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Protection of chicken against very virulent IBDV provided by *in ovo* priming with DNA vaccine and boosting with killed vaccine and the adjuvant effects of plasmid-encoded chicken interleukin-2 and interferon-γ

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The aim of this study was to examine the efficacy of *in ovo* prime-boost vaccination against infectious bursal disease virus (IBDV) using a DNA vaccine to prime *in ovo* followed by a killed-vaccine boost post hatching. In addition, the adjuvant effects of plasmid-encoded chicken interleukin-2 and chicken interferon-γ were tested in conjunction with the vaccine. A plasmid DNA vaccine (pcDNA-VP243) encoding the VP2, VP4, and VP3 proteins of the very virulent IBDV (vvIBDV) SH/92 strain was injected into the amniotic sac alone or in combination with a plasmid encoding chicken IL-2 (ChIL-2) or chicken IFN-γ (ChIFN-γ) at embryonation day 18, followed by an intramuscular injection of a commercial killed IBD vaccine at 1 week of age. The chickens were orally challenged with the vvIBDV SH/92 strain at 3 weeks of age and observed for 10 days. *In ovo* DNA immunization followed by a killed-vaccine boost provided significantly better immunity than the other options. No mortality was observed in this group after a challenge with the vvIBDV. The prime-boost strategy was moderately effective against bursal damage, which was measured by the bursa weight/body weight ratio, the presence of IBDV RNA, and the bursal lesion score. *In ovo* DNA vaccination with no boost did not provide sufficient immunity, and the addition of ChIL-2 or ChIFN-γ did not enhance protective immunity. In the ConA-induced lymphocyte proliferation assay of peripheral blood lymphocyte collected 10 days post-challenge, there was greater proliferation responses in the DNA vaccine plus boost and DNA vaccine with ChIL-2 plus boost groups compared to the other groups. These findings suggest that priming with DNA vaccine and boosting with killed vaccine is an effective strategy for protecting chickens against vvIBDV.

Keywords: adjuvant, DNA vaccine, IBDV, prime-boost vaccination

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Introduction

Infectious bursal disease virus (IBDV) causes infectious bursal disease (IBD) or Gumboro disease, an acute and highly contagious disease that affects chickens at 3 weeks of age and older. The disease has a high mortality rate, and chickens that survive IBD have a decreased immune response to vaccination, are immunosuppressed and vulnerable to a variety of secondary infections. This disease is the source of enormous economic loss in the poultry industry worldwide [23].

IBDV, a member of the genus *Avibirnavirus* of the family *Birnaviridae*, is a double-stranded RNA virus with a genome consisting of segments A and B [26]. Segment A contains two open reading frames encoding VP5 protein and a precursor polyprotein that is proteolytically cleaved to yield the major structural proteins VP2 and VP3 [14,27]. VP2 is thought to be the major host-protective antigen, as it can elicit viral-neutralizing antibodies against IBDV [5]. Segment B encodes VP1, a protein with RNA-dependent RNA polymerase activity [26].

Vaccination with live attenuated viruses and killed viruses has been used to prevent IBD. These live conventional vaccines can cause immunosuppression and some bursal atrophy, and may not fully protect chickens against the very virulent IBDV (vvIBDV) strain and antigenic variants of IBDV [34,38]. Several DNA vaccines containing the *VP2* or *VP2-VP4-VP3* genes have been tested in chickens in an effort to eliminate these side effects [2,3,12,15]. However, repeated vaccinations with a large amount of DNA, and sometimes the use of an adjuvant, were necessary to provide adequate protection against IBDV. It is difficult to compare these studies because the methods, vaccination schedule, IBDV strains used to develop the vaccine, and challenges to the vaccine differed [2,3,12,15,24].

Recent reports have indicated that a prime-boost vaccination strategy could enhance the efficacy of DNA vaccines against several pathogens [13,33]. The prime-

boost vaccination regime typically involved priming with a DNA vaccine and boosting with killed vaccines or recombinant proteins. This method generated high levels of T-cell memory, induced extremely high levels of cell-mediated immunity against pathogens [30], and increased the antibody response to the vaccine [18]. DNA vaccination against IBDV involves priming with DNA vaccine and boosting with killed IBDV vaccine or recombinant fowlpox expressing the IBDV *VP2* gene [8,11]. These prime-boost vaccinations protected chickens against challenges by standard, variant, or classical IBDV strains [8,11].

