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The pathogenesis of Newcastle disease: A comparison of selected Newcastle disease virus wild-type strains and their infectious clones

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Abstract

The effect of mutations of Newcastle disease virus (NDV) fusion (F) gene, hemagglutinin–neuraminidase (HN) gene, and phosphoprotein (P) gene and HN chimeras between the virulent Beaudette C and low virulence LaSota strains on pathogenesis and pathogenicity was examined in fully susceptible chickens. A virulent F cleavage site motif within a LaSota backbone increased pathogenicity and severity of clinical disease. A LaSota HN within a Beaudette C backbone decreased pathogenicity indices and disease severity. A Beaudette C HN within a LaSota backbone did not change either pathogenicity indices or severity of disease in chickens. Loss of glycosylation at site 4 of the HN or modified P gene of Beaudette C decreased pathogenicity indices and caused no overt clinicopathologic disease in chickens. Both pathogenicity indices and clinicopathologic examination demonstrated that the F, HN, and P genes of NDV collectively or individually can contribute to viral virulence. © 2006 Elsevier Inc. All rights reserved.

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Introduction

Newcastle disease virus (NDV) or avian paramyxovirus 1 (APMV-1) is a non-segmented, single-stranded, negative-sense RNA virus in the family Paramyxoviridae (de Leeuw and Peeters, 1999; Krishnamurthy and Samal, 1998; Phillips et al., 1998). The RNA genome consists of 15,186 bases and contains six genes encoding the six structural proteins in order from 3' to 5': nucleoprotein (NP)–phosphoprotein (P)–matrix (M)–fusion (F)–hemagglutinin–neuraminidase (HN)–large protein (L) (Chambers et al., 1986; Wilde et al., 1986). In addition,

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transcriptional editing of the P gene mRNA results in two nonstructural proteins, V and a potential W (Peeters et al., 2004; Steward et al., 1993).

The disease resulting from an NDV infection of birds varies from mild to severe with high mortality depending on virulence of the infecting strain and host susceptibility (Alexander, 1995, 2001, 2003). Because NDV strains are of a single serotype, virulence differentiation among those strains must be determined by standard pathotyping assays. The results of those tests which utilize inoculation of embryonated chicken eggs and live chickens are the basis for classifying NDV as velogenic (highly virulent), mesogenic (moderately virulent), or lentogenic (low virulent) (Alexander, 1998). Further division of the velogenic pathotype into viscerotropic velogenic (VVNDV) and neurotropic velogenic (NVNDV) pathotypes, those strains that cause an acute lethal disease with frequent visceral hemorrhage or an acute and often lethal disease with neurological and respiratory

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signs, respectively, is accomplished by intracloacal inoculation of chickens (Alexander, 1998, 2003). Pathogenesis studies to assess virus distribution in tissues and resultant lesions from an NDV infection have been completed by inoculation of chickens with a lower virus dose by a natural route in contrast to the inoculation of a high virus dose by a systemic route in pathotyping tests (Brown et al., 1999). Prior pathogenesis studies demonstrated that viruses of both velogenic pathotypes produce severe clinical disease and infect multiple tissues. Gross and histologic lesions that are the result of those infections are usually more extensive and severe with VVNDV than with NVNDV (Brown et al., 1999). No overt clinical signs were usually observed with infections from either mesogenic or lentogenic NDV. However, mesogenic isolates do cause some gross and histologic lesions that are considerably less extensive than those caused by a velogenic virus infection (Brown et al., 1999; Kommers et al., 2003). Minimal lesions, if present, occurred in birds with lentogenic infections, affecting mostly the respiratory tract (Hamid et al., 1990). In lentogenic NDV infections, viral replication is detected primarily at the inoculation sites (Kommers et al., 2003) but minimal replication can also be present in cardiac myofibers (Brown et al., 1999).

