48 h at 40°C in a 5% CO<sub>2</sub> atmosphere. The lymphocyte proliferation activity was measured using a WST-8 working solution [29]. The optical density (OD) was determined at 450 nm and the stimulation index (SI) was calculated using the following formula: SI = mean OD of ConA-stimulated cells/mean OD of non-stimulated cells.

### Antibody assay by enzyme-linked immunosorbent assay

Blood samples were collected from the birds in each experimental group both before the challenge and at 10 days after the challenge. The serum antibody titers of the experimental groups were determined using an infectious bursal disease antibody test kit (IDEXX, USA) as described elsewhere [17]. Titers >396 were considered positive.

### Statistical analysis

All the analyses were performed using the statistical package SAS 8.01 (SAS Institute, USA). The non-parametric Kruskal-Wallis rank test with a pairwise multiple comparison, which used the Dunn method for post-hoc analysis, was used to evaluate the differences in the B/B ratios between the groups [49]. One-way ANOVA was used to assess the individual differences in the serum antibody titers and lymphocyte proliferation assays. The Levene's test for homogeneity of the data was used to determine the equality of the variances among the groups [12]. A P-value <0.05 was considered significant.



**Fig. 1.** Colorimetric detection of SDS-PAGE analysis of a coupled in vitro transcription/translation reaction (Promega). Lane 1 = SDS-PAGE molecular weight standard, broad range (Invitrogen); Lane 2 = pcDNA-ChIFN- $\gamma$ . The position of the interferon gamma protein is on the right side. The sizes of the marker proteins are indicated on the left.

## Results

### In vitro transcription and translation

The protein expressed by pcDNA-ChIFN- $\gamma$  was detected using an in vitro transcription/translation and detection system (Fig. 1), and a band with a molecular weight of 17-18 kDa was observed. According to the observations reported by Song *et al.* [35], the molecular weight of the recombinant ChIFN- $\gamma$  expressed in *E. coli* was 17-18 kDa, which corresponds to the nonglycosylated ChIFN- $\gamma$  form.

### Evaluation of adjuvant in chickens

The protective efficacy against IBDV in the chickens immunized with the DNA vaccine mixed with selected adjuvants, DDA, CpG-ODN and ChIFN- $\gamma$  was examined by challenging the chickens with the IBDV SH/92 strain two weeks after the second immunization and observing them clinically for 10 days. The efficacy of the adjuvants in these chickens was evaluated by the mortality, B/B ratios, serum antibody titers, and peripheral blood and splenic lymphocyte proliferation assays (Table 1, Fig. 2 & 3).

The clinical signs (anorexia, depression, and ruffled feathers) began to appear on three days after the challenge, and the mortality peaked over a period of 4-5 days after the challenge. The groups that received the DNA vaccine plus DDA or CpG-ODN had significantly lower survival rates than the group given the DNA vaccine alone ( $p < 0.05$ ). However, the survival rates of the groups given the DNA vaccine alone and DNA vaccine plus ChIFN- $\gamma$  were similar

**Table 1.** Protection efficacy of the adjuvants, as determined by mortality and bursal weight/ body weight (B/B) ratio, at day 10 after the challenge with the virulent IBDV SH/92 strain, and by the antibody titer to the IBDV, as determined by enzyme-linked immunosorbent assay (ELISA), in serum collected from the chickens before the challenge and at day 10 after the challenge

Group <sup>A</sup>	Mortality <sup>B</sup> (%)	B/B ratio <sup>C</sup> (mean ± SD)	ELISA antibody titer <sup>D</sup> (mean ± SD)	
			Prechallenge	Post-challenge
Normal control	0/9 (0)	5.09 ± 1.75a	<396 (0/9) <sup>F</sup>	<396 (0/9)
Challenge control	9/9 (100)a	- <sup>E</sup>	<396 (0/9)	-
DNA vaccine alone	5/9 (56)c	4.56 ± 2.13ab	<396 (0/9)	10626.75 ± 6150.98a (4/4)
DNA vaccine plus DDA	9/9 (100)a	-	<396 (0/9)	-
DNA vaccine plus CpG	7/9 (78)b	1.51 ± 0.44b	<396 (0/9)	17436.50 ± 1470.08a (2/2)
DNA vaccine plus IFN- $\gamma$	6/9 (67)bc	2.04 ± 0.21ab	<396 (0/9)	14118.33 ± 1192.05a (3/3)