Late-stage chicken embryos are immunologically competent and able to respond to antigens [33], and efforts are underway to develop a safe and effective *in ovo* vaccine. *In ovo* vaccines are particularly useful for large-scale poultry industries because they reduce labor costs, contamination and deliver an accurate dose without affecting hatchability [28].

Cytokines are vital immune modulators, and their use as a genetic adjuvant has been studied for several vaccines [1,10,12,37]. For example, chicken interleukin-2 (ChIL-2) enhanced the immunogenicity of DNA vaccines against IBDV [12], but chicken IFN-γ (ChIFN-γ) did not [10,32].

In this study, we evaluated the immunity against the vvIBDV strain provided by priming with an *in ovo* DNA vaccine prepared from a vvIBDV strain followed by boosting with killed IBV vaccine. We also investigated the effectiveness of plasmid-encoded ChIL-2 and ChINF-γ as adjuvants. To our knowledge, this is the first reported test of an *in ovo* DNA vaccine with genetic adjuvants followed by a killed-vaccine boost to a vvIBDV challenge.

Materials and Methods

Chickens

Fertilized eggs of specific-pathogen-free (SPF) White Leghorn chickens (Hy-Vac, USA) were incubated. The embryos to be vaccinated were inoculated with a plasmid formulation on embryonation day 18 (Table 1). Hatched layer-type chickens were placed into isolators operated under positive air pressure and provided with *ad libitum* food and water during the experimental period.

Construction and preparation of plasmids

The IBDV DNA vaccine containing the plasmid pcDNA-VP243, encoding for the VP2, VP3 and VP4 proteins of the vvIBDV SH/92 strain, and the plasmid pcDNA-ChIFN-γ, encoding for ChIFN-γ, were prepared as previously described [15,32].

Table 1. Protective immunity against very virulent infectious bursal disease virus (vvIBDV) provided by an *in ovo* prime with DNA vaccine followed by a killed-vaccine boost

Group*	Survival † (%)	Presence of IBDV RNA ‡ (%)	B/B ratio§ (Mean ± SD)	Bursal lesion score ‖ (mean ± SD)	ELISA antibody titer (mean ± SD)¶	
					Pre-challenge	Post-challenge
DNA vaccine plus boost	10/10 (100%)	5/10 (50%)^{b}	2.22 ± 0.63^{ab}	2.6 ± 0.48	404.8 ± 435.13^{abc}	11,704 ± 3053.51^{a}
DNA vaccine with ChIL-2 plus boost	8/10 (80%)	4/8 (50%)^{b}	1.84 ± 0.50^{bc}	2.9 ± 0.32	1900 ± 3556.96^{a}	10,344.71 ± 3207.64^{ab}
DNA vaccine with ChIFN- γ plus boost	7/10 (70%)	6/7 (85.7%)^{a}	1.92 ± 0.45^{abc}	3.0 ± 0.50	486.8 ± 553.12^{ab}	7,182 ± 2459.17^{abc}
DNA vaccine without boost	2/10 (20%)	2/2 (100%)^{a}	1.15 ± 0.06^{c}	3.5 ± 0.35	< 396 (0/10)^{d}	5,798.5 ± 1887.27^{abc}
Vaccine control	7/10 (70%)	5/7 (71.4%)^{a}	1.17 ± 0.80^{bc}	3.2 ± 0.38	2679.75 ± 2488.84^{a}	11,044 ± 3613.96^{a}
Challenge control	2/10 (20%)	2/2 (100%)^{a}	1.66 ± 0.31^{bc}	3.5 ± 0.35	< 396 (0/10)^{cd}	2,724 ± 301.22^{bc}
Normal control	10/10 (100%)	0/8 (0%)^{c}	3.59 ± 0.50^{a}	0.0 ± 0.0	< 396 (0/10)^{bcd}	< 396 (0/10)^{c}