The marked strain-dependent difference in tropism and virulence observed with NDV are hypothesized to depend upon the presence of cellular proteases required for the activation of the viral fusion glycoprotein precursor (Alexander, 2001; Gotoh et al., 1992; Nagai, 1995; Nagai and Klenk, 1977). Recent studies utilizing viruses containing mutations generated by reverse genetics have supported the importance of the amino acid sequence at the F cleavage site for NDV virulence (de Leeuw et al., 2003; Panda et al., 2004b; Peeters et al., 1999; Römer-Oberdörfer et al., 2003) and viral distribution in embryos (Al-Garib et al., 2003). However, some investigators have suggested involvement of other factors (de Leeuw et al., 2003; Panda et al., 2004b). The loss of glycosylation sites from the HN protein altered NDV pathogenicity (Panda et al., 2004a), and HN chimeras generated from low virulent or virulent viruses either increased or decreased viral pathogenicity depending on the virulence of the virus that was the origin of the HN gene (Huang et al., 2004). Evidence for a P gene product, the V protein, contribution to NDV virulence was demonstrated in chickens (Huang et al., 2003), in embryonating chicken eggs (Mebatsion et al., 2001; Park et al., 2003a), and during in vitro cell culture (Huang et al., 2003; Park et al, 2003a, 2003b). These prior studies with infectious clones demonstrated the potential role of the F, HN, and P genes in NDV virulence, but the dissemination of these infectious clones and induction of pathological changes was not reported for infected mature chickens. Therefore, the purpose of this study was to extend the understanding of the role of the F, HN, and P genes in the pathogenesis of NDV by comparing the results of a clinicopathologic assessment in chickens infected via a natural route with selected wild-type NDV, their infectious clones, and those clones with various gene changes or mutations. The virulence of those viruses was also determined by standard pathogenicity assays (Alexander, 1998).

Results

Pathogenicity index tests

The wild-type viruses and their infectious clones utilized for this study are depicted in Fig. 1, and a summary of the pathogenicity test results is presented in Table 1. The indices for recombinant Beaudette C (rBC) and recombinant LaSota (rLaSo) were very similar to the wild-type viruses, wild-type Beaudette C (wtBC) and wild-type LaSota (wtLaSo), respectively, for the mean death time (MDT), the intracerebral pathogenicity index (ICPI), and the intravenous pathogenicity (IVPI) tests. A low virulent backbone with virulent F cleavage site (rLaSoVF) showed markedly increased pathogenicity indices compared to its backbone virus (rLaSo), and the pathogenicity indices were similar to that of rBC. The HN from a low virulent virus within a virulent backbone (rBC LaSoHN) had mildly decreased pathogenicity indices compared to rBC. The HN from a virulent virus within a low virulent backbone (rLaSo BCHN) had similar pathogenicity indices to rLaSo. Additional F cleavage site changes within rLaSo BCHN (rLaSoVF BCHN) markedly increased pathogenicity compared to rLaSo BCHN and rLaSo, and the virulence was similar to that of rLaSoVF and rBC. Deletion of glycosylation site 4 within the HN of rBC (rBCG4) moderately decreased pathogenicity indices compared to rBC. Both clones with P gene mutations (rBC/V-Stop and rBC/ Edit) had decreased pathogenicity compared to their parent virus (rBC), but their pathogenicity was higher than that of rLaSo.

The overt clinical signs observed in chickens following intracloacal inoculation were tremors and paralysis, typical of a neurotropic virus infection. Those with severe paralysis were euthanized and recorded as dead. No overt hemorrhagic lesions were observed in the euthanized birds following infection with this set of viruses.

Clinicopathologic assessment in chickens—clinical disease, gross pathology, histopathology, immunohistochemistry (IHC), in situ hybridization (ISH), virus isolation, and serology

Wild-type and their recombinant viruses

Morbidity, mortality, and viral distribution in tissues as detected by IHC and ISH in 4-week-old chickens are summarized in Table 2. Birds infected with wtBC exhibited the most severe overt disease of primarily nervous signs. Slight depression and head tremor were evident in one bird at 3 days post-inoculation (dpi). Whereas at 4 dpi all birds were depressed. All the infected chickens sampled after day 5 had severe nervous signs such as incoordination accompanied by leg paralysis and were euthanized by 10 dpi. In comparison, birds inoculated with rBC had a lower frequency of severe disease. All birds were slightly depressed by 4 dpi. Some birds infected with rBC had similar nervous signs to those seen in birds inoculated with wtBC. However, the number of birds euthanized because of severe disease was only 30%. Chickens infected with wtBC



