

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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54. TÍTULO VIRUS QUIMERICOS DE LA ENFERMEDAD DE NEWCASTLE QUE PRESENTAN
PROTEINAS DE SUPERFICE NO NATIVAS

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DOMICILIO NEW YORK, ESTADO DE NEW YORK, E.U.A.

74. APODERADO LUIS FELIPE CASTILLO GIBSON

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Señores
DIRECCION DE NUEVAS CREACIONES
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
Bogotá D.C.,

Re.: Código 011 – 378 – 444
Expediente No. 08 – 67.535
Fase Nacional de Solicitud
de Patente PCT
**THE MOUNT SINAI SCHOOL OF
MEDICINE OF NEW YORK
UNIVERSITY**

En relación con el expediente de la referencia y dando cumplimiento a la última providencia recaída sobre este asunto por medio del presente escrito me permito manifestarles lo siguiente:

1. Se modifica el título de esta solicitud de patente como sigue:

**VIRUS QUIMÉRICOS DE LA ENFERMEDAD DE NEWCASTLE QUE
PRESENTAN PROTEÍNAS DE SUPERFICIE NO NATIVAS**

2. De conformidad con precisas instrucciones de mi representada adjunto un nuevo capítulo reivindicatorio, revisado y corregido.
3. La Solicitante ha decidido elegir el grupo de reivindicaciones dirigidas a los virus quiméricos de la enfermedad de Newcastle (NDV) y cancelar sin perjuicio el objeto reivindicado virus quiméricos de la influenza. El juego de reivindicaciones modificadas contiene dos reivindicaciones independientes: Las Reivindicaciones 1 y 5. Así, la Solicitante asevera que esta objeción queda superada.
4. En lo que respecta a los términos de las reivindicaciones que se refieren a polipéptidos y ácidos nucleicos, la sociedad Solicitante sostiene con respeto que estos términos como, por ejemplo, proteína de fusión F, proteína de fusión

HN, proteína F, proteína HN, antígeno protector, son bien entendidos por la persona medianamente versada en la materia técnica correspondiente y se describen en la especificación de la solicitud tal como fue radicada (ver, *p.ej.*, la Sección 2.1.2 comenzando en la p. 5; la Sección 5.2 comenzando en la p. 48; y la Sección 5.3 comenzando en la p. 51). Por consiguiente, no dificultan determinar la extensión del objeto que se pretende proteger. Ni representada anota también que el requisito de caracterizar los polipéptidos y ácidos nucleicos citados en las reivindicaciones por sus secuencias respectivas de aminoácidos y nucleótidos sería indebidamente laboriosa porque limitaría de forma injusta el alcance de la protección de lo que considera su invención y complicaría innecesariamente el pliego de reivindicaciones, porque esto exigiría enumerar varias secuencias que cubren los diversos genotipos revelados del NDV, algunos de los cuales pueden variar en un único aminoácido o nucleótido. Por último, el Solicitante desea anotar que las secuencias *específicas* no son importantes para la invención. El concepto inventivo aquí reivindicado se basa por lo menos en parte en la observación de que los NDV quiméricos con proteínas de fusión HN y F son capaces de inducir inmunidad protectora contra el NDV y un antígeno extraño independiente y esto no es dependiente de ninguna secuencia particular de aminoácidos o nucleótidos.

5. En lo que respecta a las objeciones de las referencias D2 ("Peeters") y D3 ("Zimmer"), Peeters construyó un NDV quimérico sencillamente intercambiando los dominios HN entre NDV y APMV4, otro paramixovirus de la misma familia. Zimmer construyó un virus Sendai quimérico (SeV) que expresa una proteína F de fusión del virus sincicial respiratorio (RSV)/SeV – de nuevo el virus quimérico resultante se basa en dos paramixovirus. La persona medianamente versada en la técnica habría esperado que las proteínas de los paramixovirus quiméricos de Peeters y Zimmer se expresaran e incorporaran a sus respectivas columnas vertebrales de paramixovirus porque los virus que se usan vienen de la misma familia. La persona medianamente versada en la técnica no habría esperado que otro tanto fuese cierto para una columna vertebral de paramixovirus que expresa una proteína de fusión que comprende por lo menos un epítipo de un antígeno que no es un antígeno de paramixovirus (*p.ej.*, una columna vertebral de NDV – paramixovirus, y un epítipo de influenza – ortomixovirus), como lo exigen las reivindicaciones modificadas que aquí presentamos. Ver, *p.ej.*, Zimmer en 10467, primer

parágrafo, en donde explica los diferentes mecanismos por los cuales las glucoproteínas de membrana de los ortomyxovirus y paramixovirus intermedian el ingreso a la célula.

6. Hacemos notar además que Zimmer generó sus virus quiméricos para estudiar la función de las glucoproteínas de superficie RSV y SeV, y concluyó que "SeV recombinante que contiene RSV-F quimérico como la única glucoproteína que representa una herramienta útil para el análisis ulterior del papel de RSV-F en el ingreso del virus". Zimmer, en 10476, última oración; ver también Zimmer en 10468, primer párrafo completo. Zimmer no enseña ni sugiere generar virus quiméricos basados en dos virus no relacionados con el propósito de inmunizar o vacunar contra uno o los dos virus. Peeters no hace nada para subsanar esta deficiencia. En efecto, Peeters seleccionó el APMV4 porque "no ha sido asociado con enfermedad" (página 1617, columna derecha, primer párrafo completo), y (como lo reconoció el Examinador en el Oficio) la página 1623, primera párrafo de la Discusión en Peeters dice lo siguiente:

"el propósito de producir el NDV quimérico recombinante descrito en este informe no fue generar una vacuna más segura o más inmunogénica, sino generar una vacuna que permita la diferenciación serológica entre animales vacunados y animales infectados por cepas de campo del NDV."

Por consiguiente, la persona medianamente versada en la técnica no podría haber deducido obviamente de Peeters o Zimmer que se pudiera generar una vacuna más segura e inmunogénica contra dos agentes causales de enfermedad (p.ej., NDV e influenza) de diferentes familias. Más bien, estos se cuentan entre los hallazgos inesperados que subyacen el nivel inventivo de la invención que aquí se reivindica.

7. La invención que aquí se reivindica se basa, en parte, en el resultado de que los NDV quiméricos reivindicados, con proteínas de fusión F o HN del NDV que contienen antígenos que no son antígenos de paramixovirus, son capaces de inducir inmunidad protectora contra virus causantes de enfermedad no emparentados. Por ejemplo, los ejemplos de trabajo de las Secciones 6.2 y 6.3 de la especificación de la solicitud en cuestión demuestran la generación de NDV quimérico seguro e inmunogénico que porta una proteína de fusión NDV

F/influenza HF. Ver, *p.ej.*, la descripción en ¶ [00249] que hace puente entre las páginas 90 y 91; ¶ [00250] que hace puente entre las páginas 94 y 95; y ¶ [00263] que hace puente entre las páginas 96 y 97.

Como se demuestra en ¶ [00262] en la página 96 de la descripción, las proteínas de fusión de la invención que aquí se reivindica también tienen la propiedad inesperada de una mejor expresión e incorporación dentro de los virus quiméricos de NDV. Este resultado inesperado de mejor expresión e incorporación de las proteínas de fusión fue confirmado en Park *et al.*, 2006, PNAS 103:8203-8208 ("Park," una copia del cual adjuntamos a la presente), publicado en una revista revisada por pares después de la fecha de prioridad de la solicitud en estudio. En Park, se demostró que una proteína quimérica con un ectodominio HA de la influenza aviar y los dominios de transmembrana y citoplasmático de la proteína F del NDV con un sitio de ruptura de proteasa modificado tiene una mejor incorporación dentro de virus NDV modificados. Ver, *p.ej.*, Park, Resumen y el párrafo que hace puente entre las páginas 8205 y 8206. Además, tanto Park como los ejemplos de trabajo de las Secciones 6.2 y 6.3 de la especificación demuestran que un NDV modificado con una proteína F de NDV quimérico/proteína HA de influenza aviar no se generó con éxito, también despierta inmunidad protectora contra NDV e influenza aviar. Ver la especificación en, *p.ej.*, ¶ [00249] que hace puente entre las páginas 90 y 91; y ¶ [00263] que hace puente entre las páginas 96 y 97; y Park, Resumen y página 8206, primer párrafo completo. Además, otra publicación posterior a la fecha de radicación, Nayak *et al.*, 2009, PLoS ONE 4:e6509 ("Nayak," una copia del cual adjuntamos a la presente), informa sobre la mejora de incorporación y expresión de una proteína quimérica que tiene un ectodominio HA de la influenza aviar y los dominios de transmembrana y citoplasmático de la proteína F del NDV en virus NDV quiméricos. Ver, *p.ej.*, Nayak, Resumen; y página 2º, 3º y 7º párrafos completos.

8. Con base en lo anterior, la invención que aquí se reivindica es novedosa, inventiva, y no obvia con respecto a las Referencias D2 (Peeters) y D3 (Zimmer). La sociedad Solicitante por consiguiente solicita respetuosamente que se retire el rechazo en relación con estas referencias.
9. Se adjuntan, debidamente corregidos, el formulario petitorio y la carátula.

10. Igualmente se acompaña el comprobante de pago por concepto de reivindicaciones adicionales por valor e \$ 840.000.00, recibo No. 134030.

Renuncio al término faltante de traslado, y les solicito otorgar el privilegio de patente de invención dentro de este expediente.

De Ustedes Atentamente,


LUIS FELIPE CASTILLO GIBSONE
T.P. No. 68.995 del C.S.J.

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PNAS

Engineered viral vaccine constructs with dual specificity: Avian influenza and Newcastle disease

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Contributed by Peter Palese, March 29, 2006

Avian influenza viruses of the H5 and H7 hemagglutinin subtypes, and Newcastle disease virus (NDV), are important pathogens in poultry worldwide. Specifically, the highly pathogenic H5N1 avian influenza virus is a particular threat because it has now occurred in more than 40 countries on several continents. Inasmuch as most chickens worldwide are vaccinated with a live NDV vaccine, we embarked on the development of vaccine prototypes that would have dual specificity and would allow a single immunization against both avian influenza and Newcastle disease. Using reverse genetics, we constructed a chimeric avian influenza virus that expressed the ectodomain of the hemagglutinin-neuraminidase gene of NDV instead of the neuraminidase protein of the H5N1 avian influenza virus. Our second approach to creating a bivalent vaccine was based on expressing the ectodomain of an H7 avian influenza virus hemagglutinin in a fusogenic and attenuated NDV background. The insertion into the NDV genome of the foreign gene (containing only its ectodomain, with the transmembrane and cytoplasmic domains derived from the F protein of NDV) resulted in a chimeric virus with enhanced incorporation of the foreign protein into virus particles. A single immunization of chickens with this improved vaccine prototype virus induced not only a 90% protection against an H7N7 highly pathogenic avian influenza virus, but also complete immunity against a highly virulent NDV. We propose that chimeric constructs should be developed for convenient, affordable, and effective vaccination against avian influenza and Newcastle disease in chickens and other poultry.

chicken | Newcastle disease virus | pandemic | avian influenza virus | hemagglutinin

In recent years, outbreaks of high-pathogenicity avian influenza (HPAI) have been reported in Asia and Europe (1, 2). These outbreaks involving H5N1 or H7N7 influenza viruses resulted in lethal infections in domestic poultry and the death of a limited number of people (2–4). The current H5N1 viruses have been circulating among poultry in China in recent years (5, 6) and, although migratory birds are considered to be the primary reservoir of these viruses, transmission from infected poultry back to migratory birds is believed to have contributed to the increased geographical distribution of the viruses (7). Currently, the H5N1 virus has emerged from Asia, spreading across Europe and Africa (8). Wholesale culling of poultry was shown to be a successful strategy for eradicating H5N1 outbreaks in Hong Kong in 1997 and in The Netherlands in 2003 (9). Because human victims of recent HPAI outbreaks have had close contact with infected poultry (10), it follows that the prevention of interspecies transmission of avian influenza viruses (AIVs) may be accomplished by eradicating AIV in poultry through slaughter. However, for economic and practical reasons, the destruction of infected poultry alone is no longer considered the method of choice for the control of this disease. In addition, for ethical and ecological reasons, the culling of migratory wildfowl is considered an unacceptable practice. Recently, the World Organization for Animal Health and the Food and Agriculture Organization of the United Nations recommended that vaccination of poultry be considered for the control of AIV (11).

In addition, it has been reported that vaccination of chickens with inactivated H5 vaccine in a field study was successful in interrupting virus transmission (12). Recently, China has accepted vaccination as a component of its AIV control program. Accordingly, the entire poultry population in China is to be vaccinated (13).

As part of our approach to the development of vaccines with dual specificity against AIV and Newcastle disease virus (NDV), we report here on the construction of an avian H5 influenza virus in which the ectodomain of the avian neuraminidase (NA) is replaced with that of the hemagglutinin-neuraminidase (HN) protein of an NDV. The latter protein is the major antigen of NDV that has been shown to elicit a protective immune response in chickens (14, 15). Construction of this virus was made possible by using the reverse genetics technology described in ref. 16. A bivalent influenza virus expressing the parainfluenza HN ectodomain was also reported recently (17).

Our second approach to the development of a bivalent vaccine is based on the NDV platform. NDV is a highly contagious avian disease agent of the genus *Avulavirus*, belonging to the family *Paramyxoviridae* and can vary in virulence (18). Over 250 species of birds, including poultry, are susceptible to NDV (19). Mortality from either highly virulent NDV or HPAI infection can reach up to 100% in unvaccinated poultry flocks. Therefore, the development of a combined vaccine against AIV and NDV would be of benefit to the poultry industry and would improve public health by reducing the HPAI load in vaccinated poultry below the level of transmissibility to people.

We and others have previously established reverse genetics systems to manipulate the genome of NDV (20, 21, 31, 32), allowing the generation of recombinant NDVs (rNDVs) that express foreign proteins. We have previously shown that immunization with rNDV expressing the influenza A/WSN/33 (H1N1) virus (WSN) hemagglutinin (HA) protein protected mice from challenge with a lethal dose of WSN (21). On the basis of these results, we first attempted to develop a bivalent vaccine against NDV and HPAI H7 virus by using a nonfusogenic rNDV vector expressing the WT HPAI H7 HA protein (rNDV/B1-H7). However, immunization of chickens with this rNDV/B1-H7 induced only partial immunity against HPAI and highly virulent NDV (22). Here, we have developed and evaluated a bivalent vaccine candidate against both AIV and NDV for poultry that is based on the fusogenic rNDV vector expressing a chimeric HA protein. This vaccine shows improved protection against both pathogens in a chicken challenge model.

Conflict of interest statement: Mount Sinai School of Medicine owns patent positions for reverse genetics of negative-strand RNA viruses (A.G.-S. and P.P.).

Freely available online through the PNAS open access option.

Abbreviations: HPAI, high-pathogenicity avian influenza; AIV, avian influenza virus; NDV, Newcastle disease virus; NA, neuraminidase; HN, hemagglutinin-neuraminidase; rNDV, recombinant NDV; HA, hemagglutinin; WSN, influenza A/WSN/33 (H1N1) virus; cHN, chimeric HN; EID₅₀, egg 50% infectious dose; MDCK, Madin-Darby canine kidney; vRNA, viral RNA; CEF, chicken embryo fibroblast; MDT, mean death time; F, fusion protein; HI, hemagglutination inhibition; pNDV, parental NDV; vNDV, velogenic NDV.