<sup>A</sup>Normal control: without vaccine and challenge; Challenge control: without vaccine but with the challenge; DNA vaccine alone: with pcDNA-VP243 DNA vaccine and with the challenge; DNA vaccine plus DDA: with pcDNA-VP243 mixed with DDA and with the challenge; DNA vaccine plus CpG-ODN: with pcDNA-VP243 mixed with CpG-ODN and with the challenge; DNA vaccine plus chIFN- $\gamma$ : with pcDNA-VP243 and plasmid encoding chIFN- $\gamma$  and with the challenge. At week 2 after the second vaccination, all groups except the normal control group were orally challenged with  $1 \times 10^{4.8}$  ELD<sub>50</sub> of the virulent IBDV SH/92 strain.

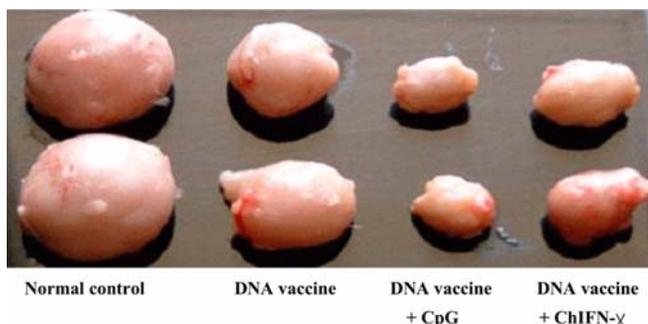
<sup>B</sup>Mortality was recorded during the 10 day period and is presented as the number of dead chickens/total number of chickens in each group. The values followed by the different lowercase superscripts are significantly different ( $p < 0.05$ ).

<sup>C</sup>B/B (Bursal/body weight) ratio was calculated by (bursa weight)/(body weight)  $\times 1000$  and presented as the mean  $\pm$  SD from each group. The values followed by different lowercase superscripts are significantly different ( $p < 0.05$ ).

<sup>D</sup>ELISA titers were determined using an ELISA kit (IDEXX, USA) according to the procedures recommended by the manufacturer. Titers greater than 396 are considered to be positive. Titer-Relates S/P at a 1:500 dilution to an endpoint titer:  $\log_{10}$  titer = 1.09 ( $\log_{10}$  S/P) + 3.36 (FlockCheck program). Each value represents the mean ELISA optical density obtained from the chickens  $\pm$  SD. The values followed by the same lowercase superscripts are not significantly different ( $p > 0.05$ ).

<sup>E</sup>None of the birds in this group survived to 10 days after the challenge.

<sup>F</sup>Number of positive serum samples/number of tested serum samples.



**Fig. 2.** The size and morphology of the representative bursa of Fabricius recovered from the chickens with or without the DNA vaccine at day 10 post-challenge with the virulent IBDV SH/92 strain. At week 2 after the second vaccination, all the groups except for the normal control group were orally challenged with  $1 \times 10^{4.8}$  ELD<sub>50</sub> of the virulent IBDV SH/92 strain.

( $p > 0.05$ ). In particular, the DNA vaccine plus DDA group showed a similar mortality to the challenge control group.