Fig. 1. Wild-type Newcastle disease viruses and their infectious clones.

or rBC had severe histologic lesions including encephalitis, lymphoid depletion, airsacculitis, and pancreatic exocrine cell necrosis. Viral proteins and mRNAs were detected systemically, predominantly in the lymphoid tissues (Fig. 2A), respiratory tissues, heart, and brain (Fig. 2B). Viral distribution as determined by IHC and ISH, within the gastrointestinal tract was detected in birds with wtBC, but not with rBC. Recombinant LaSota (rLaSo) and its parent virus (wtLaSo) induced no overt clinical signs of disease, and there were no histologic lesions in tissues. Viral infection, as detected by IHC, was restricted to inoculation sites with rLaSo and wtLaSo viruses.

Fusion cleavage activation site mutations

Severe neurological clinical signs also occurred in chickens inoculated with rLaSoVF. Nervous signs such as leg paralysis and head twitching were first observed at 4 dpi. Birds showing nervous signs initially were relatively bright and had minimal depression, but the nervous signs increased in severity with time and 60% were euthanized. Principal histologic lesions were lymphoid depletion in all lymphoid tissues and non-suppurative encephalitis. Virus was distributed to multiple systemic sites including lymphoid tissues, respiratory tissues (Fig. 2C), gastrointestinal tract, pancreas (Fig. 2D), and brain, but no virus was detected in the heart.

Table 1 Pathogenicity index results and amino acid sequence at the fusion cleavage activation site of wild-type NDV strains and their infectious clones

Gene changes	Virus	MDT ^a	ICPI ^b	IVPI °	Intracloacal signs/dead (total) ^d	F sequence ^e
	wtBC	50	1.75	2.29	4/2 (5)	Virulent
	rBC	48	1.66	2.06	2/2 (5)	Virulent
	wtLaSo	>90	0.00	0.01	ND ^f	Low virulent
	rLaSo	>90	0.19	0.00	ND	Low virulent
F	rLaSoVF	59	1.69	2.39	4/4 (5)	Virulent
HN	rBC	60	1.58	1.27	0/0 (5)	Virulent
	LaSoHN					
	rBCG4	77	1.31	0.56	0/0 (5)	Virulent
	rLaSo	>90	0.00	0.00	3/0 (10)	Low
	BCHN					virulent
F & HN	rLaSoVF	49	1.69	2.20	4/3 (5)	Virulent
	BCHN					
Р	rBC/V-	63	1.54	1.07	0/0 (5)	Virulent
	Stop					
	rBC/Edit	80	1.19	0.79	1/0 (5)	Virulent

^a Mean death time in embryonating eggs (hours) (<60: velogen; 60–90: mesogen; >90: lentogen; Alexander, 1998).

^b Intracerebral pathogenicity index in day-old chicks (0.0–0.5: lentogen; 1.0–1.5: mesogen; 1.5–2.0: velogen; Alexander, 1998).

^c Intravenous pathogenicity index in 6-week-old chickens. (0.0: lentogen; 0.0–0.5: mesogen; 2.0–3.0: velogen; Alexander, 1998).

^d Intracloacal inoculation pathogenicity test in 6-week-old chickens; numbers of birds showing clinical signs/number dead (total number inoculated). The signs observed in chickens following intracloacal inoculation were tremors and paralysis typical of a neurotropic virus infection. Those with severe paralysis were euthanized and recorded as dead. No overt hemorrhagic lesions were observed.

^e Fusion cleavage site amino acid sequence; avirulent=low virulent fusion cleavage site sequence, ¹¹²G/E-K/R-Q-G/E-R-L¹¹⁷; virulent=virulent fusion cleavage site sequence, ¹¹²R/K-R-Q-K/R-R-F¹¹⁷.

^f ND: not done.