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MICROBIOLOGY

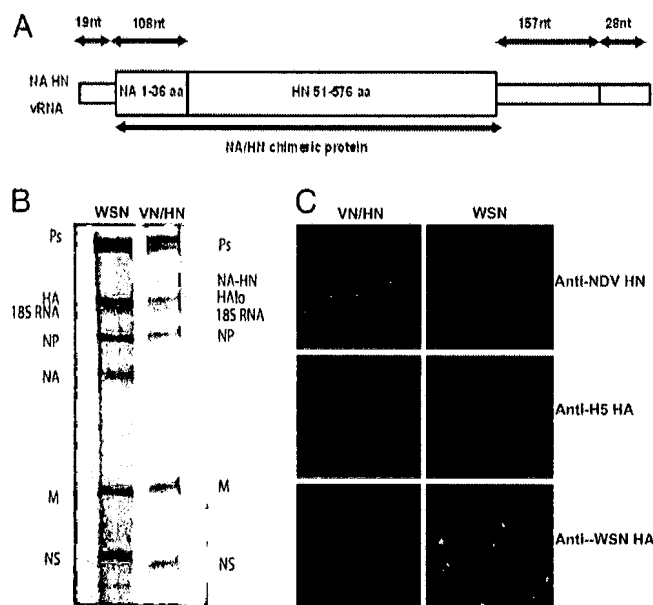


Fig. 1. Generation of a transfectant influenza virus bearing a chimeric NA-HN segment. (A) Schematic diagram of the viral RNA (vRNA) encoding the influenza virus NA NDV HN chimeric segment. The vRNA consists of the first 127 nucleotides of the WSN NA vRNA, which includes the 19 nucleotides of the noncoding region and the nucleotides encoding the cytoplasmic tail, the transmembrane domain, and the start of the NA stalk. This domain is followed by the nucleotides encoding amino acids 51–576 of the NDV HN protein, comprising the HN ectodomain and tandem stop codons (TAG TAG). The last domain represents the 185 nucleotides of the 5' end of the ORF of the WSN NA (157 nucleotides) and of its 5' noncoding region (28 nucleotides). (B) vRNAs of purified recombinant VN/HN virus. RNAs extracted from recombinant WT WSN and VN/HN viruses were separated through a 2.8% polyacrylamide gel containing 7 M urea and were visualized by silver staining. The positions of the polymerase (Ps), HA (WSN), HALo (VN 1203), NP, M, and NS segments are indicated. The RNA encoding the NA protein is absent from the VN/HN virus. A contaminating 18S ribosomal RNA band is indicated. (C) Expression of cHN protein. MDCK cells were infected with influenza VN/HN virus or with WT WSN. Infected cells were incubated for 16 hr at 37°C, fixed, and permeabilized with methanol. Specific antigens present in the virus were detected by immunofluorescence with anti-NDV HN monoclonal antibody (7B1), anti-influenza H1 HA monoclonal antibody (2G9), and polyclonal anti-influenza H5 HA serum.

Results

Generation of a Virus Expressing Influenza Virus and NDV Antigens. To obtain a candidate live bivalent vaccine virus for protection against both avian H5 influenza and NDV, we constructed a transfectant influenza virus (designated VN/HN virus) with a chimeric segment in which the encoded NA ectodomain, responsible for NA activity, was replaced with the ectodomain from the NDV HN protein (cHN) (Fig. 1A). Because of their importance in the formation of infectious virus (23, 24), the cytoplasmic tail and transmembrane domains of the influenza NA protein were retained. Numerous reports have indicated that the polybasic cleavage peptides of the H5 HA and H7 HA proteins are required for high pathogenicity of viruses in chickens (25). Thus, as a strategy to attenuate the transfectant virus while retaining the H5 antigen, we removed the polybasic cleavage site and replaced it with a consensus sequence derived from avirulent H5 HA strains. The introduction of adenosine residues into the nucleotide sequence encoding the HA cleavage peptide of avirulent H5 influenza strains has been associated with spontaneous conversion to virulence. This introduction is postulated to occur through a mechanism of polymerase slippage (26); therefore, to minimize the opportunity for the reintroduction of adenosine residues, we altered the codon usage in the region of the cleavage peptide, resulting in the formation of an avirulent HA

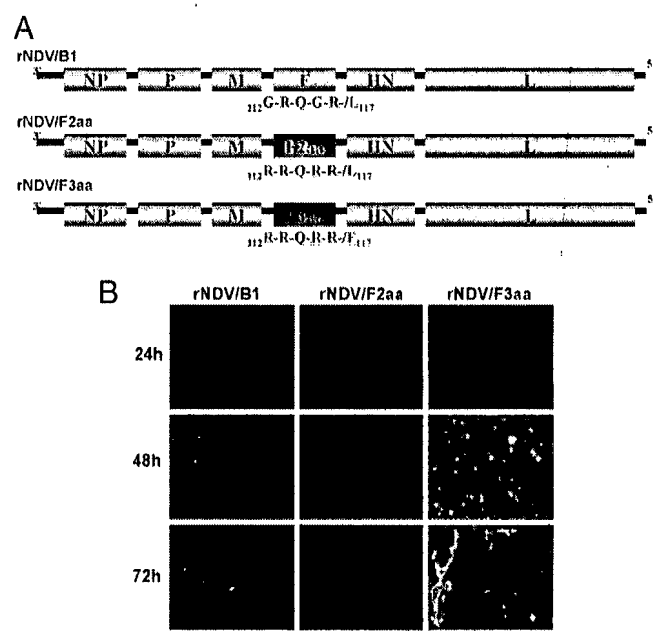


Fig. 2. Modification of the cleavage site of the F protein of rNDV. (A) Schematic representation of the rNDV/B1 genome with two or three amino acid changes in the cleavage site of their F proteins. The modified cleavage sites are known to be recognized by ubiquitous proteases. The peptide bond that is cleaved in the F protein is indicated with a slash. (B) Syncytia formation in CEF cells infected by rNDVs with modified F proteins. CEF cells were infected, with a multiplicity of infection of 0.001, with rNDV/B1, rNDV/F2aa, and rNDV/F3aa viruses. Infected cells were incubated at 37°C, and then viral spreading by cell fusion was monitored every 24 hr by immunofluorescence assay.

consensus sequence designated HALo. A transfectant virus containing HALo and cHN expressing segments derived from constructs encoding these alterations was generated by a modified version of the cDNA-based rescue of Fodor *et al.* (16).

To be of use as a vaccine, a virus must grow efficiently in eggs or cell culture. The recombinant virus was amplified in 10-day-old embryonated chicken eggs and grew to a titer of 8.0 log₁₀ egg 50% infectious dose (EID₅₀) per milliliter in this substrate. In Madin-Darby canine kidney (MDCK) cell culture, the virus grew to a titer of 5 × 10⁸ plaque-forming units per milliliter and produced plaques approximately two-thirds of the size of WSN.

The incorporation of viral RNA (vRNA) into the chimeric viral particle was examined by acrylamide gel electrophoresis of purified virion RNA. The presence of eight viral segments was observed, with the chimeric HN segment, which has a predicted size of 1,911 nt, migrating between the PA and HALo segments (Fig. 1B). The identity of the HA and cHN segments was confirmed by RT-PCR, and the segments were sequenced by using segment-specific primers. Incorporation of the cHN segment was shown to be stable for eight passages in embryonated chicken eggs by acrylamide gel electrophoresis of purified virion RNA (data not shown). Furthermore, expression of the recombinant HALo and cHN proteins during viral infection was confirmed by immunofluorescence. MDCK cells infected with VN/HN virus expressed HALo and cHN proteins 16 hr after infection, whereas, in cells infected with the control WSN, HA but not HN protein was expressed (Fig. 1C).

Generation of Fusogenic rNDV Mutants. Previously, a nonfusogenic, rNDV virus (rNDV/B1) expressing the H7 HA protein was unable to generate the required immunogenicity to completely protect chickens against H7 HPAI and highly virulent NDV (22). To overcome this limitation, we have developed two rNDV mutants, rNDV/F2aa and rNDV/F3aa, in which the cleavage site of the F protein was replaced with one containing one or two extra arginine

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Table 1. MDT of rNDVs in embryonated chicken eggs

Virus	Trypsin requirement (cell culture)	Inoculation, EID ₅₀	MDT, hr
rNDV/B1	Yes	10	113
		1	122
rNDV/F2aa	No	10	100
		1	104
rNDV/F3aa	No	10	80
		1	84
rNDV/B1-H7	Yes	10	Alive
		1	Alive
rNDV/F3aa-chimericH7	No	10	128
		1	140

MDT has been used to classify NDV strains. Velogenic NDV strains take <60 hr to kill the embryos, whereas mesogenic NDV strains and lentogenic NDV strains take 60–90 hr and >90 hr, respectively.

residues (Fig. 2A). These cleavage sites can be activated by ubiquitously expressed proteases of the furin family (27). Infection of chicken embryo fibroblast (CEF) cells with rNDV/F2aa and rNDV/F3aa, and not with rNDV/B1, resulted in the formation of syncytia (giant multinucleated cells that aid in the spread of the virus) in the absence of exogenously added protease (Fig. 2B). In addition, rNDV/F3aa induced syncytia more rapidly in CEF cells than did rNDV/F2aa. Thus, it was postulated that improved spreading of the virus in immunized animals may enhance immunogenicity against the inserted foreign protein. Hence, we selected the fusogenic rNDV/F3aa as a backbone vector to develop a bivalent vaccine designed to protect poultry against AIV and NDV.

Mean Death Time (MDT) Analysis of rNDV Platform Vectors in Embryonated Chicken Eggs. NDV can be classified as highly virulent (velogenic), intermediate (mesogenic), or low-virulent (lentogenic) on the basis of its pathogenicity for chickens. Because the presence of a fusion protein (F protein) with a multibasic cleavage site is known to be an NDV virulence factor, we assessed the pathogenicity of rNDVs with modified F protein in 10-day-old embryonated chicken eggs. The MDT of chicken embryos infected with NDVs correlates with virulence *in vivo* (19). Lentogenic strains, which cause asymptomatic infections in birds, are characterized by MDTs of >90 hr; mesogenic strains, which cause respiratory disease in birds, have MDTs between 60 and 90 hr; and velogenic strains, which cause severe disease in birds, have MDTs of <60 hr. The MDT of rNDV/F2aa was indicative of a lentogenic strain, whereas that of rNDV/F3aa was typical of a mesogenic strain. Neither of these strains had MDTs typical of a highly virulent (velogenic) strain (Table 1). On the basis of these data, rNDV/F3aa vector would represent a lower threat to birds and is thus suitable as a backbone to develop a bivalent vaccine for the protection of poultry against AIV and NDV.

Generation of a Fusogenic rNDV Vector Expressing the Ectodomain of AIV HA Protein. To construct a chimeric HA gene for insertion into a fusogenic rNDV, the ectodomain of the H7 HA protein from A/chicken/NY/13142-5/94 (H7N2) was fused with the transmembrane domain and cytoplasmic tail regions derived from the NDV F protein. This chimeric H7 HA gene was then inserted between the P and M genes of the rNDV/F3aa vector, resulting in the formation of rNDV/F3aa-chimericH7 (Fig. 3A). rNDV/F3aa-chimericH7 virus was rescued from cDNA by using the methods described in ref. 21. The presence of the inserted chimeric H7 HA gene in the viral genome was confirmed by RT-PCR and sequencing (data not shown). The growth kinetic of rNDV/F3aa-chimericH7 in embryonated chicken eggs was compared with that of the parental rNDV/F3aa (Fig. 3B). The virus expressing the chimeric H7 HA protein grew more slowly than the virus without the insert, and

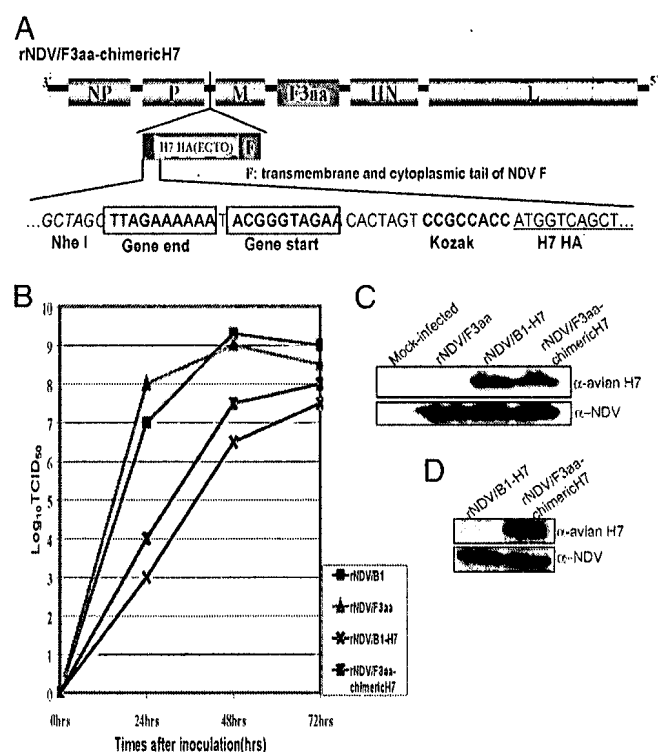


Fig. 3. Construction and characterization of the fusogenic rNDV vector expressing HPAI H7 HA protein. (A) Schematic representation of rNDV/F3aa-chimericH7 cDNA construct. The ectodomain of the H7 HA protein was fused with the transmembrane domain and cytoplasmic tail of the NDV F protein. This chimeric H7 HA gene was inserted between the P and M genes of the rNDV/F3aa vector. (B) Comparison of viral growth kinetics. Ten-day-old embryonated chicken eggs were inoculated with 100 plaque-forming units of each virus, and allantoic fluids were harvested at different time points (24, 48, and 72 hr after inoculation). Viral titers (tissue culture 50% infective dose, TCID₅₀) in Vero cells were determined by immunofluorescence assay with an anti-NDV rabbit serum and an anti-rabbit IgG FITC-labeled antibody (DAKO). (C) Expression of the WT H7 HA protein or the chimeric H7 HA protein in cells infected with rNDVs. Infected cells were harvested at 36 hr after infection, and cell lysates were used for immunoblotting analysis. (D) Incorporation of chimeric H7 HA protein into rNDV virions was increased as compared with that of WT H7 HA protein. rNDV/B1-H7 and rNDV/F3aa-chimericH7 were purified from allantoic fluids of infected embryonated chicken eggs. The viral proteins from rNDV/B1-H7 or rNDV/F3aa-chimericH7 were separated on an SDS/10% polyacrylamide gel. The viral proteins were transferred to a nitrocellulose membrane, and the H7 HA proteins were detected by chemiluminescence with an anti-chicken H7 HA polyclonal antibody and an anti-chicken IgG peroxidase-labeled antibody (Jackson ImmunoResearch).

maximal titers were about a log lower. Interestingly, the MDT of this virus was that of a lentogenic strain (≈128–140 hr) (Table 1). Expression of the chimeric H7 HA protein from rNDV/F3aa-chimericH7 was confirmed by Western blotting of infected Vero cells 36 hr after infection (Fig. 3C).

