Virus-Like Particle Vaccine Confers Protection against a Lethal Newcastle Disease Virus Challenge in Chickens and Allows a Strategy of Differentiating Infected from Vaccinated Animals

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In this study, we developed Newcastle disease virus (NDV) virus-like particles (VLPs) expressing NDV fusion (F) protein along with influenza virus matrix 1 (M1) protein using the insect cell expression system. Specific-pathogen-free chickens were immunized with oil emulsion NDV VLP vaccines containing increasing dosages of VLPs (0.4, 2, 10, or 50 μg of VLPs/0.5-ml dose). Three weeks after immunization, the immunogenicity of the NDV VLP vaccines was determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit, and a lethal challenge using a highly virulent NDV strain was performed to evaluate the protective efficacy of the NDV VLP vaccines. NDV VLP vaccines elicited anti-NDV antibodies and provided protection against a lethal challenge in a dose-dependent manner. Although the VLP vaccines containing 0.4 and 2 μg of VLPs failed to achieve high levels of protection, a single immunization with NDV VLP vaccine containing 10 or 50 μg could fully protect chickens from a lethal challenge and greatly reduced challenge virus shedding. Furthermore, we could easily differentiate infected from vaccinated animals (DIVA) using the hemagglutination inhibition (HI) test. These results strongly suggest that utilization of NDV VLP vaccine in poultry species may be a promising strategy for the better control of NDV.

Newcastle disease virus (NDV), also known as avian paramyxovirus serotype 1 (APMV-1), is an enveloped nonsegmented negative-strand RNA virus which is a member of the genus Avulavirus in the family Paramyxoviridae (1). On the basis of their pathogenicity for chickens, specifically by the intracerebral pathogenicity index (ICPI), NDVs are classified as asymptomatic, lentogenic, mesogenic, or velogenic (virulent) strains (2). Among infections by these NDV strains, only infection by a virulent NDV strain is defined as Newcastle disease (ND) (1, 2), which is one of the most devastating diseases in the poultry industry. Since its first recognition in 1926 at Newcastle-upon-Tyne in England, ND has become endemic across large geographical regions, causing enormous economic losses worldwide (1). ND outbreaks often result in approximately 100% mortality in fully susceptible poultry species, and due to the serious impact of outbreaks, ND was previously on the World Organisation for Animal Health’s (OIE) list A of diseases (http://www.oie.int/en/animal-health-in-the-world/the-world-animal-health-information-system/old-classification-of-diseases-notifiable-to-the-oie-list-a/), and it is now categorized as a disease notifiable to the OIE (http://www.oie.int/animal-health-in-the-world/oie-listed-diseases-2014/).

Currently, in order to keep ND under control, stringent vaccination policies are being maintained in many countries worldwide. Classically, inactivated or live NDV vaccines have been frequently used to control ND. These vaccines have been used routinely for decades, especially in regions where ND is endemic, and have acted as effective control measures in ND outbreaks (2). However, despite their contributions to the control of ND, these vaccines have significant limitations on differentiating infected from vaccinated animals (DIVA), and this necessitates the development of novel vaccines that allow a DIVA strategy with solid protective efficacy.

Virus-like particles (VLPs), which morphologically resemble authentic virus structures (from which they were given their name), have been suggested as a novel vaccine antigen against several viral pathogens (3–5). VLPs have been produced in prokaryotic and eukaryotic expression systems (5, 6), and VLP vaccines have been shown to confer high levels of protective efficacy against various viral pathogens. The safety, immunogenicity, protective efficacy, and mode of host immune response stimulation have been described well in recent studies (7–11). The formation of NDV VLPs was first characterized using an avian cell line (12), and the immunogenicity of the avian-cell-expressed NDV VLPs has been evaluated using a mouse model (13). A review article written by Trudy G. Morrison describes the development and the immunogenicity of NDV VLPs in detail (11). However, the protective efficacy of NDV VLP vaccine against a lethal NDV challenge in chickens has not been studied.