*DNA vaccine plus boost: vaccinated with pcDNA-VP243 vaccine, boost, and challenge; DNA vaccine with IL-2 and boost: vaccinated with pcDNA-VP243 vaccine mixed with chicken IL-2 (ChIL-2), boost, and challenge; DNA vaccine with IFN-γ and boost: vaccinated with pcDNA-VP243 vaccine mixed with chicken IFN-γ (ChIFN-γ), boost, and challenge; DNA vaccine without boost: vaccinated with pcDNA-VP243 vaccine only; Vaccine control: no DNA vaccine, only boost and challenge; Challenge control: no vaccine, only challenge; Normal control: no vaccine or challenge. †Number of surviving chickens at 10 days post-challenge/total number of chickens in each group. ‡Presence of IBDV RNA in the bursae of surviving chickens at 10 days post-challenge. Values followed by different lowercase superscripts are significantly different ($p < 0.05$). §The B/B ratio of the surviving chickens 10 days post-challenge. Values followed by different lowercase superscripts are significantly different ($p < 0.05$). ‖Bursal lesion score (mean ± SD). The bursae of surviving chickens were histologically examined at 10 days post-challenge and scored from 0 to 4 on the basis of increasing severity. ¶ELISA antibody titers (mean ± SD) measured from blood samples collected pre-challenge and at day 10 post-challenge. A titer level greater than 396 was considered to be positive. Values followed by different lowercase superscripts are significantly different ($p < 0.05$).

The *ChIL-2* gene was isolated from spleens obtained aseptically from 8-week old SPF chickens. The spleens were passed through a plastic cell strainer (Becton Dickinson Labware, USA), and the lymphocytes were separated using Histopaque-1077 (Sigma, USA). The prepared splenocytes were rinsed three times in Hanks' Balanced Salt Solution (Invitrogen, USA) and incubated for 6 h at 1 × 10^7 cells/ml, 40°C, and 5% CO_2 in RPMI-1640 medium containing 10% fetal bovine serum (FBS) (Invitrogen, USA) supplemented with 12.5 μg/ml concanavalin A (ConA, Sigma). Total RNA was isolated and purified from the harvested splenocytes using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions, and cDNA was synthesized using random primers (Invitrogen, USA). Polymerase chain reaction (PCR) fragments were synthesized from the cDNA using the primers IL-2F (5'-GCCGCCGCCATGATGTGCAAAGTACTGATCTTT-3') and IL2-R (5'-TTATTTTTGCAGATATCTC-3'), which were synthesized based on the published *ChIL-2* sequence [36]. The sequence GCCGCCGCC, which is compatible with Kozak's rule, was incorporated into the 5' end of the IL-2F primer [16]. 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 1% agarose gels.

The PCR products were purified utilizing the GENECLEAN Turbo Kit (BIO 101, USA) according to the manufacturer's instructions. The purified PCR products were inserted into the pcDNA 3.1/V5/His-TOPO vector (Invitrogen, USA) and transformed into competent *Escherichia coli* (TOP 10) cells (Invitrogen, USA). Plasmid DNA was prepared using a plasmid purification kit (Intron, Korea). The nucleotide sequence and orientation of the plasmid construct were confirmed by DNA sequencing. The verified plasmid construct was named pcDNA-ChIL-2. Large quantities of all plasmid DNAs for administration were prepared by Aldevron (USA).

In vitro transcription and translation

In vitro expression of pcDNA-ChIL-2 was performed using the TNT Quick Coupled Transcription/Translation System (Promega, USA). The protein produced from this reaction was 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 of TBS containing 0.5% Tween 20. After analysis using a translation detection system (Transcend Colorimetric Translation Detection System, Promega), streptavidin-alkaline phosphatase conjugate was added to the membranes, which were then rocked gently for 60 min. Stabilized substrate (Western Blue Stabilized Substrate; Promega, USA) was then added to visualize the bands.

Immunization and challenge protocol

SPF eggs were randomly divided into seven experimental groups (Table 1). At embryonation day 18, one of four preparations for priming *in ovo*, pcDNA-VP243 (100 μg) with pcDNA empty vector (50 μg), pcDNA-VP243 (100 μg) with pcDNA-ChIL-2 (50 μg), pcDNA-VP243 (100 μg) with pcDNA-ChINF-γ (50 μg), or sterile PBS (pH 7.4), was administered directly into the amniotic sac using a 1 inch 23-gauge needle. The hatching rates for the experimental groups were 95～100%. At 1 week of age, the chickens, with the exception of the DNA vaccine with no boost, challenge, and normal control groups, were boosted intramuscularly with commercial killed IBD vaccine containing IBDV (Gumboro D78) emulsified in an oil base (Intervet, Netherlands).

At 3 weeks of age, all of the chickens, except those in the normal control group, were orally challenged with 1 × $10^{3.8}$ 50% egg lethal dose (ELD_{50}) of the vvIBDV SH/92 strain and observed for 10 days. At day 10 post-challenge, all surviving chickens were bled and euthanized for necropsy. The bursa and body weights were determined and the bursa weight/body weight (B/B) ratios calculated [B/B × 1,000]. The bursae of Fabricius were examined for histopathological lesions, and RT-PCR was used to detect evidence of IBDV RNA.