Improved Incorporation of AIV H7 HA Protein into rNDV Virions. We postulated that expression of the chimeric H7 HA protein containing the heterologous transmembrane and cytoplasmic tail regions of the NDV F protein would be associated with enhanced incorporation into rNDV virions. To address this question, rNDV/B1-H7 and rNDV/F3aa-chimericH7 virions were purified as described in *Materials and Methods*. The amounts of H7 HA protein or NDV viral protein from rNDV/B1-H7 or rNDV/F3aa-chimericH7 were measured by Western blotting using anti-chicken AIV H7 polyclonal antibody or anti-rabbit NDV polyclonal serum. As expected, incorporation of chimeric H7 HA protein into rNDV virions was significantly increased compared with that of WT H7

Table 2. HI serology and survival of chickens before and after challenge

Vaccine group*	HI serology before challenge (GMT)		Challenge virus	No. of survivors	HI serology 14 days after challenge (GMT)	
	AIV/H7 antigen	NDV antigen			AIV/H7 antigen	NDV antigen
rNDV/F3aa-chimericH7, 1×	8/10 (11)	10/10 (49)	vNDV	10/10	9/10 (15)	10/10 (416)
rNDV/F3aa-chimericH7, 1×	7/10 (10)	10/10 (49)	HPAIV	9/10	9/9 (2,048)	9/9 (37)
rNDV/F3aa-chimericH7, 2×	8/10 (13)	9/10 (56)	vNDV	10/10	7/10 (17)	10/10 (315)
rNDV/F3aa-chimericH7, 2×	5/10 (9)	9/10 (60)	HPAIV	9/10	8/8 (955)	8/8 (30)
pNDV, 2×	0/10	10/10 (34)	vNDV	10/10	0/10	10/10 (294)
pNDV, 2×	0/10	10/10 (56)	HPAIV	0/10	NA	NA
Sham, 2×	0/10	0/10	vNDV	0/10	NA	NA
Sham, 2×	0/10	0/10	HPAIV	3/10	0/3	0/3

Chickens were vaccinated with rNDV containing the AIV H7 HA gene (rNDV/F3aa-chimericH7), pNDV, or sterile tissue culture fluid (sham), followed by challenge with vNDV and A/Human/Steele/59 (H7N7) HPAI virus (HPAIV). HI serology is shown as no. of chickens with HI-positive serum/no. of chickens vaccinated; values in parentheses are geometric mean titer (GMT). NA, not applicable.
*n = 10 birds per group; 1× = one vaccination; 2× = two vaccinations.

HA protein (Fig. 3D). These data suggest that the transmembrane and cytoplasmic tail regions of the NDV F protein play a major role in the improved incorporation of the foreign protein into the viral surface.

Immunization and Challenge of Chickens. After one or two vaccinations with rNDV/F3aa-chimericH7, 50–80% of the chickens inoculated had hemagglutination inhibition (HI) titers to H7 AIV, and 90–100% of the chickens had HI titers to NDV (Table 2). In contrast, all chickens immunized twice with the parental NDV/B1 (pNDV) had HI titers to NDV but none had titers to H7 AIV. All sterile tissue culture media (sham)-infected birds lacked HI titers to either virus. When challenged with a highly virulent strain of NDV (velogenic NDV, vNDV), 100% of rNDV/F3aa-chimericH7- and pNDV-immunized chickens were protected. By comparison, 90% of rNDV/F3aa-chimericH7-vaccinated chickens were protected from HPAI H7 virus, but none of pNDV-vaccinated chickens were protected from HPAI H7 virus. By contrast, 100% and 70% of sham-infected birds died when challenged by vNDV and HPAI H7 virus, respectively. The survivors mounted an anamnestic response evidenced as a 4-fold or greater rise in HI titer for the respective challenge virus, except for the three survivors in the sham-HPAI H7-virus challenge group which had no serological evidence of being infected.

Discussion

Vaccination against AIV in poultry can play an important role in reducing virus shedding and raising the threshold for infection and transmission. It is believed that vaccination with a high-quality vaccine against AIV can be part of an effective control program. In conjunction with culling, quarantine, improved serological surveillance, and high biosecurity, the control of outbreaks of HPAI in poultry, as well as the prevention of transmission of HPAI to humans, is possible. Because the vaccination of poultry with live vaccines against NDV is mandatory in many countries (19), the development of bivalent vaccines should be made a priority. Such vaccines would reduce the burden of vaccine production and administration in comparison with individual vaccination. For these reasons, we have focused on the development of live bivalent viruses that show potential as vaccines against the major economic pathogens AIV and NDV.

First, by reverse genetics, we produced an influenza virus (VN/HN) that expresses the ectodomain of the NDV HN protein in place of the influenza virus NA. The HN of NDV has been shown to be the major antigen eliciting a protective immune response in chickens (14, 15). We reasoned that the HN protein would confer protection against NDV and provide the necessary NA activity in

the absence of the endogenous NA protein. The VN/HN virus grew efficiently in embryonated chicken eggs and stably expressed the cHN segment, both of which characteristics are fundamental requirements for a successful vaccine. However, preliminary experiments using VN/HN to vaccinate 2-week-old White Leghorn chickens revealed that this virus is possibly too attenuated *in vivo* to induce a highly protective immune response in chickens (data not shown). Experiments are needed to examine the ability of VN/HN to induce a protective response after immunization of chick embryos *in ovo*. Such an approach requires a more highly attenuated vaccine strain than that used for vaccination of hatched chickens.

Next, we chose NDV as a bivalent vaccine vector because it possesses several properties that make it suitable for use in viral vaccine development. The RNA genome of the virus does not integrate into host cell DNA. Furthermore, the rNDV vector can stably incorporate an inserted foreign gene over multiple passages and, because it is a respiratory virus, it can provide a convenient platform for rapid, efficient, and economical mass immunization of poultry. These benefits are multiplied in the case of a multivalent vaccine; one immunization can lead to protection from two or more pathogens. Whereas simultaneous vaccination with multiple live attenuated viruses may lead to complications if the presence of one virus interferes with the growth or immunogenicity of another, such problems are circumvented entirely by the use of a single bivalent virus vaccine. Thus we propose that, by using recently developed reverse genetics techniques (20, 21), it is possible to develop a new generation of effective, economical, and convenient bivalent live attenuated vaccines.

An outbreak of HPAI H7N7 in The Netherlands in 2003, in which 30 million chickens were slaughtered, involved the transmission of H7N7 to 89 persons and led to the death of a veterinarian. In addition, an HPAI H7N3 virus emerged in Canada in 2004 that also transmitted to humans (2, 3). Accordingly, in the present study we produced rNDV that expressed the H7 HA protein, with the intention of protecting poultry from HPAI H7 and NDV, and showed its efficacy in chickens. We suggest that the HAs from genetic variants of HPAI H5N1 or HPAI H7N7 viruses, as well as from other potential pandemic strains such as H9N2, could be inserted into the rNDV fusogenic vector to develop bivalent vaccines against these AIV strains and NDV. In addition, the rNDV vector can potentially harbor two different subtypes of HA as extra transcriptional units, opening the possibility for the development of bivalent vaccines that protect poultry against multiple HPAI strains.

One limitation in the use of regular vaccines against AIV or NDV is that vaccinated poultry frequently cannot be differentiated from naturally infected birds, making serological surveillance dif-

ficult to perform. However, vaccination of birds with a bivalent vaccine like rNDV/F3aa-chimericH7, which induces an immune response against H7 HA and NDV in chickens, makes serological surveillance against avian influenza possible because vaccinated animals lack immune responses against antigens present in whole influenza. Therefore, our bivalent vaccine would be suitable for use as a "differentiating infected from vaccinated animals" (DIVA) vaccine. NDV vaccines based on lentogenic strains of NDV are widely used in poultry. A chimeric NDV/AIV bivalent vaccine would be administered in the same way as the conventional NDV vaccines, but would confer protection against both NDV and AIV.

Velogenic NDV strains are able to spread efficiently to neighboring cells upon infection of a variety of cells in the absence of exogenous proteases. This property is partially mediated by the F protein of the virus. The F protein of NDV is synthesized as an inactive precursor (F0) that needs to be cleaved into a heterodimer (F1–F2) to be functional. Cleavage of the F protein is mediated by a host protease that recognizes a cleavage site between the F1 and F2 subunits. When the F1–F2 heterodimer is expressed on the surface of infected cells, it induces fusion between the infected cell and the neighboring cells, resulting in the formation of syncytia. Because the induction of syncytia helps the spread of the virus from cell to cell, the use of a vaccine with a velogenic NDV-derived F cleavage site in poultry may raise potential safety concerns. However, when we introduced a multibasic cleavage site in the F protein of the highly attenuated rNDV/B1, the resulting viruses did not acquire the characteristics typical of velogenic strains with MDTs of <60 hr in chicken embryos.

We postulated that enhanced incorporation of the H7 HA protein into rNDV virions may afford a better humoral immune response, resulting in higher levels of protection in chickens against highly pathogenic AIV. However, it was previously observed that virion incorporation of an influenza virus WSN HA protein expressed from rNDV was lower than the homologous HA protein expressed from influenza virus (21). If the H7 HA protein contained the cytoplasmic tail and transmembrane domains of the NDV envelope glycoprotein (F), which are required for efficient viral assembly, in place of its own, this resulted in enhanced incorporation of HA into the viral particle and in improved immunogenicity of the virus. Indeed, the use of an improved NDV vector that had enhanced cell-to-cell transmission and allowed for efficient incorporation of the avian HA into virus particles resulted in 90% protection against challenge with type A HPAI strains and complete protection against velogenic NDV.

In summary, the use of reverse genetics systems for the construction of chimeric influenza or NDV viruses provides a practical strategy for the protection of poultry against existing and newly emerging viruses of HPAI, as well as against NDV. It is expected that these approaches will also prove useful in the development of bivalent vaccines for other pathogens.

Materials and Methods

Cells and Viruses. 293T and Vero cells were maintained in DMEM with 10% FBS. CEF cells, prepared from 10-day-old specific-pathogen-free embryos (Charles River Laboratories, SPAFAS, Preston, CT), and MDCK cells were maintained in Eagle's MEM with 10% FBS. WSN was generated by plasmid-based reverse genetics, as described in ref. 16, and propagated in MDCK cells. We have previously generated a full-length infectious clone of NDV, pT7NDV/B1 (21). rNDV was proliferated in embryonated chicken eggs.

Construction of vRNA Expression Plasmids from Influenza A/Vietnam/1203/04(H5N1). cDNAs derived from influenza A/Vietnam/1203/04(H5N1) RNA (kindly provided by Terrence Tumpey, U.S. Centers for Disease Control and Prevention, Atlanta) by RT-PCR were cloned between a PolI promoter and a hepatitis delta virus ribozyme present in the pPolI-SapI-RT plasmid (28). Full-length

cDNAs for seven of the eight viral segments from influenza A/Vietnam/1203/04(H5N1) virus were amplified by PCR with primers that are competent for the subsequent cloning of the full-length viral segments into pPolI-SapI-RT, allowing the production of exact negative-sense vRNAs. In this way, seven segments of influenza A/Vietnam/1203/04(H5N1) virus were cloned into pPolI-SapI-RT. To construct a chimeric vRNA segment encoding the ectodomain of the NDV HN protein (Fig. 1), the region corresponding to the ectodomain of the NDV HN (i.e., amino acids 51–576), followed by tandem stop codons, was amplified by PCR and inserted between the first 127 nucleotides and the last 185 nucleotides of the WSN NA vRNA.

Modification of HA to Remove the Polybasic Cleavage Peptide. pPolI-VN1203-HAlo was designed to produce the HA vRNA in which nucleotides 1014–1038 of the HA coding sequence were altered to a consensus sequence that was based on avirulent avian strains of influenza A H5. The nucleotide sequence was altered from CAA AGA GAG AGA AGA AGA AAA AAG AGA GGA to CAG CGG GAG ACG CGG GGA. The codon usage in this region was modified to lessen the presence of adenosine residues in the nucleotide sequence and to minimize opportunity for the reintroduction of adenosine residues by polymerase slippage, and the resultant introduction of basic residues into the HA protein cleavage site (26). Therefore, the encoded amino acid sequence in this region was altered from QRRERRKKR ↓ G to QRETR ↓ G (where the arrows indicate cleavage sites).

Generation of Transfectant Influenza Virus. To generate the chimeric influenza virus expressing the NDV HN ectodomain, 1 μg each of 15 plasmids was transfected into 293T cells in monolayer. Each transfection contained vRNA expression plasmids for the A/Vietnam/1203/04 PA, PB1, PB2, HAlo, NP, M, and NS segments; the cHN segment; and the protein expression plasmids pCAGGS-PA, PB1, PB2, NP, HA, NA, and NS1 (pCAGGS expression plasmid kindly provided by J. Miyazaki, Osaka University, Osaka) derived from WSN (29). At 48 hr after transfection, supernatants were harvested, and transfectant virus was passaged into 10-day-old embryonated chicken eggs. RNA segments of transfectant virus were visualized as described in ref. 30.

Generation of rNDVs with a Modified Cleavage Site in Their F Proteins.

To generate rNDV/F3aa, two PCR fragments were generated by using primers (regions of overlap are shown underlined) F3aa-1(+), 5'-GGA TCC CGG TTG GCG CCC TCC AGG-3' with F3aa-1(-), 5'-AAa GCG CCt CTG TCT CCg CCC TCC AGA TGT AGT CAC AG-3'; and F3aa-2(+), 5'-GGc GGA GAC AGa GGC GCt TTA TAG GCG CCA TTA TTG G-3' with F3aa-2(-), 5'-CCA TAT TCC CAC CAG CTA GAT TGT-3'; and pT7NDV/B1 as the template. The nucleotides shown in lowercase are mutated to modify the amino acid sequence of the cleavage site of the F protein from that of the NDV/B1 strain (GGRRQR ↓ L) to GRRQRR ↓ F (amino acids modified are shown in italics). These overlapping fragments were combined by PCR using primers F3aa-1(+) and F3aa-2(-). The resulting PCR fragment was cloned into pSLF3aa. The *Stu*I–*Not*I fragment (nucleotides 4646–4952) of pSLF3aa was excised to replace the corresponding fragment in the pT7NDV/B1 plasmid, resulting in the formation of the pT7NDV/F3aa plasmid, which was used to generate rNDV/F3aa virus by reverse genetics. For generation of rNDV/F2aa, PCR mutagenesis was performed by the same strategy described above, with primers F2aa-1(+), 5'-GGA TCC CGG TTG GCG CCC TCC AGG with F2aa-1(-), 5'-AAG GCG CCt CTG TCT CCg CCC TCC AGA TGT AGT CAC AG-3'; and F2aa-2(+), 5'-GGc GGA GAC AGa GGC GCCTTA TAG GCG CCA TTA TTG G-3' with F2aa-2(-), 5'-CCA TAT TCC CAC CAG CTA GAT TGT-3'; and pT7NDV/B1 as the template. Two overlapping PCR fragments were combined by PCR using primers F2aa-1(+) and F2aa-2(-),

resulting in modification of the cleavage site from GGRQGR ↓ L to GRRQRR ↓ L.