In this study, we developed NDV VLPs expressing NDV fusion (F) protein along with influenza virus matrix 1 (M1) protein using insect cell lines for the first time and evaluated the immunogenicity and protective efficacy against a lethal NDV challenge in specific-pathogen-free (SPF) chickens. Furthermore, the DIVA test...
was performed to differentiate VLP-vaccinated chickens from vaccinated and then infected chickens.

**MATERIALS AND METHODS**

**Cloning of NDV F and influenza virus M1 genes.** For amplification of the NDV F gene, viral RNA was extracted from the virulent NDV strain Kr-005/00 (kindly provided by the animal and plant quarantine agency of Korea; GenBank accession no. AV630423) using the Viral Gene-Spin viral DNA/RNA extraction kit (iNtRon Biotechnology, Republic of Korea) according to the manufacturer’s instructions. For cDNA synthesis, reverse transcription (RT) was performed on extracted viral RNA using SuperScript III (Invitrogen, USA) with random hexamers. From the cDNA, the NDV F gene was PCR amplified using a previously described primer pair (14), F-forward and F-reverse (see Table S1 in the supplemental material).

Viral RNA was extracted from influenza virus strain A/Puerto Rico/8/1934 (H1N1; GenBank accession no. KCB66600.1) as described above, and the influenza virus M1 gene was amplified as previously described (15) using the primer pair M-1 and M-1027R (see Table S1 in the supplemental material).

PCR-amplified NDV F and influenza virus M1 genes were cloned into the TA cloning vector pGEM-T (Promega, USA), and each gene sequence was determined by DNA sequencing. The two resulting plasmid vectors containing the NDV F and influenza virus M1 genes were designated vF and vM1, respectively.

**Generation of recombinant baculoviruses and production of NDV VLPs.** The F and M1 genes were further amplified from vF and vM1 by PCR using primer pairs EcoRI-F-forward/HindIII-F-reverse and EcoRI-M1-F/HindIII-M1-R, respectively (see Table S1 in the supplemental material), and cloned into the pFastBac1T (Invitrogen) bacmid transfer vector. The resulting transfer vectors containing the F and M1 genes were designated pF and pM1, respectively. MAX Efficiency DH10Bac competent Escherichia coli cells (Invitrogen) were transformed with one of the constructed transfer vector plasmids, pF and pM1, to generate recombinant bacmids according to the manufacturer’s instructions. The recombinant bacmids were transfected into Sf9 cells seeded in 6-well plates for generating recombinant baculovirus (rBV), at a density of 8 × 10^5 cells/well, using Cellfectin reagent (Invitrogen). The two resulting rBVs encoding the NDV F and influenza virus M1 proteins were designated rBV F and rBV M1, respectively.

**Production and characterization of NDV VLP and preparation of VLP vaccines.** For NDV VLP production, Sf9 cells were coinfected with rBV F and rBV M1, both at a multiplicity of infection (MOI) of 5, for 72 h. The culture medium containing VLPs was collected and clarified by low-speed centrifugation (2,000 × g, 30 min, and 4°C) to remove large cell debris, and VLPs from the clarified supernatants were pelleted (30,000 × g, 2 h, and 4°C). The pellet was resuspended in phosphate-buffered saline (PBS) solution (pH 7.2), loaded onto a 20%-35%-50% (wt/vol) sucrose density gradient, and sedimented by ultracentrifugation (150,000 × g, 1.5 h, and 4°C). After sedimentation, two major visible bands at the top of the 35% and 50% sucrose density gradients were collected. The protein concentration of each bands were determined by Bradford protein assay (Pierce, USA), and 2 μg of each band was analyzed by Western blotting. Expression of the NDV F protein was detected with chicken polyclonal sera against NDV and a horseradish peroxidase (HRP)-conjugated rabbit anti-chicken IgG secondary antibody (Bethyl, USA). Influenza virus M1 protein was detected with rabbit anti-M1 polyclonal antibodies (Immune Technology, USA) and an HRP-conjugated goat anti-rabbit IgG secondary antibody (Merck, Germany). The presence of NDV RNA was observed by transmission electron microscopy (TEM; Tecnai G2 Spirit, FEI, Netherlands, installed at Korea Basic Science Institute) using a negative staining method as previously described (16). Different concentrations of NDV VLPs collected at the top of the 35% sucrose density gradient were emulsified in the oil adjuvant Montanide ISA70 (SEPPIC, France) at a ratio of 30:70 (vol/vol) to obtain escalating doses of VLP vaccines (0.4, 2, 10, and 50 μg of VLPs/0.5 ml dose).