Detection of IBDV RNA in the bursa of Fabricius

IBDV RNA was extracted from bursae and purified using the Viral Gene-spin kit (Intron, Korea) according to the manufacturer's instructions. A 474-bp hypervariable region in the *VP2* gene was amplified by RT-PCR with the primers P2.3 (5'-CCCAGAGTCTACACCATA-3') and RP5.3 (5'-TCCTGTTGCCACTCTTTC-3') [20]. The RT-PCR conditions were as follows: 42°C for 60 min followed by heating at 94°C for 5 min; 35 cycles of 94°C for 30 sec, 51°C for 30 sec, and 72°C for 2 min; and 74°C for 15 min. The PCR products were analyzed on 1.5% agarose gels.

Histopathology of the bursa of Fabricius

Bursae collected from the surviving chickens at day 10 post-challenge were fixed in 10% buffered formalin. After routine processing, the tissues were embedded in paraffin, cut into approximately 3-μm sections, and prepared for hematoxylin and eosin (H&E) staining for histological examination. The bursal lesions were graded in five categories (0～4): 0, no lesions; 1, mild scattered cell depletion in a few follicles; 2, moderate with 1/3-1/2 of the follicles atrophied or with depleted cells; 3, diffuse with atrophy in all follicles; and 4, acute inflammation and acute necrosis typical of IBD [6]. The H&E stained tissues were examined by two veterinary pathologists who were blind to the treatment groups. When there was a discrepancy in the grading, the pathologists reached an agreement after

discussion.

Lymphocyte proliferation assay

Lymphocyte proliferation assays were performed as described [15]. Briefly, peripheral blood was collected aseptically from chickens before the challenge and at 10 days post-challenge. The blood collected from chickens of each group was pooled and then peripheral blood lymphocytes from pooled blood of each group were prepared. Lymphocytes were separated using Histopaque-1077 (Sigma, USA), washed three times, and resuspended in RPMI-1640 medium supplemented with 10% FBS. The cells were placed in 96-well flat-bottom tissue culture plates at 1.25×10^6 cells/well. ConA (12.5 μg/ml) was added to each well except for the negative-control well. The plates were incubated at 40°C for 48 h in 5% CO_2. Lymphocyte proliferation activity was measured using WST-8 working solution (Dojindo Laboratories, Japan). The optical density (OD) was determined at 450 nm, and the stimulation index (SI) was calculated as follows: SI = mean OD of ConA-stimulated cells / mean OD of unstimulated cells.

Enzyme-linked immunosorbent assay (ELISA) of antibodies

Blood samples were collected from the birds in each experimental group before the challenge and at 10 days post-challenge. Serum antibody titers were determined for the experimental groups using an Infectious Bursal Disease Antibody Test Kit (IDEXX, USA) as described [15]. Titers greater than 396 were considered positive.

Statistical analysis

All analyses were performed using SAS 9.0 statistical software (SAS Institute, USA). The non-parametric Kruskal-Wallis rank test with pairwise multiple comparison, using the Dunn method for *post-hoc* analysis, was used to evaluate the differences in the B/B ratios among the groups. A one-way ANOVA was used to assess individual differences in serum antibody titers and lymphocyte proliferation assays. Levene's test for homogeneity of the data was used to determine the equality of variances among groups [9]. p values below 0.05 were considered significant.

Results

Construction of plasmid and characterization *in vitro*

A 441-bp fragment of the *ChIL-2* gene, including Kozak's sequence, was amplified by RT-PCR (data not shown). The *ChIL-2* RT-PCR product was purified and inserted into the pcDNA3.1/V5/His-TOPO vector. The protein expressed from pcDNA-ChIL-2 was confirmed by *in vitro* transcription/translation and detection (Fig. 1). A band with a molecular weight of approximately 18.4 kDa was observed [35].

Immunization and challenge with vvIBDV

The effectiveness of a prime-boost vaccination strategy in enhancing the immunogenicity and protective effect of a DNA vaccine against IBDV was investigated. The experimental groups were immunized with DNA vaccine alone or vaccine mixed with selected genetic adjuvants at day 18 of embryonation, and boosted with killed vaccine at 1 week of age. The chickens were challenged with vvIBDV at 3 weeks of age. After 10 days of observation, the mortality rate, presence of IBDV RNA, B/B ratios, serum antibody titers, and ConA-induced peripheral blood lymphocyte proliferation were recorded (Table 1, Figs. 2 and 3).