Generation of a Fusogenic rNDV Vector Expressing the Chimeric H7 HA Protein. To construct the chimeric H7 HA gene as an extra transcriptional unit of the rNDV/F3aa, the fragment containing the transmembrane (TM) and the cytoplasmic tail (CYTO) of the NDV F gene was initially produced by PCR using primers HpaNDV F(TM+CYTO)P, 5'-cg GTT AAC CTC ATT ACC TAT ATC GTT TTG ACT-3'; and SacNhe NDVF(TM+CYTO)M, 5'-cg GAG CTC AAG CTA GCT TAT CAC ATT TTT GTA GTG GCT CTC ATC TG-3'; and the NDV F gene as a template. This PCR product was digested with SacI and HpaI and then cloned into the plasmid pNhe-NDV-GE/GS, which possesses the gene end and the gene start signals of NDV, to produce plasmid pNhe-NDV-GE/GS-NDVF(TM+CYTO). As the next step, allowing the connection of the H7 HA ectodomain with the TM and CYTO region of the NDV F, the region encoding the H7 HA ectodomain was amplified by PCR using the primers SpeH7(ECTO)P, 5'-cg ACT AGT CCG CCA CCA TGA ACA CTC AAA TTC TGG CAT TCA T-5'; HpaH7(ECTO)M, 5'-cgG TTA ACG TCT TTG TAT CCA CTA CTC AAT TTC AC-3'; and the H7 HA gene from A/chicken/NY/13142-5/94(H7N2) as the template. This PCR product was digested with SpeI and HpaI and then inserted into the cassette plasmid pNhe-NDV-GE/GS-NDVF(TM+CYTO). In a final step, the cassette plasmid was digested with NheI to cut out the chimeric H7 HA gene. This DNA fragment was cloned between the P and M genes of pT7NDV/F3aa, forming pT7NDV/F3aa-chimericH7. The rNDV/F3aa virus expressing the chimeric H7 HA protein was then rescued from pT7NDV/F3aa-chimericH7 by using reverse genetics (21).

Viral Growth Kinetics. rNDVs (100 plaque-forming units per egg) were inoculated into 10-day-old embryonated chicken eggs. Allantoic fluids were harvested to determine viral titers at different time points (24, 48, and 72 hr). The tissue culture 50% infective dose (TCID₅₀) of each virus present in the allantoic fluid was determined by immunofluorescence assay.

Immunofluorescence Assays. MDCK cells infected with transfectant influenza virus were fixed and permeabilized with ice-cold methanol. Viral antigens were detected with anti-NDV HN monoclonal antibody (7B1), anti-influenza H1 HA monoclonal antibody (2G9), and anti-influenza H5 HA polyclonal serum. For the analysis of NDV growth, confluent Vero cells were infected with the rNDVs, which were harvested at 24, 48, and 72 hr. Infected cells were fixed with 2.5% formaldehyde containing 0.1% Triton X-100. Fixed cells

were treated with anti-rabbit NDV polyclonal antibody, washed, and stained with FITC-conjugated anti-rabbit immunoglobulins (DAKO).

MDT. To check the pathogenicity of rNDVs in embryonated chicken eggs, MDT was determined. Briefly, five 10-day-old embryonated chicken eggs were infected with serial 10-fold dilutions of viruses. The eggs were incubated at 37°C and monitored two times daily for 7 days. The time to kill embryos was recorded. The highest dilution that killed all embryos was determined to be the minimum lethal dose. The MDT was calculated as the mean time for the minimum lethal dose to kill the embryos.

Western Blotting. Dishes (100 mm in diameter) of confluent Vero cells were infected with rNDVs at a multiplicity of infection of 1. Cell extracts were prepared in RIPA buffer (0.15 mM NaCl/0.05 mM Tris-HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% SDS) at 36 hr after infection. Equal amounts of protein were analyzed by SDS/PAGE. H7 HA and NDV-derived protein were immunoblotted by using anti-rabbit NDV polyclonal or anti-chicken AIV H7 polyclonal sera. The incorporation of H7 HA protein into virions was also determined by Western blotting. For this Western blot, virus particles from the allantoic fluid of infected embryonated chicken eggs were purified through a sucrose cushion, and the total amount of protein from each virus was measured with the Bradford assay kit (Bio-Rad). Equal amounts of protein were analyzed by immunoblotting as described above.

Immunization and Challenge of Chickens. White Leghorn chickens were vaccinated once or twice by eyedrop in the conjunctival sac with 10^{5.7-6.1} mean chicken EID₅₀ of rNDV/F3aa-chimericH7, or twice with 10^{5.7-6.3} EID₅₀ of pNDV, or twice with sterile tissue culture media (sham) at 2 and 4 weeks of age. At 6 weeks of age, the chickens were challenged intranasally with the Fontana strain of vNDV (10^{5.1} EID₅₀ per bird) or A/Human/Steele/59 (H7N7) HPA1 (10^{5.1} EID₅₀ per bird). The survivors were bled and killed at 14 days after challenge. HI serological titers were determined by using standard procedures. All challenge experiments were done at the U.S. Department of Agriculture (USDA) poultry research laboratory in Athens, GA, in accordance with USDA guidelines.

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Immunization of Chickens with Newcastle Disease Virus Expressing H5 Hemagglutinin Protects against Highly Pathogenic H5N1 Avian Influenza Viruses

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Abstract

Background: Highly-pathogenic avian influenza virus (HPAIV) and Newcastle disease virus (NDV) are the two most important poultry viruses in the world. Natural low-virulence NDV strains have been used as vaccines over the past 70 years with proven track records. We have previously developed a reverse genetics system to produce low-virulent NDV vaccine strain LaSota from cloned cDNA. This system allows us to use NDV as a vaccine vector for other avian pathogens.

Methodology/Principal Finding: Here, we constructed two recombinant NDVs (rNDVs) each of which expresses the hemagglutinin (HA) gene of HPAIV H5N1 strain A/Vietnam/1203/2004 from an added gene. In one, rNDV (rNDV-HA), the open reading frame (ORF) of HA gene was expressed without modification. In the second, rNDV (rNDV-HAF), the ORF was modified so that the transmembrane and cytoplasmic domains of the encoded HA gene were replaced with those of the NDV F protein. The insertion of either version of the HA ORF did not increase the virulence of the rNDV vector. The HA protein was found to be incorporated into the envelopes of both rNDV-HA and rNDV-HAF. However, there was an enhanced incorporation of the HA protein in rNDV-HAF. Chickens immunized with a single dose of either rNDV-HA or rNDV-HAF induced a high titer of HPAIV H5-specific antibodies and were completely protected against challenge with NDV as well as lethal challenges of both homologous and heterologous HPAIV H5N1.

Conclusion and Significance: Our results suggest that these chimeric viruses have potential as safe and effective bivalent vaccines against NDV and HPAIV. These vaccines will be convenient and affordable, which will be highly beneficial to the poultry industry. Furthermore, immunization with these vaccines will permit serological differentiation of vaccinated and avian influenza field virus infected animals.

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Introduction

Avian Influenza (AI) is an economically-important disease of poultry worldwide. The etiologic agent is a segmented negative-strand RNA virus in the genus *Influenza virus A* of the family *Orthomyxoviridae*. Avian influenza virus (AIV) strains are divided into subtypes based on serological reactions of the two surface glycoproteins, the hemagglutinin (HA) and neuraminidase (NA). There are 16 different HA (H1-H16) and 9 different NA (N1-N9) subtypes [1,2]. In addition, AIV strains are further classified into two different pathotypes, low pathogenic (LP) and highly pathogenic (HP). Historically, HPAIV of subtypes H5 and H7 have caused severe disease and high mortality in poultry. Since 1996, HPAIV strains of subtype H5N1 have emerged recently in Asia have caused disease in poultry around the world, resulting in the culling or death of more than 500 million poultry in more than

62 countries. H5N1 HPAIV also has caused zoonotic infections in humans, resulting in 406 confirmed cases and 254 deaths to date [3,4]. The transmission of H5N1 HPAIV from birds to humans remains a serious threat to public health. Therefore, control of H5N1 virus infections in poultry is not only important for the poultry industry, but also for preventing H5N1 infection in humans.

Traditionally, HPAI is controlled by elimination of infected flocks. Due to economical reasons, culling of infected flocks is no longer a practical method for control of AI in either developed or developing countries. Although vaccination has been recommended by the World Organization for Animal Health (OIE) and the Food and Agriculture Organization (FAO) to control AI, few effective AI vaccines are available [5]. For the most part, these consist of inactivated preparations of whole influenza viruses. However, inactivated vaccines are not commonly used because of

the high cost and the difficulty in “differentiating infected from vaccinated animals” (DIVA). Live attenuated vaccines are not used because of concern that the vaccine viruses may, through either mutation or genetic reassortment with circulating strains, become virulent [6]. To overcome these difficulties, several strategies have been developed based on *in vivo* expression of HA by DNA vaccines [7,8] or by vectors based on adenovirus [9,10,11], fowl pox virus [12,13,14,15], baculovirus [16,17,18], infectious laryngotracheitis virus (ILT) [19,20], or Newcastle disease virus (NDV) [21,22,23,24].

Of all AI vaccine strategies, HA protein expressed *in vivo* using NDV as a vector has shown the most promising results [21,22,23]. NDV is a non-segmented, negative-strand RNA virus belonging to genus *Australivirus* in the family *Paramyxoviridae*. Virulent NDV strains cause a severe respiratory and neurologic disease in poultry world wide. Naturally-occurring avirulent NDV strains have been successfully used as vaccines for more than 70 years. Development of reverse genetic systems has allowed manipulation of the genome of NDV [25,26,27] and generation of recombinant NDVs that express foreign proteins from added genes [24,28,29]. Several groups have used NDV reverse genetic systems to develop vaccines against AIV subtypes H5 [21,23] and H7 [22].

There have been two independent studies to develop bivalent vaccines against NDV and HPAI H5 virus [21,23], with different levels of success. In one study, the HA gene from an H5N2 virus was inserted between the F and HN genes of NDV [23]. This recombinant NDV protected chickens against both NDV and H5N2 virus, but some vaccinated chickens showed signs of clinical disease when challenged with highly- pathogenic H5N2 virus. In the other study, the HA gene from an H5N1 virus was inserted between the P and M genes of NDV [21]. This recombinant NDV protected chickens against both NDV and two different strains of H5N1 viruses isolated in China.

In this study, we have attempted to develop improved bivalent vaccines against NDV and HPAI H5 virus by using recombinant NDV (rNDV) vaccine strain LaSota as a vector to express the HA protein from an HPAI H5N1 virus. Two different rNDVs were constructed expressing different forms of the HA protein: one vector (rNDV-HA) expressed an unmodified form of HA, while the other (rNDV-HAF) expressed a version of HA in which the HA ectodomain was fused with the transmembrane and cytoplasmic domains derived from the F protein of NDV. This resulted in a chimeric HA protein with enhanced incorporation of foreign proteins into NDV particles. The protective efficacies of both these bivalent vaccines were compared in chickens by challenge with a lethal dose of highly-virulent NDV and homologous and heterologous H5N1 HPAIV. Our results showed that a single immunization with either of the candidate bivalent vaccines completely protected against both pathogens in chickens. A further enhanced incorporation of the H5 HA into NDV particles was observed in rNDV-HAF vaccine than into rNDV-HA vaccine, indicating a better ability of this virus to induce neutralization antibody response in chickens.

Results

Generation of rNDVs expressing two versions of the HA protein of H5N1 AIV

Two different rNDVs were constructed expressing different versions of the HA protein of HPAIV strain A/Vietnam/1203/2004, a virus isolated from a fatal human infection [30]. In each construct, the HPAIV coding sequence was placed under the control of NDV transcriptional signals and inserted, by reverse genetics, between the P and M genes in the NDV LaSota avirulent

vaccine strain (Figure 1A). One construct, rNDV-HA, was designed to express the unmodified HA protein; the other (rNDV-HAF), was designed to express a chimeric HA protein in which the ectodomain of HA was fused with the transmembrane domain and cytoplasmic tail region of the NDV F protein (Figure 1A, constructs 1 and 2, respectively). Both recombinant viruses were recovered using the reverse genetics method described previously [31]. The structure of each H5A insert in the genome of these viruses was confirmed by RT-PCR and nucleotide sequence analysis (data not shown).

Expression of the native and chimeric forms of the HA protein by rNDV

The expression of the two versions of the H5 HA protein in DF1 cells infected with rNDV-HA and rNDV-HAF were analyzed by Western blot using polyclonal chicken H5N1 AIV antiserum (Figure 1B). Immunoblot analysis detected three bands in lysates of cells infected with rNDV-HA or rNDV-HAF: these represented (i) the inactive HA precursor HA0, with an apparent molecular weight of ~65,000 for rNDV-HA (Figure 1B, Lane 3), and ~66,000 for rNDV-HAF (Figure 1B, Lane 4), (ii) the N-terminally-derived proteolytic cleavage product HA1 with an apparent molecular weight of ~40,000 for both viruses, and (iii) the C-terminally-derived proteolytic cleavage HA2 of apparent molecular weight ~25,000 for rNDV-HA and ~28,000 for rNDV-HAF. The differences in apparent size reflected the different transmembrane and cytoplasmic domains. As expected, HA was not detected in DF1 cells that were mock-infected (Figure 1B, Lane 1) or infected with rNDV empty vector (Figure 1B, Lane 2).

We also used immunofluorescence to localize expression of the native and chimeric HA proteins in DF-1 cells infected with the recombinant viruses. Intracellular expression was investigated in cells that were fixed and permeabilized with Triton detergent (Figure 1D). Positive staining was observed for cells infected with rNDV-HA or rNDV-HAF using chicken antiserum for NDV (Figure 1D, upper panel) or H5N1 AIV (Figure 1D, lower panels).