**Immunization of animals and determination of immunogenicity.** A total of 50 6-week-old SPF White Leghorn chickens (Namduck Sanitec, Republic of Korea) were divided into five groups (10 chickens per group) and marked individually. Four groups of chickens were intramuscularly immunized (0.5 ml per chicken) with escalating doses (0.4, 2, 10, and 50 μg of VLPs/chicken) of NDV VLP vaccine. As a mock-vaccinated control group, another 10 SPF chickens were injected with an emulsified solution of PBS with ISA70.

All animal procedures performed in this study were reviewed, approved, and supervised by the Institutional Animal Care and Use Committee (IACUC) of Konkuk University. Three weeks after a single immunization, sera were collected for determination of serum NDV-specific antibody levels using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Median Diagnostics, Republic of Korea), which is precoated with NDV strain LaSota (GenBank accession no. JPF905101.1). ELISA was performed according to the manufacturer’s instructions, with minor modifications. Briefly, serum samples were diluted 50-fold and added into the wells. After 30 min at 20°C, the plate was washed, and HRP-conjugated anti-chicken IgG antibody was added to each well. The plate was washed after 30 min at 20°C, the substrate tetramethylbenzidine peroxidase (TMB) was added, and the plate was incubated for 15 min at 20°C. The reaction was stopped by the addition of stop solution, and the optical density at 450 nm (OD450) was measured using an ELISA reader (Tecan, Switzerland) for calculation of the sample-to-positive (S/P) value of each sample (determined by the equation (OD450 sample – mean OD450 negative)/(mean OD450 positive – mean OD450 negative), where OD450 negative is the value for the negative control provided by the manufacturer and OD450 positive is the value for the positive control provided by the manufacturer).

**Lethal NDV challenge and assessment of protection.** Three weeks after a single immunization, chickens were intramuscularly challenged with 1 ml of 10^5.5 50% egg infective doses (EID50)/ml of the virulent NDV strain Kr-005/00. To assess the protective efficacy of the NDV VLP vaccines, mortality and clinical symptoms were observed daily for 14 days postchallenge (dpc). Clinical symptoms were classified as follows: normal (score of 0), mild depression (score of 1), neurological signs and/or severe depression (score of 2), or death (score of 3). The average clinical scores for each group were calculated daily.

To determine the challenge virus shedding, oropharyngeal and cloacal swab samples were collected and suspended in 1 ml of PBS supplemented with gentamicin (400 μg/ml) at 3, 5, 7, and 10 dpc. Viral RNA was extracted from 150 μl of this suspension using the Viral Gene-Spin viral DNA/RNA extraction kit (iNtRon Biotechnology), and the amount of NDV RNA was quantified by the cycle threshold (Ct) value using M gene-based real-time reverse transcription-PCR (rRT-PCR), as previously described (17). For extrapolation of Ct values of rRT-PCR to infectious units, serial dilutions of oropharyngeal and cloacal swab samples from the mock-vaccinated control group at 3 dpc were calculated as the EID50/ml. In parallel, the corresponding virus doses were analyzed by rRT-PCR, and the EID50 of virus was plotted against the Ct values of viral dilutions. The resulting calibration curves (see Fig. S1 in the supplemental material) were highly correlated (r^2 > 0.99) and used for converting Ct values to EID50.