Bursal atrophy in the chickens receiving the DNA vaccine plus CpG-ODN or ChIFN- $\gamma$  was severe compared with that observed with the DNA vaccine alone and with the normal control group (Fig. 2). The bursae of the challenge control and the DNA vaccine plus DDA groups were not evaluated because all the chickens in this group died within the post-challenge period. The B/B ratio for the DNA vaccine plus CpG-ODN group was not significantly lower than that observed with the DNA vaccine alone ( $p > 0.05$ ) (Table 1). The B/B ratio for the DNA vaccine plus ChIFN- $\gamma$  was lower

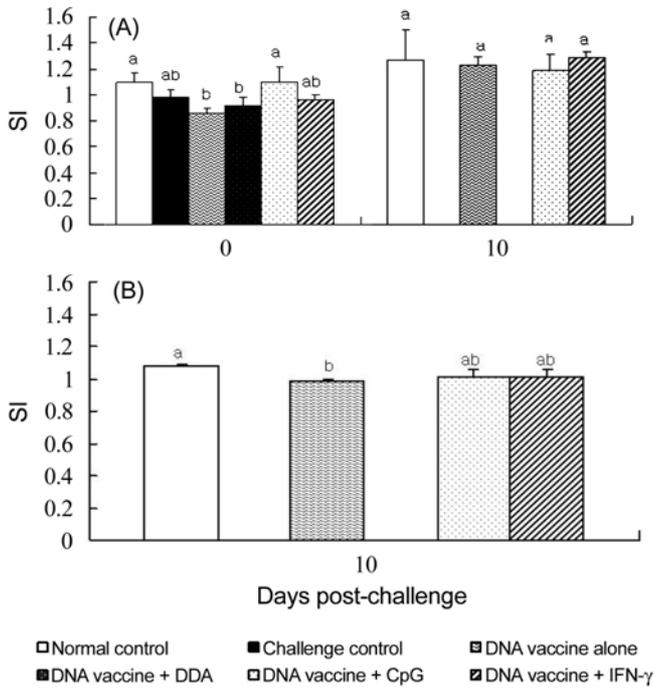
than for the DNA vaccine alone or normal control but the difference was not significant ( $p > 0.05$ ).

None of the chickens in all groups had any detectable antibodies to the IBDV before the challenge. However, all the surviving chickens of each group except for the normal control group showed detectable antibodies to the IBDV at day 10 after the challenge (Table 1). Similar ELISA antibody titers were detected in all groups except for the normal control group ( $p > 0.05$ ).

The kinetic changes in ConA-induced peripheral blood and splenic lymphocyte proliferation in the chickens in each group were measured using the WST-8 assay before and after the challenge with the IBDV SH/92 strain (Fig. 3). The SI of the peripheral blood in all groups was similar immediately before the challenge except for the normal control group ( $p > 0.05$ ). The SI at day 10 post-challenge in the DNA vaccine plus CpG-ODN or ChIFN- $\gamma$  groups was almost identical to those of the group receiving the DNA vaccine alone. The SI for the splenic lymphocytes on day 10 after the challenge were higher in the DNA vaccine plus CpG-ODN and ChIFN- $\gamma$  groups than in the DNA vaccine control group but the difference was not significant ( $p > 0.05$ ).

## Discussion

Various compounds have been examined and recommended as adjuvants for stimulating the immune response. However, the commercially available adjuvants are still mostly limited



**Fig. 3.** The mitogenic responses of peripheral blood (A) and splenic (B) lymphocytes prepared from randomly selected chickens with or without DNA vaccines after the challenge with the virulent IBDV SH/92 strain. Within the same day, the values followed by the different lowercase superscripts are significantly different ( $p < 0.05$ ). All the birds in the challenge control and DNA vaccine plus DDA groups died within 10 days after the challenge.

to the conventional adjuvants that contain oil emulsions and insoluble salts of aluminum [13]. Although mineral oil emulsions are used in a wide range of food animals, their undesirable side effects, such as deterioration in the meat quality at the injection sites and the risk of mineral oil residues in the meat from these animals, highlights the need for new adjuvants [13]. This study evaluated DDA, CpG-ODN and ChIFN- $\gamma$ , which have been reported to be immunomodulators against various infectious diseases in many animal models [7,14,16,22,42], as adjuvants for pcDNA-VP243 vaccine against IBDV [17].