Incorporation of AIV HA protein into the rNDV virion

The recombinant viruses (rNDV, rNDV-HA and rNDV-HAF) were partially purified from allantoic fluid by sucrose density gradient centrifugation. Incorporation of AIV HA into the rNDV envelope was analyzed by Western blot assay (Figure 1C) and immunogold electron microscopy (Figure 2). Western blot analysis of purified viruses with anti H5N1 AIV antibodies did not detect any bands for rNDV, as expected (Figure 1C, Lane 1), whereas HA1 and HA2 bands were detected in both rNDV-HA and rNDV-HAF (Figure 1C, Lanes 3 and 2). The inactive precursor HA0 that was detected in infected cells (Figure 1B) was not detected in purified viruses (Figure 1C). There were two-fold more HA1 and HA2 proteins in virions of rNDV-HAF, compared to rNDV-HA, as determined by densitometry analysis of the bands. The band intensities were normalized against NDV HN protein as an endogenous control. This suggested that fusion of the transmembrane domain and cytoplasmic tail region of NDV F protein with the HA ectodomain enhanced incorporation of HA protein into NDV particles. This was consistent with the results of immunoelectron microscopy (Figure 2). Antiserum to NDV directed the deposition of gold particles on the envelopes of rNDV, rNDV-HA, and rNDV-HAF, as expected (Figure 2, upper panels). Antiserum to H5N1 AIV directed deposition to the envelopes of rNDV-HA and rNDV-HAF (Figure 2, lower panel). As expected, there was no gold particle deposit on the envelope of rNDV. There was significantly more gold particle deposit on the

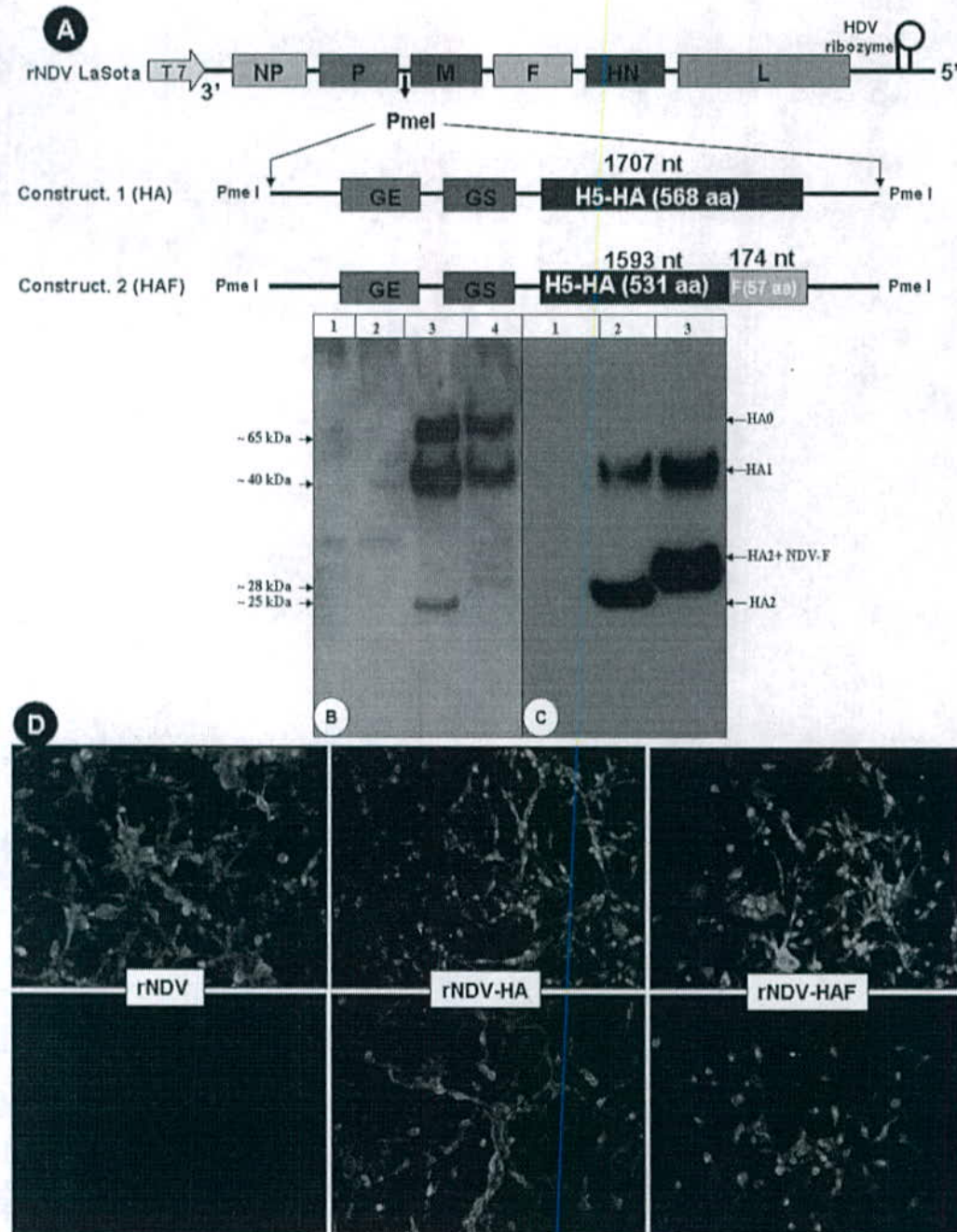


Figure 1. Construction and characterization of recombinant NDVs expressing unmodified or chimeric versions of the H5 HPAIV HA protein. (A) Schematic diagram depicting the rNDV LaSota genome with insertion of an added gene engineered to express unmodified HA (construct 1) or chimeric HA consisting of the HA ectodomain fused to the transmembrane and cytoplasmic domains of the NDV F protein (construct 2). In the inserted sequence, the foreign HA coding sequence was preceded by an NDV gene junction containing gene-end (GE) and gene-start (GS) transcription signal: this results in the HA ORF being under the control of a set of transcription signals such that it is expressed as a separate mRNA. (B) Expression and processing of H5 HA and chimeric HA in DF1 cells infected with the respective rNDVs and analyzed by Western blot analysis using chicken polyclonal H5N1 AIV antibodies. HA specific bands were not detected in lysates of mock-infected DF1 cells (Lane 1) or cells infected with rNDV empty vector (Lane 2). In contrast, lysates of cells infected with rNDV-HA (Lane 3) or rNDV-HAF (Lane 4) contained uncleaved inactive precursor (HA0) and proteolytically cleaved HA1 and HA2/HA2-F proteins. (C) Incorporation of the H5 HA protein in purified rNDV particles, detected by Western blot analysis. HA-specific bands corresponding to the cleaved HA1 and HA2/HA2-F species were detected in preparations of purified particles of rNDV-HA (Lane 2) and rNDV-HAF (Lane 3), but not the empty vector rNDV (lane 1). (D) Immunofluorescence analysis of DF1 cells infected with rNDV, rNDV-HA and rNDV-HAF and processed for intracellular expression of NDV proteins (upper panels) and H5 HA protein (lower panel), detected using chicken polyclonal anti-NDV and anti-H5N1 AIV antibodies. doi:10.1371/journal.pone.0006509.g001

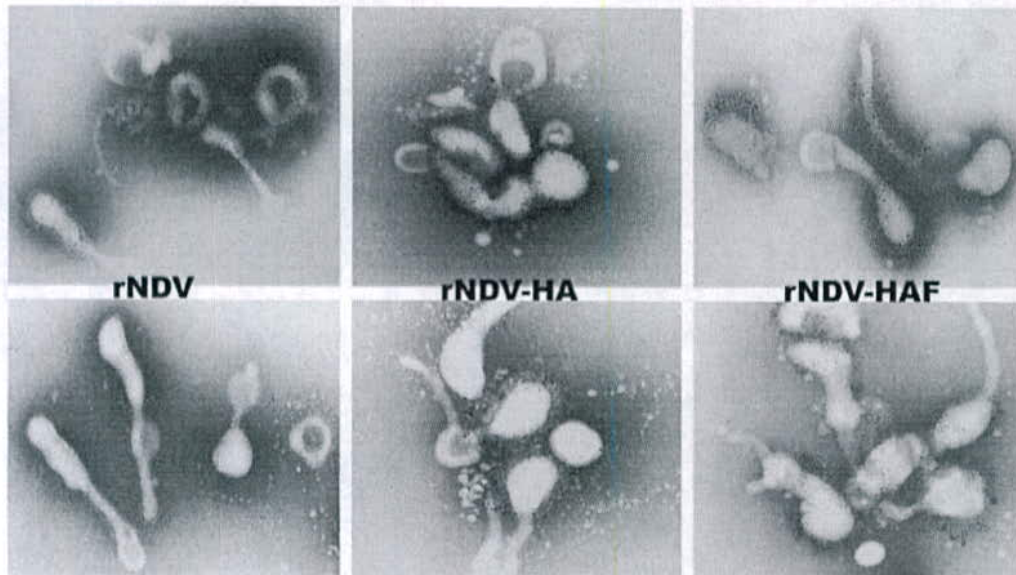


Figure 2. Immunoelectron microscopy of purified virions of rNDV, rNDV-HA and rNDV-HAF, analyzed using mouse monoclonal antibodies against the NDV HN protein (upper panel) or chicken polyclonal antibodies against H5N1 AIV (lower panel).
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envelope of rNDV-HAF than on rNDV-HA with same amount of anti H5N1 AIV antibodies (Figure 2, lower panel). This suggested that the chimeric version of the HA protein was incorporated more efficiently into the NDV envelope than the native HA protein.

Biological characterization of rNDV expressing the native or chimeric HA protein

The multicycle growth kinetics of rNDV-HA and rNDV-HAF were compared with those of the parental rNDV. Our results demonstrated similar kinetics of growth between rNDV and rNDV-HA, whereas rNDV-HAF grew more slowly and achieved a maximal titer that was approximately one log lower than those of parental rNDV titers (Figure 3A). Similar plaque sizes were observed for rNDV and rNDV-HA, but a slightly smaller plaque size was observed for rNDV-HAF (Figure 3B). The pathogenicities of wild type NDV strain LaSota (wt LaSota) and parental rNDV, rNDV-HA and rNDV-HAF were evaluated by the mean death time (MDT) test in embryonated chicken eggs. The MDTs for these viruses were 105 h (wt NDV), 110 h (rNDV), 112 h (rNDV-HA) and >120 h (rNDV-HAF). According to OIE guidelines, an NDV strain is considered lentogenic or avirulent, if the MDT value is >90 h [32], and thus the two rNDVs expressing the two versions of HA are avirulent viruses. Indeed, the presence of the added native or chimeric HA gene conferred attenuation to the NDV vector.

Immunogenicity and protective efficacy in chickens against highly-virulent NDV challenge

To evaluate protective efficacy, groups of chickens were inoculated by the oculonasal route with wt NDV LaSota (the naturally attenuated vaccine strain that was the parent for the NDV vectors), or with the recombinant rNDV-HA and rNDV-HAF viruses, or were left unimmunized as a control group (Table 1). The birds were challenged 21 days later by the oculonasal route with highly-virulent NDV strain Texas-GB. All of the chickens that had been immunized with biologically-derived

wt LaSota, rNDV-HA, or rNDV-HAF were completely protected from NDV challenge, with no evidence of virus shedding and without any disease signs (Table 1). In contrast, all of the chickens in the unvaccinated control group died within 3 days after challenge (Table 1). Each of the NDV immunizations induced a substantial titer of NDV-specific HI serum antibodies when assayed on day 21 prior to challenge. However, the mean antibody titers induced by rNDV-HA and rNDV-HAF were approximately one and two log₂ less than that of wt NDV, respectively (Table 1). This modest reduction in immune response to the vector might

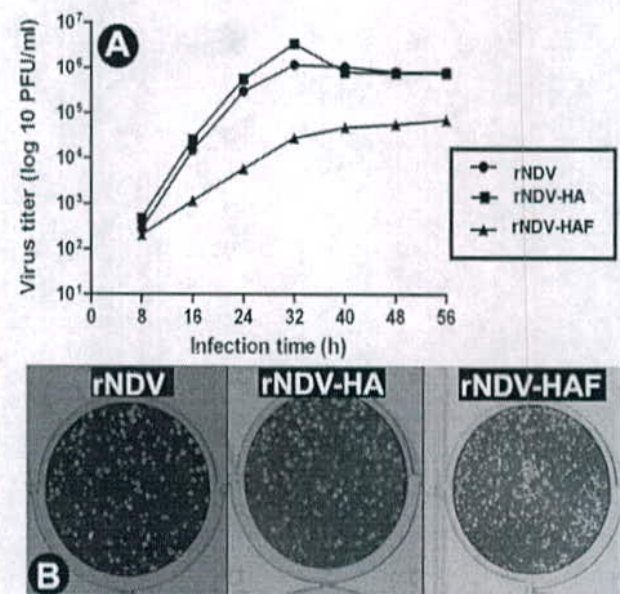


Figure 3. Multicycle growth kinetics (A) and plaque morphology (B) of the rNDV, rNDV-HA and rNDV-HAF viruses in DF-1 cells.
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Table 1. NDV-specific serum antibody responses following immunization with NDV-HA or NDV-HAF and survival of chickens following virulent NDV strain Texas-GB challenge¹.

Chicken group	Response to immunization		Protection of chickens post challenge ²		
	No. of birds seropositive	Mean NDV serum antibody HI titer (log ₂)	Oral shedding	Cloacal shedding	No. of survivors
Uninfected control	0/10	0.00	10/10	4/10	0/10
wtNDV (LaSota)	10/10	6.18±0.91	0/10	0/10	10/10
rNDV-HA	10/10	5.50±0.70	0/10	0/10	10/10
rNDV-HAF	10/10	4.10±0.63	0/10	0/10	10/10

¹Chickens in groups of 10 were vaccinated on day 0 with wtNDV, rNDV-HA, or rNDV-HAF. On day 21, serum samples were taken and the birds were challenged with 10⁴ ELD₅₀ of virulent NDV strain Texas GB.
²Shedding of challenge virus was determined by inoculation of oropharyngeal (oral) and cloacal swab samples into of embryonated chicken eggs followed by HA tests of allantoic fluid. All of the uninfected control birds died by day 3, and oral and cloacal samples were taken at the time of death. For the other birds, samples were taken on day 3.
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reflect increased attenuation of the rNDVs associated with the insertion of the HA gene, as indicated above by the MDT assay, or might reflect an effect on expression of the NDV HN protein (the antigen-specificity of the antibodies measured by the HI assay) due to the presence of the upstream foreign gene, or both.

Protective efficacy in chickens against homologous and heterologous HPAIV challenge

As a result of genetic drift, there exists considerable antigenic diversity in the HA protein among H5N1 viruses, resulting in distinct clades and subclades within the HA of the H5N1 lineage viruses. Currently, two clades are circulating among poultry, namely clade 1 that includes the A/Vietnam/1203/2004 strain represented in the NDV vectors, and clade 2.2 that includes the A/Egret/Egypt/01/2006 virus. Therefore, we were interested in evaluating the ability of the NDV-HA and -HAF vectors to provide protection against challenge with the clade 1 and clade 2.2 HPAIV. Groups of chickens were vaccinated with rNDV, rNDV-HA, rNDV-HAF, or were left unvaccinated. The pre-challenge sera collected at 21 days post-vaccination had mean NDV HI antibody titers (log₂) of 5.30±0.94, 5.15±0.95 and 4.50±0.70 for rNDV, rNDV-HA and rNDV-HAF, respectively (Table 2). Thus, the reduction in NDV-specific

antibodies associated with the presence of the foreign HA gene was less than in the preceding experiment shown in Table 1. The mean HPAIV HI serum antibody titers (log₂) in birds immunized rNDV, rNDV-HA or rNDV-HAF were 0, 4.57±1.06 and 4.53±1.36, respectively, against the homologous A/Vietnam/1203/2004 virus, and were 0, 2.92±1.09, and 2.38±1.35, respectively, against the heterologous A/Egret/Egypt/01/2006 virus (Table 2). The rNDV-HA- and rNDV-HAF-vaccinated birds were completely protected from intranasal challenge by 100 CLD₅₀ doses of either the homologous or heterologous HPAIV, with no clinical disease signs or deaths (Table 2). In contrast, all of the chickens in the unvaccinated control and rNDV-vaccinated (empty vector) groups died within 24–48 h following challenge with the A/Vietnam/1203/2004 virus, and within 48–72 h following challenge with the A/Egret/Egypt/01/2006 virus. The birds that died from the latter virus had signs of greenish white diarrhea and hemorrhagic signs in the legs and combs.

Shedding of vaccine and challenge viruses in infected chickens

Shedding of NDV vaccine and HPAIV challenge viruses was monitored by taking oral and cloacal swab samples on day 3 post-

Table 2. NDV- and HPAIV-specific serum antibody responses following immunization with rNDV-HA and rNDV-HAF and survival of chickens after homologous and heterologous HPAIV challenge.