Eggs given the vaccine and control eggs both had a hatchability rate of above 95%, indicating that the DNA vaccine did not affect embryo hatchability. The clinical signs of IBD (anorexia, depression, and ruffled feathers) began to appear at three days after the challenge, and these chickens died at 2 ~ 3 days after the first clinical signs. The DNA vaccine and boost group had a 100% survival rate, higher than those of the other groups. The DNA vaccine with ChIL-2 plus boost group also had a much lower mortality rate than the other groups. The DNA vaccine without boost group had a 20% survival rate, which was identical to that of the challenge control group.

IBDV RNA was detected in the bursa of Fabricius in every group except the normal control group, but it was present at significantly lower levels in the DNA vaccine with boost and DNA vaccine with ChIL-2 plus boost groups in comparison to the other groups ($p < 0.05$).

The damage caused to the bursa of Fabricius by the

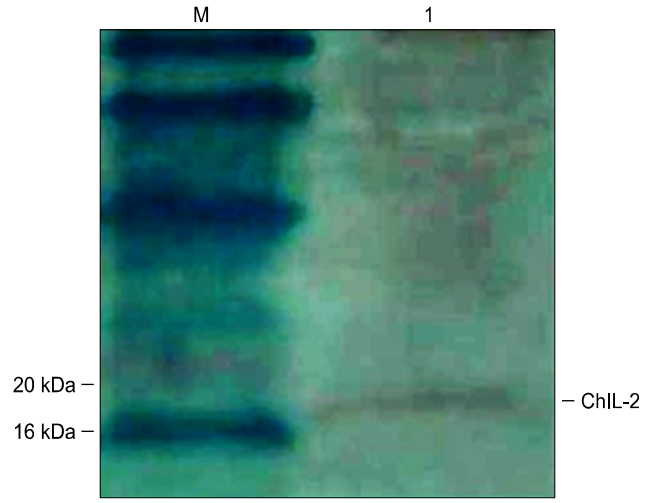

Fig. 1. Colorimetric translation detection of an SDS-PAGE analysis of a coupled in vitro transcription/translation reaction. Lane M = SDS-PAGE molecular weight standard, broad range (Invitrogen); Lane 1 = pcDNA-chicken IL-2 (ChIL-2). The position of the chicken interleukin 2 protein is on the right side. The sizes of the marker proteins are on the left.