Hemagglutinin-neuraminidase gene chimeras and mutation

Chickens infected with either rBC LaSoHN or rBCG4 exhibited no signs of clinical disease. In comparison to the parent strain (rBC), rBC LaSoHN virus induced less severe histologic lesions with slightly decreased viral distribution. Moderate encephalitis (Fig. 2E) was detected in birds with rBC LaSoHN at 10 dpi, but no viral nucleoprotein/mRNA was present in the brain. No overt histologic lesions or viral nucleoprotein/mRNA were present in tissues from birds infected with rBCG4. Infections with rLaSo BCHN produced no overt clinical signs of disease and no significant histologic lesions. Viral distribution was restricted to the inoculation site. In contrast, birds infected with rLaSoVF BCHN had severe nervous signs such as leg paralysis and head tremor (at and after 5 dpi) with 60% mortality. Principal histologic lesions were pancreatic exocrine cell necrosis, encephalitis, and lymphoid depression predominantly in the thymus. Virus was distributed systemically, including lymphoid tissues (Fig. 2F), gastrointestinal tract, pancreas, respiratory tissues, heart (Fig. 2G), and the brain as detected by either or both IHC or ISH.

Phosphoprotein gene mutations

Both viruses with mutated P gene (rBC/V-Stop and rBC/ Edit) displayed reduced virulence in chickens compared to the parent virus (rBC). Although neither P gene mutation clone caused any apparent clinical disease in chickens, rBC/ V-Stop had slightly more histologic lesions than did rBC/ Edit in the brain. Birds with rBC/V-Stop showed mild to moderate encephalitis, whereas birds with rBC/Edit did not have encephalitis. Viral distribution of both clones detectable with IHC or ISH was restricted to inoculation sites (Fig. 2H).

Virus isolation from swabs and serology

Virus was isolated from both oral and cloacal swabs of birds infected with wtBC, rBC, rLaSoVF, rBC LaSoHN, rBCG4, rLaSoVF BCHN, and rBC/V-Stop and from only oral swabs of birds infected with wtLaSo, rLaSo, and rLaSo BCHN. No virus was isolated from either oral or cloacal swab of birds inoculated with rBC/Edit or from any of the phosphate buffer saline (PBS)inoculated birds.

The hemagglutination-inhibition (HI) titers of pre-infection and all PBS inoculated chickens were 2 or less. An HI titer of greater than 8 was considered positive. All blood samples collected at days 10 and 14 dpi of wtBC, rBC, rLaSo, rLaSoVF, rBC LaSoHN, rLaSo BCHN, and rLaSoVF BCHN were HI positive. Four of six birds sampled at 10 and 14 dpi from wtLaSo, rBCG4, and rBC/V-Stop groups had seroconverted, but only two of those sampled from the rBC/Edit group had seroconverted, one at day 10 and one at day 14.

Nucleotide and predicted amino acid sequence analysis

Sequence analysis performed on isolates from oral swabs at 5 dpi confirmed that wtBC, rBC, rLaSoVF, rBC LaSoHN, rBCG4, rLaSoVF BCHN, and rBC/V-Stop had the virulent F cleavage motif ¹¹²RRQKRF¹¹⁷ whereas rLaSo had the amino acid sequence ¹¹²GRQGRL¹¹⁷ typical of the low virulence F cleavage activation site. The HN gene of rBC LaSoHN was confirmed as the same as that of LaSota. It was confirmed that the HN gene of rLaSo BCHN and rBCG4 was the same as that of Beaudette C, and rBCG4 contained the deletion at glycosylation site 4.

Discussion

This study is the first to investigate the influence of NDV gene mutations or substitutions involving the F, P, and HN genes on the pathogenesis of disease in chickens inoculated via a natural route of infection. The virulence and pathogenesis of two NDV isolates, Beaudette C (virulent) and LaSota (low virulent), along with their infectious clones were compared to determine associations among the virus strain's pathogenicity indices along with their ability to disseminate and cause disease in chickens.