Groups: Immunizing virus/ Challenge HPAIV (no. of birds) ¹	Response to immunization (samples taken on 21 day)			
	Mean NDV serum HI antibody (log ₂)	No. of birds seropositive to the indicated HPAIV	Mean serum HI antibody (log ₂) to indicated HPAIV	No. of survivors following challenge on day 21 with the indicated HPAIV
Uninfected/Viet (5)	(0) ²	0/5	0	0/5
Uninfected/Egypt (5)		0/5	0	0/5
rNDV/Viet (5)	(5.30±0.94) ²	0/5	0	0/5
rNDV/Egypt (5)		0/5	0	0/5
rNDV/Viet (10)	(5.15±0.95) ²	10/10	4.57±1.06	10/10
rNDV/Egypt (10)		10/10	2.92±1.09	10/10
rNDV/Viet (10)	(4.50±0.70) ²	10/10	4.53±1.36	10/10
rNDV/Egypt (10)		10/10	2.38±1.35	10/10

¹Chickens in groups of 5 or 10, as indicated, were vaccinated on day 0 with wtNDV, rNDV-HA, or rNDV-HAF. On day 21, serum samples were taken and the birds were challenged with 100 CLD₅₀ of the indicated HPAIV. Groups are named according to the immunizing NDV vector and the HPAIV strain used in the HI assays and challenge. Viet = homologous A/Vietnam/1203/2004; Egypt = heterologous A/Egret/Egypt/01/2006.
²Mean of the viruses in #1 and #2.
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immunization and days 3, 5, and 7 post-challenge. The presence of infectious NDV vector or HPAIV challenge virus was determined by inoculation of embryonated chicken eggs and confirmation of virus replication by HIA assay. No cloacal shedding was observed for any of the NDV vaccine viruses post-immunization, consistent with the attenuated nature of the NDV vector (Table 3). All of birds immunized with rNDV and rNDV-HA were positive for oral shedding, but only 14 out of 20 birds immunized with rNDV-HAF were positive for oral shedding. Analysis of shedding of NDV vaccine virus was performed to confirm immunization and its replication in chickens. Although the exact time at which vaccine virus stopped shedding was not determined, NDV lentogenic vaccine strains known to shed 5 days post immunization. Following challenge with homologous or heterologous HPAIV, no HPAIV shedding was observed in rNDV-HA- and rNDV-HAF-immunized birds at 3, 5 and 7 days post-challenge. In contrast, both oral and cloacal shedding of challenge viruses were observed in all (oral) or most (cloacal) of the unvaccinated and rNDV (empty vector) -vaccinated birds, respectively, of the challenge virus. The shedding results of only day 3 post-challenge are shown in Table 3.

Comparison of pre- and post-HPAIV challenge serum antibody responses by the DIVA strategy

The pre-challenge sera collected from birds at 21 days post-immunization with rNDV-HA and rNDV-HAF were compared with sera collected at 10 days post-challenge with HPAI A/Vietnam/1203/2004 or A/Egret/Egypt/01/2006 viruses. Unvaccinated control and rNDV (empty vector) vaccinated chickens did not survive the HPAIV challenge, hence pre- and post challenge comparison was not carried out. The comparison was carried out using a commercially available AIV NP ELISA that provides the basis to differentiate between birds vaccinated with an NDV-based vector (which would not stimulate antibodies against HPAIV NP) and infected birds (in which an NP-specific response would occur). Pre- and post-challenge sera also were analyzed by a commercial NDV ELISA test and an HI assay using the respective homologous or heterologous HPAIV strain. There was little or no change in NDV antibody titers detected by NDV ELISA following HPAIV challenge, as might be expected (Table 4). However, in both rNDV-HA- and rNDV-HAF- vaccinated birds, the HPAIV HI titers were increased by one and two log₂ units following A/Vietnam/1203/2004 and A/Egret/Egypt/01/2006 challenge, respectively. However, both the pre- and post-challenge sera of rNDV-HA- and rNDV-HAF-vaccinated birds were negative for AIV NP antibody by AIV NP ELISA. The lack of a detectable response to the AIV NP protein following HPAIV challenge suggested that there was little or no challenge virus replication.

Neutralization of diverse HPAIV strains with sera from birds immunized with NDV-HA or NDV-HAF

The ability of sera from vaccinated chickens (from the study described in Tables 2–4) to neutralize homologous and heterologous strains of HPAIV was assessed by virus neutralization (VN) and HI tests (Table 5). Both rNDV-HA and rNDV-HAF elicited serum antibodies that reacted with H5N1 clade 1 viruses (A/Vietnam/1203/2004, A/Hong Kong/213/2006), clade 2.2 virus (A/Egret/Egypt/01/2006) and an H5N2 virus isolated from chickens (A/Pennsylvania/8/3/83) by HI tests, and which neutralized each of these viruses by VN tests. This result indicated that the antibodies induced by rNDV-HA and rNDV-HAF have abilities to neutralize a broad range of HPAI H5 subtype viruses.

Discussion

In the past twelve years, HPAI H5N1 viruses have not only devastated poultry industries but also have become a major public health concern [3,4]. These viruses have shown continuous increases in genetic and antigenic heterogeneity, as well as dissemination in Asia, Africa and Europe. In order to limit the infection of H5N1 viruses in poultry, there is a need to develop vaccines that provide broad and effective immunity and protection against these viruses. One vaccine that has shown promising results is based on using NDV as a vector to express the HPAIV HA protein from an added gene [21,22,23]. Newcastle disease is an economically important disease of poultry, and vaccination of poultry with live NDV vaccines is readily achieved and is mandatory in many countries. Therefore, development of an efficacious live bivalent vaccine against AIV and NDV has several advantages, including price per dose, ease of administration, and ability to differentiate infected from vaccinated animals.

Our goal is to develop NDV as a vaccine vector for protection of birds against HPAIV H5 subtype virus infections. Previously, two research groups have published results describing generation of NDV recombinant vaccines against HPAI H5 subtype virus [21,23]. In the present study, we have generated improved recombinant NDV vaccines that provide greater protection in chickens against homologous and heterologous HPAI H5 virus challenges than those previously observed. Veits *et al.*, [23] generated an rNDV in which the HA gene from an H5N2 virus was inserted between F and HN genes of NDV clone 30, a derivative of strain LaSota. Although their rNDV protected chickens from lethal NDV and highly-pathogenic H5N2 virus challenges, some vaccinated chickens showed signs of disease when challenged with H5N2 virus, indicating only partial protection. Ge *et al.*, [21] constructed an rNDV in which the HA gene from an HPAI H5N1 virus isolated from an wild bird in China was

Table 3. Frequency of shedding of the NDV-based vaccine viruses and HPAIV challenge viruses¹.

Chicken groups	rNDV-based vaccine virus		HPAIV challenge virus			
	Oral	Cloacal	A/Vietnam/1203/2004		A/Egret/Egypt/01/2006	
			Oral	Cloacal	Oral	Cloacal
Uninfected control	-	-	5/5	4/5	5/5	4/5
rNDV	10/10	0/10	5/5	4/5	5/5	3/5
rNDV HA	20/20	0/10	0/10	0/10	0/10	0/10
rNDV HAF	14/20	0/10	0/10	0/10	0/10	0/10

¹From the experiment in Table 2. Shedding was determined by collecting oral and cloacal swabs on 3 day post vaccination and 3 day post challenge and inoculation into embryonated chicken eggs followed by HA tests for virus growth.
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Table 4. Comparison of pre-challenge (day 21) and post-challenge (day 31) NDV- and HPAIV-specific serum antibody titers¹.

Groups: Immunizing virus/Challenge HPAIV (no. of birds) ²	Mean NDV serum ELISA titer ^{2a}		Mean serum HI antibody (log ₂) to the indicated HPAIV ³		Mean HPAIV NP ELISA titer (OD) ⁴	
	Pre-	Post-	Pre-	Post-	Pre-	Post-
rNDV-HA/Viet (10)	1.39±0.38	1.39±0.38	4.40±0.96	5.80±0.78	0.074±0.012	0.082±0.013
rNDV-HA/Egypt (10)	1.25±0.37	1.22±0.23	2.90±1.10	4.90±1.42	0.071±0.008	0.084±0.007
rNDV-HAF/Viet (10)	1.25±0.37	1.19±0.46	4.45±1.78	5.18±1.71	0.071±0.004	0.088±0.033
rNDV-HAF/Egypt (10)	1.12±0.42	1.10±0.16	2.80±1.61	4.90±0.73	0.068±0.005	0.095±0.033

¹Continuation of the experiment in Tables 2 and 3.
²Viet = A/Vietnam/1203/2004; Egypt = A/Egret/Egypt/01/2006.
^{2a}Commercial NDV ELISA assay (Synbiotic Corporation, San Diego, CA).
³The challenge virus was used for the HI antibody assays.
⁴Commercial HPAIV NP ELISA assay (Synbiotic Corporation, San Diego, CA). Negative control OD = 0.083 and Positive control OD = 0.867.
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inserted between the P and M genes of NDV strain LaSota. The recombinant virus protected chickens from the disease following challenges with NDV and homologous and heterologous H5N1 HPAIV. However, the heterologous challenge virus was detected from the lung, spleen, kidney and brain of one of three chickens at 3 days post-challenge. In that study, both the challenge viruses used were selected from the same geographic area and having 95% similarity in HA gene. Therefore, there is a need to improve NDV-vectored AIV vaccines to provide complete protection against homologous and heterologous challenge viruses isolated from different geographic areas.

In our study, we have generated two different rNDV expressing the H5 HA protein. In one rNDV, the HA gene was expressed in native, unmodified form, while in the other it was modified to contain the transmembrane and cytoplasmic region of NDV F protein in place of its own. This was done with the idea that this would provide for enhanced incorporation into the NDV particle. The unmodified and modified HA genes were inserted separately between the P and M genes of NDV strain LaSota. Both of the recombinant vaccine viruses induced high levels of serum antibody that efficiently neutralized NDV as well as four different strains of HPAI H5 subtype viruses, which included three H5N1 viruses isolated from a humans and one H5N2 virus isolated from a

poultry outbreak in the U.S. Both of the recombinant viruses completely protected chickens against otherwise-lethal NDV and HPAI H5N1 virus challenges. The challenge viruses used were isolated from Vietnam and Egypt. None of the vaccinated animals shed detectable amounts of challenge virus, demonstrating the efficacy of our vaccines. Furthermore, none of the vaccinated chickens had a detectable serum antibody response to the NP protein of AIV after 10 days of challenge, suggesting that there was little or no challenge virus replication in vaccinated chickens.

An ideal AI vaccine should elicit robust protective immune responses against a broad range of homologous and heterologous H5 influenza strains. Therefore, it is necessary to select the HA gene from a virus that can represent most H5 strains of that nature. In our study, we chose the HA gene from A/Vietnam/1203/2004 virus for generation of recombinant NDV. This H5N1 virus belongs genetically to clade 1 and persists in Asia. The heterologous H5N1 challenge virus A/Egret/Egypt/01/2006 used in this study belongs to clade 2.2 and persists in Africa. There is approximately 7% amino acid divergence between the HAI portions of the two viruses [33]. Single vaccinations of rNDV-HA or -HAF completely protected chickens against both clade 1 and clade 2.2 challenge viruses. Analysis of the pre-challenge serum neutralizing antibody responses of vaccinated

Table 5. Serum antibodies induced by NDV-HA or NDV-HAF: HI and virus-neutralizing (VNT) antibody titers against homologous and heterologus HPAIV strains.

Serum antibody titers against the indicated HPAIV strains				
Immunizing virus	Vietnam/1203/2004 (H5N1)	Egret/Egypt/2006 (H5N1)	Hongkong/213/2006 (H5N1)	Pennsylvania/8/3/1983 (H5N2)
Mean HPAIV HI titer (log ₂) ¹				
rNDV	0	0	0	0
rNDV- HA	4.57±1.06	2.92±1.09	5.19±0.93	2.98±1.28
rNDV- HAF	4.53±1.36	2.38±1.42	5.19±1.23	2.96±1.61
Mean VNT titer (log ₂) ¹				
rNDV	0	0	0	0
rNDV- HA	5.19±1.23	2.60±0.99	6.78±0.93	5.23±0.81
rNDV- HAF	5.30±1.46	3.19±1.47	6.87±1.07	5.42±1.23

¹Sera are from the experiment in Table 2, taken 21 days following a single immunization with the indicated virus expressing the HA of the Vietnam/1203/2004 strain. The numbers of animals in the groups were: rNDV (n = 10), rNDV-HA (n = 20), rNDV-HAF (n = 20). Note that the text uses VN for virus neutralization, rather than VNT.
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chickens to H5N1 viruses showed that there was higher neutralizing activity against the homologous clade 1 virus compared to the heterologous clade 2.2 virus, which was an indication of the antigenic differences between the two clades. Nonetheless, the protection conferred by the NDV-HA and NDV-HAF vaccine viruses was sufficiently broad to protect against both HPAIV challenge viruses.

It was previously observed that replacement of the transmembrane and cytoplasmic tail regions of the H7 HA protein with those of the NDV F protein enhanced incorporation of HA into NDV particles [22]. In that study, enhanced incorporations of the H7 HA protein into NDV particles improved the immunogenicity of the virus. However, this result could not be interpreted because the authors simultaneously change the F protein cleavage site from a mono-basic to a poly-basic motif, which likely would have the effect of increasing replication, spread, and immunogenicity. Nonetheless, only 90% of the vaccinated chickens were protected against challenge with the HPAIV H7 virus. In our study, we have followed the same strategy and examined the incorporation of H5 HA protein into NDV particles. Our Western blot and immune electron microscopy results confirmed that replacement of the transmembrane and cytoplasmic tail domain of H5 HA protein with those of the NDV F protein enhanced incorporation into NDV particles and improved the immunogenicity of the virus by completely protecting (100%) the chickens against NDV and HPAI H5 challenges.

Because expression of the H5 HA protein of a highly pathogenic HPAIV, and in particular its incorporation into the NDV virion, may alter the virulence of the NDV vector, we examined the pathogenicity of the recombinant viruses by the MDT method in embryonated chicken eggs [32]. Our results did not show any increase in pathogenicity of our recombinant viruses compared to that of the parent strain LaSota, which is currently used as a vaccine. However, comparison of growth kinetics of the recombinant virus showed that the virus expressing the chimeric H5 HA protein grew about a $\log_{10}$ lower, than the NDV parent or the virus expressing the unmodified H5 HA protein. Similar slow growth kinetics were observed for the virus expressing the chimeric H7 HA protein [22]. It is possible that enhanced incorporation of chimeric HA may have affected the assembly process of NDV.

A concern we wanted to examine is whether enhanced incorporation of the chimeric H5 HA protein may decrease the incorporation of the NDV F protein into the NDV virion. The F protein of NDV is a major immunogenic protein and any reduction in the amount of this protein may alter the efficacy of the NDV vaccine. Analysis of the proteins of purified parental and recombinant viruses by coomassie blue staining did not show any difference in the amount of F protein between parental and recombinant viruses, suggesting that the incorporation of the chimeric H5 HA protein did not take the place of the F protein on the NDV virion (data not shown).

In this study, both of the recombinant NDV vaccine viruses, rNDV-HA and rNDV-HAF, induced similar levels of HI and VN antibodies and provided complete protection against challenges with highly virulent NDV and HPAI H5 viruses. However, further studies involving larger numbers of chickens and additional heterologous challenge viruses will be necessary to determine which of the two vaccine viruses is superior: namely, the NDV-HA virus that replicates somewhat better but incorporates a lesser amount of HPAIV HA, or the NDV-HAF virus that replicates less well (and is more attenuated) but incorporates a greater amount of HA. Our results demonstrated that the NDV-vectored AIV vaccines generated in this study could be used as bivalent vaccines against NDV and HPAI H5 virus in chickens.