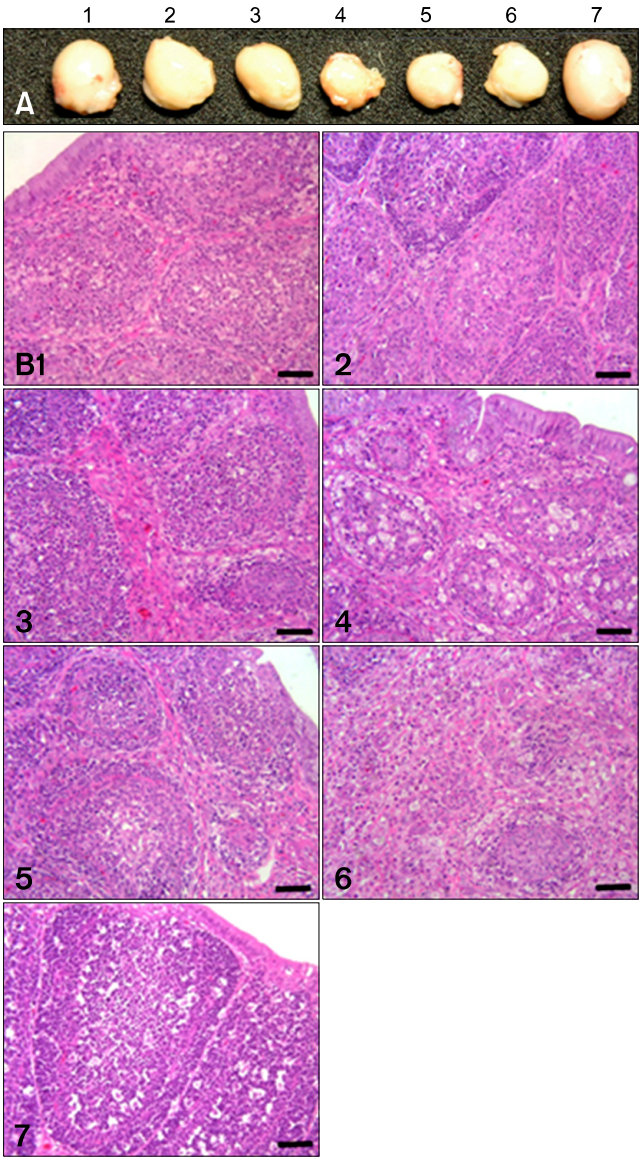

Fig. 2. The size (A) and hematoxylin-eosin-stained sections (B) of the representative bursa of Fabricius recovered from chickens either with or without DNA vaccine and adjuvants at day 10 post-challenge with very virulent IBDV SH/92 strain. Groups are: 1. DNA vaccine plus boost; 2. DNA vaccine with chicken IL-2 plus boost; 3. DNA vaccine with chicken IFN-γ plus boost; 4. DNA vaccine without boost; 5. Vaccine control; 6. Challenge control; 7. Normal control. Scale bars = 50 μm.

vvIBDV challenge was determined using the B/B ratio and a histological analysis of lesions in the bursa of Fabricius collected from the surviving chickens at 10 days post-challenge. The B/B ratios in the DNA vaccine with boost and DNA vaccine with ChIFN-γ plus boost groups were higher than in the other groups, and not significantly lower than that of the normal control group. Bursal atrophy was noted in all of the chickens that survived vvIBDV infection. The bursal lesions were characterized by lymphoid depletion

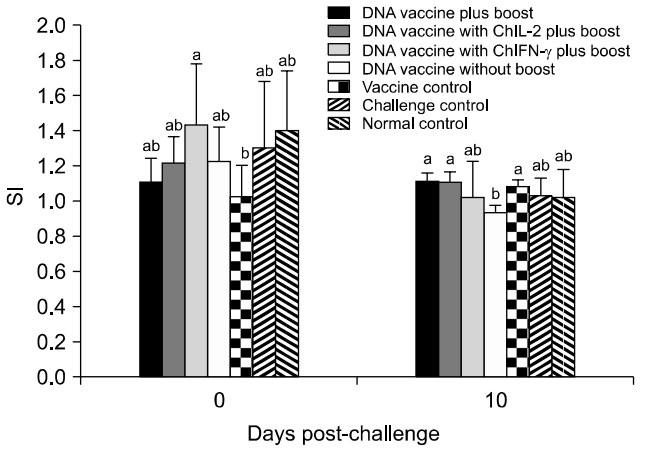

Fig. 3. The mitogenic responses of peripheral blood lymphocytes prepared from chickens before and after being challenged with the very virulent IBDV SH/92 strain. Cells were stimulated with Con A (1.25 μg/well) and each value was presented as the mean of the ELISA optical density obtained from randomly selected chickens ± SD. Within same day, values followed by different lowercase superscripts are significantly different ($p < 0.05$). Stimulation index (SI) = (mean OD of ConA-stimulated cells) / (mean OD of unstimulated cells).

and edema in the follicles, fibroplasias in the interfollicular connective tissues, and proliferation of the reticular epithelial cells. The DNA vaccine plus boost group had a lower bursal lesion score than the other groups; in particular the DNA vaccine without boost, vaccine control, and challenge control groups. The DNA vaccine with ChIL-2 plus boost and DNA vaccine with ChIFN-γ plus boost groups had similar bursal lesion scores, which were higher than those of the DNA vaccine plus boost group. Bursal atrophy and lesions were also noted in the DNA vaccine with boost group, but most of the lymphatic nodules were still present and had a considerable number of differentiated lymphocytes. In contrast, in the challenge control group, several lymphatic nodules were lost and replaced by the stroma of reticular epithelial cells. No protective effect was observed in the DNA vaccine without boost group.

Antibodies to IBDV were detectable in all groups before the challenge, with the exception of the DNA vaccine without boost, challenge control, and normal control groups. The DNA vaccine with ChIL-2 plus boost and the vaccine control groups had the highest antibody titers. Ten days after the challenge, all surviving chickens, except those in the normal control group, had detectable IBDV antibody levels. ELISA antibody titers in the DNA vaccine plus boost and vaccine control groups were significantly higher than those in the challenge control group ($p < 0.05$, Table 1).

The kinetic changes in ConA-induced peripheral blood lymphocyte proliferation in each group of chickens were measured using the WST-8 assay before and after the vvIBDV SH/92 strain challenge (Fig. 3). Immediately

prior to the challenge, the peripheral blood lymphocyte activity was significantly higher in the DNA vaccine with ChIFN-γ plus boost group than in the vaccine control group ($p < 0.05$). The peripheral blood lymphocyte activity in the DNA vaccine plus boost and DNA vaccine with ChIL-2 plus boost groups was significantly higher than in the DNA vaccine without boost group 10 days post-challenge ($p < 0.05$).