**DIVA.** For serological differentiation of NDV VLP-vaccinated chickens from vaccinated and then infected chickens, the hemagglutination inhibition (HI) test was used because the NDV VLPs developed in this study do not contain hemagglutinin-neuraminidase (HN) protein, and thus HI antibodies were expected to be raised only after NDV infection. Serum samples were collected at 0 dpc, which is 3 weeks postvaccination (wpv), and at 14 dpc from all surviving chickens in the groups vaccinated with 2 μg and 50 μg of VLP vaccine. Additionally, five 6-week-old SPF chickens were intramuscularly immunized with commercial inactivated oil emulsion ND vaccine containing inactivated LaSota antigen (KBNP, Republic of Korea) and challenged with the virulent NDV strain Kr-005/00 at 3 wpv. From these five SPF chickens, serum samples were taken on May 14, 2021 by guest http://cvi.asm.org/ Downloaded from
at 0 and 14 dpc. Collected serum samples were analyzed for the presence of HI antibodies according to the OIE standard method (2) using NDV LaSota antigen.

Statistical analysis. Analysis of variance (ANOVA) with a Tukey-Kramer post hoc test was performed for comparison of serum antibody titers between groups. Fisher’s exact test was used for statistical analysis of the mortality and morbidity results. To compare differences of HI titers between the groups vaccinated with 2 and 50 μg of VLP vaccine, an unpaired t-test was used. Results with P values of <0.05 were considered to be statistically significant.

RESULTS

Characterization of NDV VLPs. Both bands positioned at the top of the 35% and 50% sucrose density gradients were found to contain F and M1 proteins by Western blotting (Fig. 1A), which identified bands at 48 kDa and 28 kDa, indicating the NDV F1 and influenza virus M1 proteins, respectively. Identification of the F1 protein, the cleavage product of F protein, indicates that F protein expressed in Sf9 cells could be cleaved by host proteases. The band positioned at the top of the 35% sucrose density gradient was found to contain both F and M1 proteins, with higher concentrations than that of the 50% sucrose density gradient (Fig. 1A), and was used for this study. Examination of negatively stained preparations by TEM revealed the presence of VLPs with a diameter of approximately 70 nm (Fig. 1B).

Immune responses to NDV VLP vaccines. Three weeks after a single immunization, as shown in Fig. 2, VLP-vaccinated groups showed significantly increased antibody responses, except for the group immunized with 0.4 μg of VLP vaccine. Antibody responses to the VLP vaccine between groups showed dose-dependent increases up to 10 μg. Groups immunized with 10 or 50 μg showed antibody responses superior to those of other groups, with statistical significance (P < 0.001).

Protection against lethal NDV challenge and reduced viral shedding in VLP-vaccinated chickens. As shown in Fig. 3 and in Table S2 in the supplemental material, mock-vaccinated chickens showed severe clinical signs and 100% mortality within 4 days after challenge, which ensures that a proper challenge was accomplished. The group immunized with 0.4 μg of VLP vaccine showed 100% mortality with severe clinical signs, and the group vaccinated with 2 μg showed partial protection (20% mortality) with moderate clinical signs. Importantly, two surviving chickens from the group vaccinated with 2 μg showed neurological signs (score of 2), including head tilt and posterior paresis, indicating that the 2 μg of NDV VLP vaccine was not effective enough to confer high levels of protective efficacy against lethal NDV infection in chickens. However, chickens vaccinated with 10 or 50 μg of vaccine were fully protected from mortality and morbidity, except one chicken from each group which showed only moderately reduced activity (clinical score of 1), as shown in Fig. 3B and in Table S2 in the supplemental material. Moreover, chickens vaccinated with 10 and 50 μg of vaccine not only survived but also showed significantly lower levels of challenge virus shedding than the mock-vaccinated group, it showed higher levels of viral shedding than did chickens vaccinated with 10 or 50 μg. The low levels of protective efficacy in the group vaccinated with 2 μg was in accordance with the result that the group vaccinated with 2 μg showed significantly lower levels of antibodies than the groups vaccinated with 10 and 50 μg (Fig. 2) and showed 20% mortality after the challenge (Fig. 3A). Interestingly, as shown in Fig. 4, the group vaccinated with 2 μg showed significantly higher levels of seroconversion, as measured by HI test, than did the group vaccinated with 50 μg at 14 dpc, reinforcing that the suppression of challenge virus replication was greater in the chickens vaccinated with 50 μg than in those vaccinated with 2 μg. All chickens immunized with 0.4 μg of VLP vaccine not only succumbed to death but also could not reduce challenge virus shedding.