Materials and Methods

Viruses and cells

Madin-Darby Canine Kidney (MDCK), human epidermoid carcinoma (HEp-2), and chicken embryo fibroblast (DF1) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). MDCK and HEp-2 cells were grown in Eagle's minimal essential medium (EMEM) containing 10% fetal bovine serum (FBS) and maintained in EMEM with 5% FBS. DF1 cells were grown in Dulbecco's minimal essential medium (DMEM) with 10% FBS and maintained in DMEM with 5% FBS. Recombinant NDV (rNDV) strain LaSota was generated as previously described [31,34]. The HPAIV strains, A/Vietnam/1203/2004 (H5N1) and A/Hong Kong/213/2006 (H5N1), were obtained from the Centers for Disease Control and Prevention (CDC), Atlanta GA. The HPAIV strain A/Egret/Egypt/01/2006 (H5N1) was obtained from Dr. Daniel Perez and the HPAIV strain H5N2 strain (A/Pennsylvania/8/3/1983) was a field isolate maintained in our virus repository. The viruses were propagated in 9-day-old specific pathogen free (SPF) embryonated chicken eggs.

Virus titration

The titration of rNDVs and AIVs was carried out by infecting DF1 and MDCK cells, respectively. The rNDV titers were determined by plaque assay in DF1 cells using a 0.8% methylcellulose overlay and 5% allantoic fluid. The infected cells were incubated at 37°C for 3 to 4 days, until the development of plaques was apparent. The cell monolayers were then fixed with methanol and stained with crystal violet for the enumeration of plaques. The HPAIV titers in 50% end point tissue culture infectious dose ($TCID_{50}$) units/ml were determined on monolayers of MDCK cells using the Reed and Muench method [36]. For both NDVs and AIVs, HA titers were determined using chicken RBCs [32]. Egg infective dose 50 (EID_{50}) was determined by infecting 5 eggs per group for each 10-fold serial dilution. Following 24 h of infection, eggs were harvested for allantoic fluid and the presence of virus was confirmed by HIA test. Similarly, chicken embryo lethal dose 50 (ELD_{50}) was determined by observing 50% mortality in embryos at end point dilutions after 48 h of infection. The 50% end point titer was determined by the Reed and Muench method [36]. For HPAIV challenge viruses, the chicken lethal dose of 50 (CLD_{50}) was determined by infecting 3 chickens (5-wk-old) per group, and the 50% end point for the lethal dose was determined by the Reed and Muench method [36].

Generation of rNDVs containing HPAIV HA coding sequence

The construction of a full-length cDNA of the antigenomic RNA of NDV strain LaSota was reported earlier [31,34]. The present study used a previously-described derivative that had been modified to contain a unique Pme I restriction enzyme site between the P and M genes [31]. The HA open reading frame (ORF) from HPAIV strain A/Vietnam/1203/2004 (H5N1) virus was PCR-amplified using primers (H5HAPmeI sense- 5'-CCGTTTAAACTTA GAAAAAATACGGGTAGAAAGGCATA CGCCACCAT GGAG-3' and antisense 5'-GGGTTTAAACTTAGATACAAATCCT GCAC TGG-3') that introduced an NDV gene junction, including gene end (underlined), intergenic, and gene start (underlined) signals, on the upstream side and Pme I sites (italic) on both sides. The H5 HA gene was further modified to create a second version in which the HA ectodomain was fused to the transmembrane and cytoplasmic domains of the NDV F protein by overlapping PCR using primer H5HAPmeI sense and H5HAF antisense primer (5'-GGGTTTAACTTAGATACAA-

ATCCTGCACTGG-3'). The cDNA templates used for overlapping PCR were amplified by H5HA PmeI sense primer and antisense primer (5'-GTAATGAGAGCAGATGTGCTTTGGTATATCCCTATGCTCTCC-3', region corresponding H5 HA is underlined) from H5 HA template and also amplified by sense primer (5'-GGAGAGCATAGGGATATACCAAAGCACATCTGCTCTCATTAC-3', region corresponding NDV is underlined) and H5HAF antisense from NDV F cDNA template. Altering the transmembrane and cytoplasmic domains changed the length of the HA ORF from 1707 nt to 1767 nt. The PCR products were cloned into full-length NDV plasmid at the unique Pme I site between the P and M genes. The total genome length was maintained as a multiple of six, which is required for efficient NDV replication [35]. In order to decrease the possibility of any mutation or deletion of parts of the NDV genome during propagation in bacteria, the transformed bacteria were grown at 30°C. The HA genes of the resulting plasmids, pNDV-HA and pNDV-HAF, were sequenced to confirm the correct HA gene structure and the absence of any deletion or undesired mutation. Our results showed that the plasmids pNDV-HA and pNDV-HAF were quite stable in bacteria and no mutation or deletion was detected. The NDV recombinants were recovered by transfecting these plasmids along with support plasmids into HEp-2 cells by the previously described procedure [31,34].

Expression of H5 HA in cells infected with rNDVs

The HA protein expression by the rNDVs was examined by Western blot and immunofluorescence assays. DF1 cells were infected at a multiplicity of infection (MOI) of 0.01 and were harvested at 48 h post-infection, lysed and analyzed by Western blot using polyclonal chicken H5N1 (post-infection) antiserum. To examine the incorporation of H5 HA and H5 HAF into NDV particles, Western blot analysis was carried out using purified virus and polyclonal chicken H5N1 (post-infection) antiserum. The band intensities in the western blot were normalized using an NDV HN monoclonal antibody. Immunofluorescence assays were performed to evaluate cell surface and intracellular expression of HA protein. Briefly, DF1 cells were infected with the recombinant viruses at an MOI of 0.01 PFU. At 48 h post-infection, the infected DF1 cells were fixed with 4% para-formaldehyde. For internal H5 HA expression, cells were permeabilized with 0.2% Triton X 100 in PBS for 10 minutes. The primary antibodies used were chicken polyclonal anti-H5 HA and the secondary antibodies used were goat anti-chicken conjugated with FITC. The cells were analyzed with a fluorescent microscope.

Immunoelectron microscopy

The rNDVs purified by sucrose density gradient from allantoic fluid of infected embryonated eggs were adsorbed to parlodion-film carbon-coated nickel grids. The grids were blocked in PBS containing 2% globulin-free BSA and incubated with chicken anti-NDV HN monoclonal antiserum or chicken anti-H5 HA polyclonal antiserum. Grids were washed in blocking solution and incubated in goat anti-chicken or anti-mouse IgG antiserum conjugated to 6 nm gold beads (Aurion, Costerweg 5, The Netherlands). The grids were then negatively stained with 2% ammonium molybdate for 3 min and examined under a transmission electron microscope.

Growth characteristics of the recombinant viruses in DF1 cells

The growth kinetics of rNDV, rNDV-HA and rNDV-HAF were determined by multicycle growth curves in DF1 cells. DF1

cells in duplicate wells of six-well plates were infected with viruses at a MOI of 0.01 PFU. After 1 h of adsorption, the cells were washed with DMEM and then covered with DMEM containing 5% FBS and 5% allantoic fluid. The cell culture supernatant samples were collected and replaced with an equal volume of fresh medium at 8-hour intervals until 64 hours post-infection. The titers of virus in the samples were quantified by plaque assay in DF1 cells.

Pathogenicity of recombinant viruses in embryonated chicken eggs

The pathogenicity of the recombinant viruses were determined by the MDT test in 9-day-old embryonated chicken eggs according to a standard protocol [32]. Briefly, 10-fold serial dilutions of fresh infective allantoic fluid ranging from 10^{-6} to 10^{-9} dilutions were made with sterile PBS. 0.1 ml of each diluent was injected into the allantoic cavities of five 9-day-old SPF embryonated chicken eggs (Bee Eggs Company, PA) and incubated at 37°C. Each egg was examined three times daily for 7 days, and times of embryo death were recorded. The minimum lethal dose is the highest virus dilution that causes all embryos inoculated with that dilution to die.

Immunization and challenge experiments

In experiment one (Table 1), groups of 2-week-old SPF chickens were immunized through the ocular route with 10^6 EID₅₀ of biologically-derived wild type NDV LaSota or recombinant virus rNDV (empty vector), rNDV-HA or rNDV-HAF, and an additional group of birds was left uninfected. Three days post-immunization, oropharyngeal (oral) and cloacal swabs were collected for detection of NDV shedding. Three weeks post-immunization, pre-challenge serum samples were collected from all of the birds, and all of the birds were challenged with 10^4 ELD₅₀ of virulent NDV strain Texas-GB through the ocular route. Oral and cloacal swabs were collected on day 3 post-challenge for shedding. In experiment two (Tables 2–5), groups of birds were immunized in the same way except that the biologically-derived LaSota control was replaced by its recombinant version rNDV. Three weeks post-immunization, serum samples were collected and the animals were challenged through the intranasal route with 100 CLD₅₀ of the homologous HPAIV A/Vietnam/1203/2004 virus or the heterologous HPAIV A/Egypt/01/2006 virus. To monitor shedding of the challenge viruses, oral and cloacal swabs were collected on day 3 post-infection. Isolation of NDV vaccine vector or HPAIV challenge virus from swabs was carried out by inoculation in 9-day-old embryonated chicken eggs. The chickens were observed daily for 10 days for disease symptoms and mortality, and the presence of NDV or HPAIV was confirmed by HA assay using chicken erythrocytes. Post-challenge serum was collected from the surviving birds before sacrifice after 10 days of challenge. All challenge studies were performed in a BSL3 (Ag) containment facility certified by the USDA and CDC.

Serological analysis

The antibody levels of serum samples collected from chickens vaccinated with rNDVs were evaluated by hemagglutination (HA), HA inhibition (HI), virus neutralization (VN) and enzyme-linked immunosorbent assays (ELISA) using standard protocols [36]. Cross-reactivity of immunized chicken sera were conducted by VN and HI tests against four homologous and heterologous strains of influenza viruses. The homologous clade 1 H5N1 strains included (A/Vietnam/1203/2004 and A/Hongkong/213/2006), and het-

crologous strains included clade 2.2 H5N1 virus (A/Egypt/Egypt/01/2006) and the H5N2 virus (A/Pennsylvania/8/3/1983). A commercial AIV NP ELISA kit and NDV ELISA kit (Synbiotic Corporation, San Diego, CA) were used to detect antibodies against the NP antigen of type A influenza viruses and whole NDV antigen of NDVs.

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Author Contributions

Conceived and designed the experiments: BN DRP PLC SKS. Performed the experiments: BN SNR SK. Analyzed the data: BN SNR SK SKS. Contributed reagents/materials/analysis tools: MSK MMF LEA KCE DRP. Wrote the paper: BN PLC SKS.

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Reivindicaciones

1. Un NDV quimérico, que comprende un genoma empacado que comprende una secuencia de nucleótidos que codifica una proteína de fusión F que tiene los dominios de transmembrana y citoplasmático de una proteína F y por lo menos un epítipo de un ectodominio de un antígeno protector de un agente infeccioso diferente de NDV o de un antígeno asociado con una enfermedad que está anclado por el C terminal, de manera que la proteína de fusión F se expresa e incorpora dentro del NDV quimérico, que se caracteriza porque el antígeno no es un antígeno de paramixovirus.

2. El NDV quimérico de la reivindicación 1, que se caracteriza porque el agente infeccioso es un patógeno infeccioso.

3. El NDV quimérico de la reivindicación 1 o 2, en el cual el genoma comprende una secuencia de nucleótidos que codifica una proteína F, de manera que la proteína F se expresa e incorpora dentro del NDV quimérico.

4. El NDV quimérico de la reivindicación 1 o 2, en el cual la secuencia de nucleótidos que codifica la proteína de fusión F reemplaza la secuencia de nucleótidos que codifica la proteína F del NVD y la proteína de fusión F suplente la función de la proteína F.

5. Un NDV quimérico, que comprende un genoma empacado que comprende una secuencia de nucleótidos que codifica una proteína de fusión HN que tiene los dominios de transmembrana y citoplasmático de una proteína HN y por lo menos un epítipo de un ectodominio de un antígeno protector de un agente infeccioso diferente de NDV o de un antígeno asociado con una enfermedad que está anclado por el N terminal, de manera que la proteína de fusión HN se expresa e incorpora dentro del NDV quimérico, que se caracteriza porque el antígeno no es un antígeno de paramixovirus.

6. El NDV quimérico de la reivindicación 5, que se caracteriza porque el agente infeccioso es un patógeno infeccioso.

7. El NDV quimérico de la reivindicación 5 o 6 en el cual el genoma comprende una secuencia de nucleótidos que codifica una proteína HN, de manera que la proteína HN se expresa e incorpora dentro del NDV quimérico.

8. El NDV quimérico de la reivindicación 5 o 6 en el cual la secuencia de nucleótidos que codifica la proteína de fusión HN reemplaza la secuencia de nucleótidos que codifica la proteína HN del NDV y la proteína de fusión HN suplente la función de la proteína HN.

9. El NDV quimérico de cualquiera de las reivindicaciones 1 a 4, que se caracteriza porque el antígeno es el ectodominio de un antígeno de hemaglutinina de un virus de la influenza.

10. El NDV quimérico de cualquiera de las reivindicaciones 5 a 8, que se caracteriza porque el antígeno es el ectodominio de un antígeno de neuraminidasa de un virus de la influenza.

11. El NDV quimérico de la reivindicación 9, que se caracteriza porque el virus de la influenza es un virus de la influenza aviar.

12. El NDV quimérico de la reivindicación 10, que se caracteriza porque el virus de la influenza es un virus de la influenza aviar.

13. El NDV quimérico de cualquiera de las reivindicaciones 1 a 12, que se caracteriza porque los dominios de transmembrana y citoplasmático de la proteína de fusión F o la proteína de fusión HN provienen de la cepa LaSota del NDV.

14. El NDV quimérico de cualquiera de las reivindicaciones 1 a 3 o 5 a 13, que se caracteriza porque la proteína F es genéticamente modificada en el sitio de ruptura, de manera que la actividad fusogénica está aumentada.

15. El NDV quimérico de la reivindicación 14, que se caracteriza porque el sitio de ruptura genéticamente modificado comprende a un sitio de ruptura de múltiples bases.

16. El NDV quimérico de cualquiera de las reivindicaciones 1 a 15 que se caracteriza porque la proteína de fusión F contiene bien sea ningún residuo de aminoácidos del ectodominio de la proteína F, o bien contiene un fragmento del ectodominio de la proteína F que no retiene la actividad del ectodominio de la proteína F; o que se caracteriza porque la proteína de fusión HN contiene bien sea ningún residuo de aminoácidos del ectodominio de la proteína HN, o bien contiene un fragmento del ectodominio de la proteína HN que no retiene la actividad del ectodominio de la proteína HN.

17. El NDV quimérico de la reivindicación 16, que se caracteriza porque la proteína de fusión F no contiene residuos de aminoácidos del ectodominio de la proteína F.

18. El NDV quimérico de la reivindicación 16, que se caracteriza porque la proteína de fusión F contiene un fragmento del ectodominio de la proteína F que no retiene la actividad del ectodominio de la proteína F.

19. El NDV quimérico de la reivindicación 16, que se caracteriza porque el fragmento del ectodominio de la proteína F está entre 1 y 15 residuos del ectodominio de una proteína F del NDV que se encuentra inmediatamente adyacente al dominio de transmembrana

de la proteína F; o que se caracteriza porque el fragmento del ectodominio de la proteína HN está entre 1 y 15 residuos del ectodominio de una proteína HN del NDV que se encuentra inmediatamente adyacente al dominio de transmembrana de la proteína HN.