The pre-challenge serum antibody titers of the chickens, whether vaccinated or not, were all negative as tested by ELISA, but all the post-challenge serum samples revealed high antibody titers except for the normal control group. The commercial IBDV antibody test kit used in these experiments was specifically designed to evaluate the immune status of the chickens for the IBDV, and only those serum samples with antibody titers  $>369$  were considered positive. Although it was reported that protection against IBDV correlates with the levels of neutralizing antibodies in chickens [26], the antibody titers for the DNA vaccine alone and for our adjuvant groups were not in proportion to their mortality or B/B ratios. However, there were no significant differences

between the groups receiving the DNA vaccine alone and those receiving the DNA vaccine plus CpG-ODN or ChIFN- $\gamma$ . This is despite the fact that the adjuvant groups had higher mortality rates than the group given the DNA vaccine alone. It appears that other protective mechanisms might have been induced in the chickens from the vaccine-only group. The chickens were partially or fully protected against the IBDV challenge, even though the VP2-VP4-VP3-expressing plasmid, which was constructed with the classical IBDV strain STC, induced only low or undetectable levels of antibodies in the chickens before the challenge [3]. In this study, the high post-challenge serum antibody titers appeared to have been induced by the challenge virus.

The lymphocyte proliferation assay is widely used to evaluate the cell-mediated immune responses in chickens in the normal and disease states [27,29]. The DNA vaccine alone and DNA vaccine plus CpG-ODN or ChIFN- $\gamma$  produced in higher levels of peripheral lymphocyte proliferation in response to ConA at 10 days post-challenge than the pre-challenge. However, the differences between these groups were not statistically significant. In addition, the splenic lymphocyte proliferation assay at day 10 post-challenge showed a similar of mitogenic response between the 3 groups. In a previous study with the DNA vaccine [17], the level of peripheral blood lymphocyte proliferation in the challenge control group decreased after the challenge.

DDA has been identified as an effective enhancer of the immune response to bacteria [15] and viruses [18,19] in mammalian models. In chickens, DDA enhanced the antibody response after a secondary boost with the Newcastle disease virus vaccine [33] and augmented the humoral and T-cell immunity to coccidial antigens [23]. DDA improved the pseudorabies virus-specific humoral immune and cell-mediated responses after DNA vaccination against the pseudorabies virus [41]. However, DDA had an adverse effect on the efficacy of the pcDNA-vp243 vaccine based on the mortality and B/B ratio when co-administered with the DNA vaccine. The amount or preparation method of DDA might have to be modified in future studies because several researchers have used various DDA formulations in their experiments.

The efficacy of CpG-ODNs as immunomodulators has been reported in many studies [20,24,36,37,47,48]. According to these studies, CpG-ODNs activate and induce the maturation of several cells such as B cells, macrophages, NK cells and dendritic cells which play important roles in the immune system. However, the CpG-ODNs used in this study worsened the DNA vaccine effect. Although the co-administration of DNA vaccine with CpG-ODNs (ODN2007) in chickens surviving after challenge induced an almost identical antibody titer and a similar stimulation index score in the lymphocyte proliferation assay compared to those obtained with DNA vaccine alone, co-administration also increased the mortality rate and induced bursal atrophy. This