These results suggest that a single immunization of chickens with 10 or 50 μg of NDV VLP vaccine could fully protect chickens after a lethal NDV challenge and could effectively reduce challenge virus shedding.
DIVA. As expected, HI antibody responses were not observed in prechallenge sera from VLP-vaccinated chickens. However, as shown in Fig. 4, HI antibodies were detected after NDV challenge from all VLP-vaccinated chickens, which allowed the DIVA. In contrast, the sera from chickens vaccinated with inactivated vaccine (n = 5) were all positive for HI antibodies both pre- and postchallenge. Therefore, VLP vaccine and the companion HI test could allow the utilization of the DIVA strategy, which is not applicable to the commercial inactivated ND vaccine.

DISCUSSION

The insect cell expression system has been recognized as a versatile recombinant protein expression tool, and it is now widely accepted as a proven technology in the industry. The key advantage of this recombinant protein manufacturing platform is that it can perform most of the posttranslational modifications (e.g., glycosylation, disulfide bond formation, and phosphorylation) and thus can produce biologically active proteins while offering the potential for low manufacturing costs. Moreover, insect cell expression systems have been widely used for the development of VLP vaccines against both human and animal viruses (15, 16, 18–20).

In the present study, we generated NDV VLPs using the insect cell expression system and evaluated its possible use as a DIVA vaccine in SPF chickens for the first time. To the best of our knowledge, this is the first study reporting the generation of NDV VLPs using the insect cell expression system. Even a single immunization with 10 or 50 μg of NDV VLP vaccine elicited significant levels of antibodies against NDV, fully protected chickens from a lethal NDV challenge, and strongly reduced challenge virus shedding. Moreover, we could differentiate VLP-vaccinated chickens from VLP-vaccinated and then infected chickens using the standard HI test, which is one of the most commonly utilized methods for detection of antibodies against NDV.

For the generation of VLPs in the insect cell expression system, adoption of a core protein from a well-established VLP production system is a frequently used strategy, resulting in so-called chimeric VLPs. For example, Haynes et al. used the murine leukemia virus gag protein for influenza VLP generation (21), and both Quan et al. and Wang et al. used influenza virus M1 protein for the generation of respiratory syncytial virus (RSV) VLPs (22) and porcine reproductive and respiratory syndrome virus (PRRSV) VLPs (23), respectively, in insect cell expression systems. In this study, we adopted influenza virus M1 as a core protein for the generation of NDV VLPs incorporating NDV F protein. In accordance with previous studies on the generation of RSV or PRRSV VLPs using influenza virus M1 protein (22, 23), coexpression of NDV F and influenza virus M1 protein in insect cells resulted in the generation of spherical particles of uniform size, indicating successful formation of NDV VLPs. These results may support the further utilization of influenza virus M1 as a core protein for the development of VLP vaccines in the insect cell expression system against various viruses.

Induction of complete prevention of virus infection, so-called sterilizing immunity, is an ideal goal for vaccination. Although 10 or 50 μg of NDV VLP vaccine in this study fully protected chick-

### TABLE 1 Challenge virus excretion from oropharyngeal swab samples

<table>
<thead>
<tr>
<th>Vaccine dose (μg)</th>
<th>3 dpc</th>
<th>5 dpc</th>
<th>7 dpc</th>
<th>10 dpc</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>2/10 (0.58)</td>
<td>1/10 (0.36)</td>
<td>4/10 (0.60)</td>
<td>0/10 (—)</td>
</tr>
<tr>
<td>10</td>
<td>1/10 (0.32)</td>
<td>2/10 (0.52)</td>
<td>7/10 (1.14)</td>
<td>1/10 (0.20)</td>
</tr>
<tr>
<td>2</td>
<td>8/10 (2.15)</td>
<td>7/8 (2.62)</td>
<td>5/8 (0.88)</td>
<td>1/8 (0.19)</td>
</tr>
<tr>
<td>0.4</td>
<td>10/10 (3.36)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Mock</td>
<td>10/10 (3.62)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Log EID₅₀ equivalents were determined with the use of rRT-PCR. Numbers in parentheses are averages of viral titers shed from each group. NA, not applicable (all chickens in the group were dead); —, not detected.