20. El NDV quimérico de la reivindicación 19, que se caracteriza porque el fragmento del ectodominio de la proteína F está entre 1 y 15 residuos del ectodominio de una proteína F del NDV que se encuentra inmediatamente adyacente al dominio de transmembrana de la proteína F.

21. El NDV quimérico de cualquiera de las reivindicaciones 1 a 20, que se caracteriza porque la secuencia que codifica la proteína de fusión se inserta entre los genes P y M del genoma NDV.

22. El NDV quimérico de cualquiera de las reivindicaciones 1 a 21, que se caracteriza porque el NDV quimérico tiene una columna vertebral de la cepa LaSota del NDV.

23. El NDV quimérico de cualquiera de las reivindicaciones 1 a 22, que se caracteriza porque el NDV quimérico está atenuado.

24. Un método para producir una formulación inmunogénica, el cual método comprende:

- (a) propagar el NDV quimérico de cualquiera de las reivindicaciones 1 a 23 en un huevo embrionado o una línea celular que es susceptible de infectarse con el NDV; y
- (b) recoger la progenie del virus,

que se caracteriza porque el virus se cultiva hasta obtener cantidad suficiente y en condiciones suficientes para que el virus esté libre de contaminación, de manera que la progenie del virus es apropiada para usar en una formulación inmunogénica.

25. Una molécula de ADN recombinante que codifica el NDV quimérico de cualquiera de las reivindicaciones 1 a 23.

26. Una formulación inmunogénica que comprende el NDV quimérico de cualquiera de las reivindicaciones 1 a 23.

27. La formulación inmunogénica de la reivindicación 26 para usar en la prevención de una enfermedad asociada con una infección viral.

28. La formulación inmunogénica de la reivindicación 27, que se caracteriza porque la infección viral es causada por el NDV.

29. La formulación inmunogénica de la reivindicación 27 o 28, que se caracteriza porque la infección viral es causada por el virus de la influenza.

30. La formulación inmunogénica de la reivindicación 29, que se caracteriza porque el virus de la influenza es el virus de la influenza aviar.

31. La formulación inmunogénica de la reivindicación 26 para usar en la inducción de una respuesta inmunitaria al NDV y al antígeno en un ave.

32. La formulación inmunogénica de la reivindicación 26 para usar en la inducción de una respuesta inmunitaria al antígeno en un ser humano.

33. El NDV quimérico de cualquiera de las reivindicaciones 1 a 23 en la preparación de un medicamento para inducir una respuesta inmunitaria al NDV y al antígeno en un ave.

34. El NDV quimérico de cualquiera de las reivindicaciones 1 a 23 en la preparación de un medicamento para inducir una respuesta inmunitaria al antígeno en un ser humano.

35. El NDV quimérico de cualquiera de las reivindicaciones 1 a 23 en la preparación de un medicamento para prevenir una enfermedad asociada con una infección viral.

36. El NDV quimérico de la reivindicación 35, que se caracteriza porque la infección viral es causada por el NDV.

37. El NDV quimérico de la reivindicación 35 o 36, que se caracteriza porque la infección viral es causada por el virus de la influenza.

38. El NDV quimérico de la reivindicación 37, que se caracteriza porque el virus de la influenza es el virus de la influenza aviar.

26

 Industria y Comercio SUPERINTENDENCIA	Espacio reservado para el adhesivo de radicación
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DIRECCIÓN DE NUEVAS CREACIONES
SOLICITUD FASE NACIONAL - PCT

1	TIPO SE SOLICITUD	☒ Patente de invención ☒ Capítulo I	☐ Patente de Modelo de Utilidad ☐ Capítulo II
2	DATOS SOLICITUD INTERNACIONAL PCT (86)		
Solicitud Internacional No.		PCT/ PCT/US2006/045859	Fecha 01/12/2006
Publicación Internacional No.		WO WO 2007/064802 A1	Fecha 07/06/2007
3	TÍTULO DE LA INVENCION (200 caracteres o espacios máximos)		
VIRUS QUIMÉRICOS DE LA ENFERMEDAD DE NEWCASTLE QUE PRESENTAN PROTEÍNAS DE SUPERFICIE NO NATIVAS			
4	CLASIFICACIÓN INTERNACIONAL (CIP)		
A61K 39/00			
5	SOLICITANTE (S) ☐ Esta persona también es inventor. Para datos adicionales utilizar hoja de información complementaria		
THE MOUNT SINAI SCHOOL OF MEDICINE OF NEW YORK UNIVERSITY		IDENTIFICACIÓN	TIPO
6	DATOS DEL SOLICITANTE		
DIRECCIÓN One Gustave L. Levy Place		No. TELÉFONO	
CIUDAD New York, Estado de New York, E.U.A.		CORREO ELECTRÓNICO	
PAÍS		NACIONALIDAD O LUGAR DE CONSTITUCIÓN	
7	INVENTOR (ES) Para datos adicionales utilizar hoja de información complementaria		
	APELLIDOS	NOMBRES	NACIONALIDAD
1	Peter PALESE - Adolfo GARCIA-SASTRE		E.U.A.
2			E.U.A.
3			
4			
5			
DIRECCIÓN DE CORREO ELECTRÓNICO:			
8	DATOS INVENTOR (ES)		
	PAÍS RESIDENCIA	DEPARTAMENTO/ESTADO	CIUDAD DIRECCIÓN
1	E.U.A.	NJ	414 Highwood Avenue
2	E.U.A.	NY	16 East 96th Street Ap. 3G
3			
4			
5			
OTRO(S) SOLICITANTE(S) Y/O (OTRO(S)) INVENTOR(ES)			
☐ Los demás solicitantes y/o (demás) inventores se indican en una hoja a continuación			
9	☐ REPRESENTANTE LEGAL ☒ APODERADO		
LUIS FELIPE CASTILLO GIBSONE		IDENTIFICACION C.C. 79.397.879 de TP 68.995 del Bogotá C.S.J.	
DIRECCIÓN Carrera 13 No. 37-43 Piso 12		No. TELÉFONO 285 7460	
CIUDAD Bogotá, D.C.		CORREO ELECTRÓNICO info@castillograu.c om	
PAÍS Colombia		No. RADICACIÓN O PORTOCOLO DE PODER GENERAL	

10	DECLARACIONES DE PRIORIDAD ☒ SI ☐ NO		
	(33) PAIS DE ORIGEN	CÓDIGO PAÍS	(31) NÚMERO
	1 E.U.A.		50/741,833 - 60/802,864
	2		02.12.2005 - 22.05.2006
	3		

11	DECLARACIÓN SOBRE USO DE RECURSOS GENÉTICOS O BIOLÓGICOS
<i>Declaro que el objeto de la presente solicitud de patente fue obtenido a partir de recursos genéticos o biológicos de los que cualquiera de los países miembros de la Comunidad Andina es país de origen.</i> ☐ SI ☒ NO Nota: En caso afirmativo deberá anexar copia del contrato de acceso de recursos genéticos o productos derivados, o certificado o número de registro, expedido por la Autoridad competente.	

12	DECLARACION SOBRE USO DE CONOCIMIENTOS TRADICIONALES
<i>Declaro que el objeto de la presente solicitud de patente fue obtenido a partir de conocimientos tradicional de comunidades indígenas, afroamericanas o locales de países miembros de la Comunidad Andina.</i> ☐ SI ☒ NO Nota: En caso afirmativo deberá anexar la licencia o autorización de uso de conocimiento tradicional, o certificado o número de registro expedido por la Autoridad competente.	

13	REDUCCIÓN DE TASAS
Declaro que carezco de medios económicos para presentar la solicitud de la patente. ☐ SI ☒ NO Nota: En caso de ser persona natural y carecer de medios económicos, y por lo tanto, aplique la reducción de tasas a que se refiere la resolución vigente en tarifas, debe firmar la presente solicitud bajo la gravedad de juramento.	
Micro, pequeñas y medianas empresas ☐ Universidades públicas o privadas ☐ Entidades sin ánimo de lucro ☐	

Debe aportar los documentos que se indican en el numeral 17 de anexos

14	AUTORIZACION DE NOTIFICACIÓN EN LINEA ☐ SI ☒ NO
Manifiesto que he leído y entendido perfectamente los términos y condiciones de uso de medios electrónicos para las notificaciones en línea a través de internet de los actos administrativos proferidos por la Superintendencia de Industria y Comercio que deben ser notificados personalmente y, en consecuencia, autorizo el servicio de notificación a través de internet.	

15	COMPROBANTE DE PAGO O PAGO ELECTRÓNICO	No.	Fecha.
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16	FIRMA DEL SOLICITANTE, DEL APODERADO O DEL REPRESENTANTE LEGAL
<i>Junto a cada firma, indicar el nombre del firmante y su calidad (si tal calidad no es obvia al leer el petitorio)</i> LUIS FELIPE CASTILLO GIBSONE	

17	ANEXOS																																																																																						
Documentación Técnica Solicitud Internacional en Castellano <table style="width: 100%;"> <tr> <td style="width: 5%;">1</td> <td style="width: 10%;">☒</td> <td style="width: 45%;">Descripción</td> <td style="width: 40%;">No. De folios:</td> </tr> <tr> <td>2</td> <td>☒</td> <td>Reivindicaciones</td> <td>No. Reivindicaciones:</td> </tr> <tr> <td>3</td> <td>☐</td> <td>Dibujos y/o figuras</td> <td>No. Folios:</td> </tr> <tr> <td>4</td> <td>☒</td> <td>Resumen</td> <td></td> </tr> <tr> <td>5</td> <td>☐</td> <td>Certificado de depósito de material biológico si fuera el caso</td> <td></td> </tr> <tr> <td>6</td> <td>☐</td> <td>Listado de secuencias de nucleótidos y/o aminoácidos en forma digital si fuera el caso</td> <td></td> </tr> <tr> <td>7</td> <td>☐</td> <td>Arte final 12 x 12</td> <td></td> </tr> <tr> <td>8</td> <td>☐</td> <td>Anexo formato digital</td> <td></td> </tr> </table>	1	☒	Descripción	No. De folios:	2	☒	Reivindicaciones	No. Reivindicaciones:	3	☐	Dibujos y/o figuras	No. Folios:	4	☒	Resumen		5	☐	Certificado de depósito de material biológico si fuera el caso		6	☐	Listado de secuencias de nucleótidos y/o aminoácidos en forma digital si fuera el caso		7	☐	Arte final 12 x 12		8	☐	Anexo formato digital		Documentación Jurídica <table style="width: 100%;"> <tr> <td style="width: 5%;">9</td> <td style="width: 10%;">☐</td> <td style="width: 85%;">Poderes, si fuera el caso.</td> </tr> <tr> <td>10</td> <td>☐</td> <td>Documento que legalmente pruebe la cesión del inventor al solicitante o a su causante</td> </tr> <tr> <td>11</td> <td>☐</td> <td>Copia del contrato de acceso de recursos genéticos o productos derivados, o certificado número de registro, si fuera al caso</td> </tr> <tr> <td>12</td> <td>☐</td> <td>Copia de la licencia o autorización de conocimientos Tradicionales, o certificado o número de registro, si fuera el caso</td> </tr> <tr> <td>13</td> <td>☐</td> <td>Reducción de tasas</td> </tr> <tr> <td colspan="3">Micro, pequeñas o medianas empresas</td> </tr> <tr> <td></td> <td>☐</td> <td>Copia simple de la declaración de renta del año inmediatamente anterior, o en su defecto prueba documental idónea</td> </tr> <tr> <td></td> <td>☐</td> <td>Documento de constancia de cumplimiento con lo establecido en la ley 905 de 2004</td> </tr> <tr> <td colspan="3">Universidades públicas o privadas</td> </tr> <tr> <td></td> <td>☐</td> <td>Copia acto de reconocimiento institucional emitido por el Ministerio de Educación</td> </tr> <tr> <td colspan="3">Entidades sin ánimo de lucro</td> </tr> <tr> <td></td> <td>☐</td> <td>Copia de regido vigente en Cámara de comercio</td> </tr> <tr> <td></td> <td>☐</td> <td>Hoja de información complementaria</td> </tr> <tr> <td></td> <td>☐</td> <td>Otros, especificar</td> </tr> <tr> <td>16</td> <td>☒</td> <td>Comprobante de pago de la tasa de presentación de la solicitud</td> </tr> <tr> <td>17</td> <td>☐</td> <td>Comprobante de pago por reivindicación de prioridad</td> </tr> <tr> <td>18</td> <td>☐</td> <td>Comprobante de pago de la tasa de concepto de excedente de palabras en la publicación</td> </tr> <tr> <td>19</td> <td>☐</td> <td>Comprobante de pago por reivindicación adicional a 10</td> </tr> </table>	9	☐	Poderes, si fuera el caso.	10	☐	Documento que legalmente pruebe la cesión del inventor al solicitante o a su causante	11	☐	Copia del contrato de acceso de recursos genéticos o productos derivados, o certificado número de registro, si fuera al caso	12	☐	Copia de la licencia o autorización de conocimientos Tradicionales, o certificado o número de registro, si fuera el caso	13	☐	Reducción de tasas	Micro, pequeñas o medianas empresas				☐	Copia simple de la declaración de renta del año inmediatamente anterior, o en su defecto prueba documental idónea		☐	Documento de constancia de cumplimiento con lo establecido en la ley 905 de 2004	Universidades públicas o privadas				☐	Copia acto de reconocimiento institucional emitido por el Ministerio de Educación	Entidades sin ánimo de lucro				☐	Copia de regido vigente en Cámara de comercio		☐	Hoja de información complementaria		☐	Otros, especificar	16	☒	Comprobante de pago de la tasa de presentación de la solicitud	17	☐	Comprobante de pago por reivindicación de prioridad	18	☐	Comprobante de pago de la tasa de concepto de excedente de palabras en la publicación	19	☐	Comprobante de pago por reivindicación adicional a 10
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28

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO NIT : 800.176.089-2 - / -					
 Industria y Comercio SUPERINTENDENCIA	RECIBO DE CAJA			No. 12 - 134030	
				Bogotá D.C., Diciembre 04 de 2012 - 12:36:55	
RECIBIDO DE : CASTILLO GRAU & ASOCIADOS LTDA.				NI 800.098.704	
*** Soporte del Pago ***					
TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	VR. PAGO
CONSIGNACION	BANCO DE BOGOTA	062754387	409420900	04/12/2012	840.000.00
*** Conceptos Pagados ***					
CANT.	RENTISTICO	CONCEPTO	Vr.UNDITARIO	Vr. CONCEPTO	
1	50005-01-09 OTROS SERVICIOS DE PROPIEDAD INDUSTRIAL	99 OTROS SERVICIOS ADMINISTRATIVOS	840.000.00	840.000.00	
				\$840.000.00	
=====					
SON: **OCHOCIENTOS CUARENTA MIL PESOS MONEDA CORRIENTE***					
Responsable: 					
Recibo de Caja Aplicado al Expediente No. _____					
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO  No. 08-067535- -00006-0000 Fecha: 2012-12-04 12:42:42 Dep. 2020 DIR.NUEVASCR Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI Act. 444 RESPUESTAREQUE Folios: 28					
Sede Centro: Carrera 13 No. 27 - 00 Pisos 3,4,5 y 10 Bogotá, D.C.- Colombia Web: www.slc.gov.co e-mail: info@slc.gov.co Conmutador: (571) 5870000 Fax: (571) 5870284 Línea: 018000-910165 Call Center: (571) 6513240 Diciembre 4 de 2012 - 12:36:56					