suggests that the CpG-ODN was not an effective immunomodulator for the pcDNA-VP243 DNA vaccine. Another CpG-ODN (ODN2135) previously showed a high stimulation index in a proliferation assay of PBMC in the peripheral blood from chickens [32] in another experiment. However, there was no significant difference between the pcDNA-VP243 alone and pcDNA-VP243 plus ODN2135 (data not shown). Conflicting results obtained with the co-administration of DNA vaccines and CpG-ODNs were reported [43,44]. The co-administration of CpG oligodeoxynucleotide with the DNA vaccines against IBDV carrying VP2 genes enhanced the protective immune response of the DNA vaccine in chickens [43]. However, the DNA vaccine, the CpG oligodeoxynucleotide, and the formulations used were different from these experiment. An ODN dose-dependent reduction in gene expression from a plasmid was reported in mice [44]. Different formulations (different times or sites) of the CpG-ODN and DNA vaccine also did not augment the antibody responses, possibly due to competition between the CpG-ODN and DNA vaccine for entry into the cells in addition to the lower nuclear resistance and poor stability of CpG-ODN *in vivo*. Several types of CpG-ODNs have been studied *in vitro* and *in vivo* [32]. The other CpG-ODNs might augment immune response to the pcDNA-VP243 DNA vaccine.

The effects of IFN- $\gamma$  on the immune response appear to be dependent upon the animal species and age, the types of combined antigen, the nature and dose of IFN- $\gamma$ , and the particular promoter/enhancer of the plasmid expressing the foreign gene. The co-administration of ChIFN- $\gamma$  expressed using a baculovirus system with the inactivated *Salmonella enteritidis* antigen enhanced the level of protection against *Salmonella enteritidis* challenge without increasing the antibody production [39]. The co-administration of the recombinant ChIFN- $\gamma$  with sheep red blood cell antigens resulted in stronger antibody responses and allowed a lower dose of the antigen to be used more effectively than in the chickens that received the antigen alone [25]. Harms *et al.* [10] reported a down-regulatory effect of IFN- $\gamma$  on both the SV40 and CMV promoter/enhancer-driven transgenes in most cell lines tested, and an up-regulatory effect of IFN- $\gamma$  on the MHC I promoter/enhancer-driven transgene in all the cell lines tested. In particular, the addition of IFN- $\gamma$  in myoblasts drastically reduced the SV40 and CMV promoter-driven expression in myoblasts.

The effect of IFN- $\gamma$  depended upon the promoter driving expression of the viral antigen. The immune responses to the antigen-expressing vectors carrying a viral promoter such as the SV40 were lower in presence of IFN- $\gamma$ . However, IFN- $\gamma$  had no effect on a vector expressing the antigen under the control of the MHC class II promoter [46]. In this experiment, the ChIFN- $\gamma$ -expressing plasmid under the control of the CMV promoter given at separate site did not increase the level of protection against the vvIBDV by the IBDV DNA

vaccine. In another experiment, the ChIFN- $\gamma$ -expressing plasmid co-injected with the IBDV DNA vaccine at the same site had an adverse effect on the level of protection against the vvIBDV in chickens (data not shown). The inhibitory effect of IFN- $\gamma$  was stronger with the co-injected DNA vaccine than with the separately injected DNA vaccine, which suggests that the effect of IFN- $\gamma$  might occur locally. These results agree with the results of most other researchers using the CMV or SV40 promoter/enhancer-driven transgenes, although the antigens expressed in the plasmids were different.

These studies showed that CpG-ODN and ChIFN- $\gamma$  had no significant adjuvant effect and DDA had a negative effect on the DNA vaccine of pcDNA-VP243. Further studies will be needed to identify the optimal adjuvants for the DNA vaccine against the IBDV.

## Acknowledgments

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (R05-2003-000-10500-0). The authors are grateful to Dr. Son Il Pak for excellent assistance in statistical analysis.