Discussion

Recently, several adjuvants and the prime-boost vaccination strategy have been used to improve the protective immunity of IBDV DNA vaccines [8,11,32]. This study investigated whether priming with an *in ovo* DNA vaccine with genetic cytokines followed by heterologous boosting with killed vaccine offered protection against vvIBDV. Because the vvIBDV strain produces a high rate of mortality in chickens, it is important to develop an effective vaccine against this virus [39]. Lymphoid necrosis and depletion are still observed in chickens protected by vaccination with attenuated live IBDV vaccine strains [38].

The DNA vaccine plus boost strategy was more effective than the other treatments as measured by the B/B ratio, the bursal lesion score, and the presence of IBDV RNA in the bursae, as well as the survival rate. We found that 100% of the chickens in the *in ovo* DNA vaccine plus boost group and 80% of the chickens in the *in ovo* DNA vaccine with ChIL-2 plus boost group survived after the challenge with vvIBDV. In a previous study using an identical DNA vaccine (pcDNA-VP243), 2-week-old chickens were injected twice at 2-week intervals with 200 μg of the vaccine, then challenged with vvIBDV 2 weeks after the second immunization. Their survival rate was 70% [15], showing that priming with an *in ovo* DNA vaccine and boosting with killed vaccine provides better protection than post-hatch DNA vaccination. There was considerably less bursal atrophy and lower bursal lesion score in the *in ovo* DNA vaccine plus boost and *in ovo* DNA vaccine with ChIL-2 plus boost groups, indicating that these strategies provided more effective protection from the virus, viral spreading, and cellular destruction than the others.

Several studies have investigated the efficacy of the heterologous prime-boost strategy to produce humoral and cell-mediated immunity against several pathogens. Priming with a DNA vaccine followed by a killed or live-vaccine or recombinant-protein boost have been tested against IBDV, infectious bronchitis virus, and influenza virus [8,11,13, 18]. Chickens primed with IBDV DNA vaccine and boosted with recombinant fowlpox expressing the *VP2* gene were protected against vvIBDV, but chickens that received the DNA vaccine or recombinant fowlpox alone were not protected, as indicated by bursal damage and B/B ratios [8]. Post-hatch priming with IBDV DNA vaccine and boosting with killed vaccine have been reported to protect chickens against homologous or heterologous IBDV [11]. In that experiment, as there were no mortalities in the experimental groups, vaccine efficacy was measured by gross bursal lesions and the B/B ratios.

The DNA priming vaccine can be administered either *in ovo* or in hatched chickens [8,11,13]. *In ovo* vaccinations are usually performed at embryonation day 18 and have been investigated as an alternative to post-hatch vaccination for several avian pathogens [8,13]. In this method, the appropriate expression of genes inserted into the plasmid vector is essential for the production of protective immunity in the embryos. Chicken embryos in the late stage have an immunological response to antigens, and *in ovo* immunization would produce immunity earlier than post-hatch inoculation and allow rapid and massive vaccination using the automatic egg injection system [4]. *In ovo* vaccination of chickens with an intermediate strain of IBDV produced active immunity and quick recovery from bursal damage and provided protection similar to that of post-hatching vaccination [4]. The S1 protein, including the *IBV S1* gene, was expressed in the bursa and heart of chicken embryos following the delivery of DNA vaccine into the allantoic sac, with the expression of the IBV and NDV viral proteins detected in the liver and muscle of embryos that received plasmid vector containing the viral gene [13]. In our experiment, the chickens that received *in ovo* DNA vaccine with no boost had low protective immunity, although the priming effect of the *in ovo* DNA vaccine was confirmed in the DNA vaccine plus boost group. Our results were consistent with those of another study using *in ovo* vaccination with a recombinant plasmid containing the *VP2* gene of IBDV, in which vaccination without boosting failed to provide complete protection against the viral challenge [8]. The incomplete protection may be explained by DNase activity detected in the amniotic fluid. [13] DNA vaccine is generally delivered into the amniotic fluid, and it is possible that the DNase degraded the plasmid DNA. Cationic liposomes or neutral lipids could be used to overcome degradation of the DNA vaccine by DNase [29].