### TABLE 2 Challenge virus excretion from cloacal swab samples

<table>
<thead>
<tr>
<th>Vaccine dose (μg)</th>
<th>3 dpc</th>
<th>5 dpc</th>
<th>7 dpc</th>
<th>10 dpc</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>1/10 (0.29)</td>
<td>2/10 (0.56)</td>
<td>3/10 (0.48)</td>
<td>2/10 (0.41)</td>
</tr>
<tr>
<td>10</td>
<td>1/10 (0.27)</td>
<td>3/10 (0.79)</td>
<td>5/10 (1.01)</td>
<td>3/10 (0.50)</td>
</tr>
<tr>
<td>2</td>
<td>8/10 (1.62)</td>
<td>6/8 (1.88)</td>
<td>7/8 (1.41)</td>
<td>4/8 (0.62)</td>
</tr>
<tr>
<td>0.4</td>
<td>10/10 (2.88)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Mock</td>
<td>10/10 (2.71)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Log EID₅₀ equivalents were determined with the use of rRT-PCR. Numbers in parentheses are averages of viral titers shed from each group. NA, not applicable (all chickens in the group were dead).
ens from a lethal NDV challenge and strongly reduced challenge virus shedding, sterilizing immunity could not be achieved, since chickens vaccinated with 10 or 50 μg showed detectable levels of challenge virus shedding, especially at 7 dpc. It is expected that increasing the immunogenicity of the NDV VLP vaccine might confer sterilizing immunity against lethal NDV infection, and further studies on enhancing immunogenicity of the NDV VLP vaccine (e.g., incorporation of a molecular adjuvant into VLPs) will be required. Especially, application of a priming-boosting vaccination regimen in combination with viral vector vaccines expressing the F gene (24) and the NDV VLP vaccine developed in this study is strongly expected to confer sterilizing immunity while still allowing DIVA, although this needs to be studied.

Vaccination is being considered as an attractive control measure to prevent animal diseases. For effective vaccination programs, adequate surveillance in vaccinated animals is essential to determine whether the field virus is circulating in vaccinated animals (25). Therefore, vaccine development should be accompanied by development of proper DIVA strategies, which is often time-consuming and troublesome because of technical challenges. The NDV VLP vaccine developed in this study did not require the development of a companion DIVA test and enabled DIVA without performing complicated and expensive laboratory tests, since the HI test was successfully adopted as a DIVA test in DIVA without performing complicated and expensive laboratory tests. Serum samples were analyzed with the standard HI test for the presence of HI antibodies against NDV strain LaSota. Error bars represent standard deviations. ***, P < 0.001 by unpaired t test.

FIG 4 DIVA test using HI test. Serum samples were taken 0 and 14 days postchallenge from chickens immunized with 50 μg of VLP vaccine, 2 μg of VLP vaccine, or commercial inactivated NDV vaccine. Serum samples were analyzed with the standard HI test for the presence of HI antibodies against NDV strain LaSota. Error bars represent standard deviations. ***, P < 0.001 by unpaired t test.

the production cost of 10 μg of NDV VLP vaccine developed in this study was approximately 3 times higher than that of conventional inactivated whole-virus NDV vaccine produced in SPF eggs. Since this cost analysis of NDV VLP production was performed based on a laboratory-scale production process, optimization of a large-scale manufacturing process is expected to significantly reduce the production cost of NDV VLP vaccine. Moreover, as performed in our previous VLP study (15), omitting the costly purification step and the use of crude NDV VLP antigen might also be applicable for the reduction of NDV VLP vaccine production cost. For the industrialization of veterinary VLP vaccines with economic feasibility, dedicated studies on reducing VLP vaccine production cost are required.

In conclusion, we developed an NDV VLP vaccine using the insect cell expression system for the first time, which was highly immunogenic and fully protected chickens from lethal NDV infection, with strongly reduced challenge virus shedding. Furthermore, DIVA was successfully performed with a simple serological test, the HI test. These results strongly suggest that utilization of NDV VLP vaccine in poultry species may be a promising strategy for the better control of NDV.

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We declare that we have no conflict of interest.

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