## References

1. **Cao M, Sasaki O, Yamada A, Imanishi J.** Enhancement of the protective effect of inactivated influenza virus vaccine by cytokines. *Vaccine* 1992, **10**, 238-242.
2. **Cao YC, Yeung WS, Law M, Bi YZ, Leung FC, Lim BL.** Molecular characterization of seven Chinese isolates of infectious bursal disease virus: classical, very virulent, and variant strains. *Avian Dis* 1998, **42**, 340-351.
3. **Chang HC, Lin TL, Wu CC.** DNA mediated vaccination against infectious bursal disease in chickens. *Vaccine* 2001, **20**, 328-335.
4. **Chettle N, Stuart JC, Wyeth PJ.** Outbreak of virulent infectious bursal disease in East Anglia. *Vet Rec* 1989, **125**, 271-272.
5. **Chow YH, Chiang BL, Lee YL, Chi WK, Lin WC, Chen YT, Tao MH.** Development of Th1 and Th2 populations and the nature of immune response to hepatitis B virus DNA vaccines can be modulated by co-delivery of various cytokine genes. *J Immunol* 1998, **160**, 1320-1329.
6. **Digby MR, Lowenthal JW.** Cloning and expression of the chicken interferon- $\alpha$  gene. *J Interferon Cytokine Res* 1995, **15**, 939-945.
7. **Dzata GK, Confer AW, Wyckoff JH 3rd.** The effects of adjuvants on immune response in cattle injected with a *Brucella abortus* soluble antigen. *Vet Microbiol* 1991, **29**, 27-48.
8. **Farrar MA, Schreiber RD.** The molecular cell biology of interferon- $\alpha$  and its receptor. *Annu Rev Immunol* 1993, **11**, 571-611.
9. **Fodor I, Horvath E, Fodor N, Nagy E, Rencendorsh A, Vakharia VN, Dube SK.** Induction of protective immunity