Both humoral immunity and cell-mediated immunity are involved in the protection against IBDV in chickens [31]. The commercial IBDV antibody kit used in this experiment was designed to evaluate the status of immunity to IBDV, and only serum samples with antibody titers greater than 396 were considered positive. Before the challenge, all groups that received the booster, including the vaccine control group that received killed vaccine alone, exhibited antibody titers greater than 396, indicating that the booster produced humoral immunity against IBDV. Surviving chickens of the boosted groups, including the vaccine control, had higher antibody titers after the challenge than the challenge control group, suggesting that the prime-boost strategy was effective. Chickens that received the

DNA vaccination followed by boosting with killed vaccine after hatching had higher antibody titers than chickens boosted with homologous DNA vaccine [11]. Cell-mediated immunity involving T cells appears to contribute to the protection against IBDV [15,34,40]. In a previous study, we showed that lymphocytes collected from chickens immunized against IBDV by DNA vaccination continued to proliferate when stimulated with ConA [15]. Chickens with severely compromised antibody-producing ability following treatment with cyclophosphamide retained memory T cells and the immune response that destroys IBDV in the absence of antibodies [40]. However, compromising functional T cells by neonatal thymectomy and Cyclosporin A resulted in a lack of protection against IBDV following immunization with an inactivated IBDV vaccine [34]. Further, priming with *in ovo* DNA vaccine and boosting with recombinant fowlpox has been reported to produce immunity in chickens, with no antibody detected before or after the viral challenge [8]. In our study, the SI was higher after the challenge in the DNA vaccine plus boost and the DNA vaccine with ChIL-2 plus boost groups compared to the other groups. This finding indicates that peripheral blood lymphocyte activity was maintained after the challenge, and that the cell-mediated immune response involving T cells contributed to the immunity. Therefore, it was likely that the high level of protection in the DNA vaccine plus boost group was the result of both humoral and cell-mediated immunity.

Cytokines can be used to enhance the efficacy of conventional or genetic vaccines that do not produce a sufficient immune response when used alone. Interleukin 2 and INF-γ are the primary adjuvants investigated for use in poultry vaccines and several studies have investigated the efficacy of INF-γ as an adjuvant against pathogens [10,22, 32]. Duck INF-γ used as an adjuvant increased the protective efficacy of a DNA vaccine against duck hepatitis B virus [22]. However, ChINF-γ co-administered with IBDV DNA vaccine in hatched chickens did not enhance protective immunity against IBDV [10,32]. The present study showed that co-delivery of ChIL-2 or ChINF-γ with IBDV DNA vaccine did not enhance immunity to vvIBDV, and that the adjuvants partially decreased the protective efficacy compared with DNA vaccine plus boost alone. Our results for ChINF-γ in an *in ovo* vaccination trial were similar to those of previous studies performed in hatched chickens [10,32], with the promoter-driven expression of SV40 and CMV in myoblasts significantly reduced by the addition of INF-γ [7]. Therefore, ChINF-γ expressed by pcDNA-ChINF-γ co-delivered with the DNA vaccine may inhibit the expression of viral genes under the control of the CMV promoter. It appeared that the effects of INF-γ on the immune response are likely to be dependent on the animal species, the types of combined antigens, and the promoter of the plasmid expressing the cloned gene [7,10,22,32].

The immune-enhancing function of ChIL-2 was not observed in this *in ovo* IBDV DNA vaccination scheme, although others have observed that ChIL-2 increased the protective immune response of IBDV DNA vaccine or live IBDV vaccine in chickens, and immunization with bicistronic DNA vaccine expressing IBDV-VP2 and ChIL-2 to 2-week-old chickens showed effective protection against IBDV [12,17,37]. *In ovo* immunization with ChIL-2 plus a plasmid encoding 3-1E Eimeria gene enhanced protective intestinal immunity against coccidiosis in chickens, but subcutaneous injection did not increase host immunity [21,25]. Therefore, the effects of IL-2 on the immune response appear to be affected by the types and combined methods of vaccines and adjuvants, the route and time of inoculation, the promoter of the plasmid expressing the cloned genes, and animal species.

In summary, we have demonstrated that *in ovo* DNA vaccination followed by a killed vaccine boost completely protected chickens against mortality after challenge with vvIBDV. Further studies may be needed to improve the efficacy of DNA vaccines by varying parameters such as the interval between priming and boosting, the vaccine used for boosting, and the use of new chemical or genetic adjuvants. We are currently examining these factors to improve the protective immune response to vaccination in chickens.

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