- in chickens immunised with plasmid DNA encoding infectious bursal disease virus antigens. *Acta Vet Hung* 1999, **47**, 481-492.
10. **Harms JS, Oliveira SC, Splitter GA.** Regulation of transgene expression in genetic immunization. *Braz J Med Biol Res* 1999, **32**, 155-162.
  11. **Heath AW, Playfair JHL.** Cytokines as immunological adjuvants. *Vaccine* 1992, **10**, 427-434.
  12. **Hicks CR, Turner KV.** *Fundamental Concepts in the Design of Experiments*. 5th ed. pp. 195-196, Oxford University Press, New York, 1996.
  13. **Hilgers LA, Nicolas I, Lejeune G, Dewil E, Boon B.** Effect of various adjuvants on secondary immune response in chickens. *Vet Immunol Immunopathol* 1998, **66**, 159-171.
  14. **Hilgers LA, Snippe H.** DDA as an immunological adjuvant. *Res Immunol* 1992, **143**, 494-503.
  15. **Hofhuis FM, Van der Meer C, Kersten MCM, Rutten VPMG, Willers JMN.** Effects of dimethyldioctadecylammonium bromide on phagocytosis and digestion of *Listeria monocytogenes* by mouse peritoneal macrophages. *Immunology* 1981, **43**, 425-431.
  16. **Katz D, Inbar I, Samina I, Peleg BA, Heller DE.** Comparison of dimethyl dioctadecyl ammonium bromide, Freund's complete adjuvant and mineral oil for induction of humoral antibodies, cellular immunity and resistance to Newcastle disease virus in chickens. *FEMS Immunol Med Microbiol* 1993, **7**, 303-313.
  17. **Kim SJ, Sung HW, Han JH, Jackwood D, Kwon HM.** Protection against very virulent infectious bursal disease virus in chickens immunized with DNA vaccines. *Vet Microbiol* 2004, **101**, 39-51.
  18. **Kraaijeveld CA, Ia Riviere G, Benaissa-Trouw BJ, Jansen J, Harmsen M, Snippe H.** Effect of the adjuvant dimethyl dioctadecyl ammonium bromide on the humoral and cellular immune responses to encephalomyocarditis virus. *Antiviral Res* 1983, **3**, 137-149.
  19. **Kraaijeveld CA, Snippe H, Harmsen M, Khader Boutahar-Trouw B.** Dimethyl dioctadecylammonium-bromide as an adjuvant for delayed type hypersensitivity and cellular immunity against Semliki Forest virus in mice. *Arch Virol* 1980, **65**, 211-217.
  20. **Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, Koretzky GA, Klinman DM.** CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* 1995, **374**, 546-549.
  21. **Kwon YK, Mo IP, Seong HW, Kang MI, Koh HB, Lee JG, Yang CK.** Studies on the pathogenicity of infectious bursal disease virus (SH/92) isolated in Korea. *RDA J Agric Sci* 1995, **37**, 637-647.
  22. **Lillehoj HS, Choi KD.** Recombinant chicken interferon-gamma-mediated inhibition of *Eimeria tenella* development in vitro and reduction of oocyst production and body weight loss following *Eimeria acervulina* challenge infection. *Avian Dis* 1998, **42**, 307-314.
  23. **Lillehoj HS, Lindblad EB, Nichols M.** Adjuvanticity of dimethyl dioctadecyl ammonium bromide, complete Freund's adjuvant and *Corynebacterium parvum* with respect to host immune response to coccidial antigens. *Avian Dis* 1993, **37**, 731-740.
  24. **Lipford GB, Sparwasser T, Bauer M, Zimmermann S, Koch ES, Heeg K, Wagner H.** Immunostimulatory DNA: sequence-dependent production of potentially harmful or useful cytokines. *Eur J Immunol* 1997, **27**, 3420-3426.
  25. **Lowenthal JW, O'Neil TE, Broadway M, David A, Strom G, Digby MR, Andrew M, York JJ.** Coadministration of IFN- $\alpha$  enhances antibody responses in chickens. *J Interferon Cytokine Res* 1998, **18**, 617-622.
  26. **Lukert PD, Saif YM.** Infectious bursal disease. In: Calnek BW, Barnes HJ, Beard CW, McDougald LR, Saif YM (eds.). *Diseases of Poultry*. pp. 721-738, Iowa State University Press, Ames, 1985.
  27. **Martin A, Lillehoj HS, Kaspers B, Bacon LD.** Mitogen-induced lymphocyte proliferation and interferon production following coccidia infection. *Avian Dis* 1994, **38**, 262-268.
  28. **Min W, Lillehoj HS, Burnside J, Weining KC, Staeheli P, Zhu JJ.** Adjuvant effects of IL-1 $\beta$ , IL-2, IL-8, IL-15, IFN- $\alpha$ , IFN- $\gamma$  TGF- $\beta$ 4 and lymphotactin on DNA vaccination against *Eimeria acervulina*. *Vaccine* 2001, **20**, 267-274.
  29. **Miyamoto T, Min W, Lillehoj HS.** Lymphocyte proliferation response during *Eimeria tenella* infection assessed by new, reliable, nonradioactive colorimetric assay. *Avian Dis* 2002, **46**, 10-16.
  30. **Oshop GL, Elankumaran S, Heckert RA.** DNA vaccination in the avian. *Vet Immunol Immunopathol* 2002, **89**, 1-12.
  31. **Qin L, Ding Y, Pahud DR, Chang E, Imperiale MJ, Bromberg JS.** Promoter attenuation in gene therapy: interferon-gamma and tumor necrosis factor-alpha inhibit transgene expression. *Hum Gene Ther* 1997, **8**, 2019-2029.
  32. **Rankin R, Pontarollo R, Ioannou X, Krieg AM, Hecker R, Babiuk LA, van Drunen Littel-van den Hurk S.** CpG motif identification for veterinary and laboratory species demonstrates that sequence recognition is highly conserved. *Antisense Nucleic Acid Drug Dev* 2001, **11**, 333-340.
  33. **Rijke EO, Loeffen AHC, Luticken D.** The use of lipid amines as immunopotentiators for viral vaccines. In: Bizzini B, Bonmassar E (eds.). *Advances in Immunomodulation*. pp. 433-443, Pythagora Press, Rome-Milan, 1998.
  34. **Snyder DB, Vakharia VN, Savage PK.** Naturally occurring-neutralizing monoclonal antibody escape variants define the epidemiology of infectious bursal disease viruses in the United States. *Arch Virol* 1992, **127**, 89-101.
  35. **Song KD, Lillehoj HS, Choi KD, Zarlenga D, Han JY.** Expression and functional characterization of recombinant chicken interferon-gamma. *Vet Immunol Immunopathol* 1997, **58**, 321-333.
  36. **Sparwasser T, Koch ES, Vabulas RM, Heeg K, Lipford GB, Ellwart JW, Wagner H.** Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur J Immunol* 1998, **28**, 2045-2054.
  37. **Sparwasser T, Miethke T, Lipford G, Borschert K, Hacker H, Heeg K, Wagner H.** Bacterial DNA cause septic shock. *Nature* 1997, **386**, 336-337.
  38. **Srivastava IK, Liu MA.** Gene vaccines. *Ann Intern Med*

- 2003, **138**, 550-559.
39. **Takehara K, Kobayashi K, Ruttanapumma R, Kamikawa M, Nagata T, Yokomizo Y, Nakamura M.** Adjuvant effect of chicken interferon-gamma for inactivated *Salmonella* Enteritidis antigen. *J Vet Med Sci* 2003, **65**, 1337-1341.
  40. **Vandenbroeck K, Nauwynck H, Vanderpooten A, Van Reeth K, Goddeeris B, Billiau A.** Recombinant porcine IFN-gamma potentiates the secondary IgG and IgA responses to an inactivated suid herpesvirus-1 vaccine and reduces postchallenge weight loss and fever in pigs. *J Interferon Cytokine Res* 1998, **18**, 739-744.
  41. **van Rooij EM, Glansbeek HL, Hilgers LA, te Lintelo EG, de Visser YE, Boersma WJ, Haagsmans BL, Bianchi AT.** Protective antiviral immune responses to pseudorabies virus induced by DNA vaccination using dimethyldioctadecylammonium bromide as an adjuvant. *J Virol* 2002, **76**, 10540-15045.
  42. **Verthelyi D, Kenney RT, Seder RA, Gam AA, Friedag B, Klinman DM.** CpG oligodeoxynucleotides as vaccine adjuvants in primates. *J Immunol* 2002, **168**, 1659-1663.
  43. **Wang X, Jiang P, Deen S, Wu J, Liu X, Xu J.** Efficacy of DNA vaccines against infectious bursal disease virus in chickens enhanced by coadministration with CpG oligodeoxynucleotide. *Avian Dis* 2003, **47**, 1305-1312.
  44. **Weeratna R, Brazolot Millan CL, Krieg AM, Davis HL.** Reduction of antigen expression from DNA vaccines by coadministered oligodeoxynucleotides. *Antisense Nucleic Acid Drug Dev* 1998, **8**, 351-356.
  45. **Xiang Z, Ertl HC.** Manipulation of the immune response to a plasmid-encoded viral antigen by coinoculation with plasmids expressing cytokines. *Immunity* 1995, **2**, 129-135.
  46. **Xiang ZQ, He Z, Wang Y, Ertl HC.** The effect of interferon-gamma on genetic immunization. *Vaccine* 1997, **15**, 896-898.
  47. **Yamamoto S, Yamamoto T, Shimada S, Kuramoto E, Yano O, Kataoka T, Tokunaga T.** DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells and inhibits tumor growth. *Microbiol Immunol* 1992, **36**, 983-997.
  48. **Yi AK, Klinman DM, Martin TL, Matson S, Krieg AM.** Rapid immune activation by CpG motifs in bacterial DNA. Systemic induction of IL-6 transcription through an antioxidant-sensitive pathway. *J Immunol* 1996, **157**, 5394-5402.
  49. **Zar JH.** *Biostatistical Analysis*. 3rd ed. pp. 228-231, Prentice Hall, Boston, 1996.