Each wild-type virus (wtBC and wtLaSo) and its infectious clone (rBC and rLaSo, respectively) had very similar

2	2	7
5	5	/

Table 2	
Clinical-pathologic results (morbidity, mortality, and viral distribution) for chickens inoculated with wild-type NDV strains and their infectious clones,	
14-day-observation	

	wtBC	rBC	wtLaSo	rLaSo	rLaSoVF	rBC LaSoHN	rBCG4	rLaSo BCHN	rLaSoVF BCHN	rBC/V-Stop	rBC/Edit
No. sick/dead (of 10) ^a	10/6	10/3	0/0	0/0	8/6	0/0	0/0	0/0	6/6	0/0	0/0
No. positive (of 21) ^b	21	10	0	2	10	7	0	1	12	3	2
Spleen	5 °	5			10				5		
Thymus	6	5			10	5					
Bursa	5	5			10				5		
H. gland ^d	6				4				5	2	5
Pancreas	6				8				8		
Proven ^e	6										
Intestine	6								8		
Air sac	5	5							5		
Trachea	5								5		
Lung	5				5				5		
Heart	5	5							5		
Esophagus	2										
Pharynx	2	5		14	5	2			5		
Crop	2				8	10					
Brain	5	14			5				8		
Liver	2	14									
Kidney	5										
Comb	5					10					
Marrow ^f	5					10					
Eyelid	5	10		5	2	5			5	2	
Turbinate	2	10				2		5		14	5

^a Numbers of birds with clinical signs/number with severe disease, typically severe paralysis, which were euthanized and recorded as dead. Total number of birds is 10.

^b Numbers of IHC- and/or ISH-positive tissues. Total number is 21.

^c Days post-inoculation when first positive cell by either IHC or ISH was detected.

^d Harderian gland.

^e Proventriculus.

f Bone marrow.

pathogenicity indices of high virulence. However, in the clinicopathologic assessment study, birds infected with rBC had lower mortality with less extensive viral distribution, in comparison to wtBC. Sequence analysis of viruses isolated from swabs confirmed that rBC and wtBC had identical amino acid sequences at the fusion cleavage site. However, nucleotide and amino acid differences between the rBC and the wtBC were reported previously (Krishnamurthy et al., 2000). These included a total of five nucleotide changes: T5464C, A7915G, C10167T, A10877C, and C12239T. Three of these nucleotide (nt) differences resulted in amino acid (aa) changes: nt 7915 in the HN gene, resulting in change of amino acid threonine to alanine (aa position 502); nt 10167 in the L gene, resulting in change of amino acid threonine to methionine (aa position 596); and nt 10877 in the L gene, resulting in change of amino acid methionine to leucine (aa position 833). Although rBC demonstrated decreased virulence in clinicopathologic assessment with 4-week-old chickens, it still induced systemic infection and had virulent pathogenicity indices. These differences between wtBC and rBC could be due to the previously reported changes in the HN and L genes.

In contrast, both wtLaSo and rLaSo were very similar to each other as both had low pathogenicity indices did not cause any overt disease in 4-week-old chickens, and viral distribution was restricted to the inoculation site.

Fusion gene

The pathogenicity indices of rLaSoVF and rLaSoVF BCHN were higher than those of rLaSo and rLaSo BCHN and similar to those of rBC. Chickens infected with rLaSoVF or rLaSoVF BCHN showed severe clinical signs of disease and tissue damage with systemic viral distributions similar to the disease seen from an rBC infection. In contrast, only mild disease was evident in birds infected with rLaSo or rLaSo BCHN. Therefore, it is evident that modifying the F cleavage site of a low virulent virus to the virulent motif resulted in an increase of the pathogenicity indices (Fig. 3A) and severity of clinicopathologic disease (Fig. 3B). These results indicate that the amino acid sequence at the fusion cleavage site played a major role in the virulence of these viruses as originally described among wild-type viruses (Glickman et al., 1988; Nagai et al., 1976).

Further evidence for the importance of the sequence of the fusion cleavage site in virus dissemination is the observation that chickens infected with wild-type or recombinant viruses which had a virulent fusion cleavage site, except for rBC/Edit, shed virus recoverable in both oral and cloacal swabs. In contrast, those infected with viruses that had the low virulence motif at that site shed virus recoverable only from oral swabs.

Hemagglutinin-neuraminidase gene

Chimeras created by rBC and rLaSo HN gene exchange among the rBC, rLaSo, and rLaSoVF backbones had variable

effects. Both HN proteins are comprised of 577 amino acids (GenBank accession nos. AAG30929 and AAC28376, respectively). The substitution of the LaSota HN to create rBC LaSoHN reduced the ICPI although the pathogenicity





Fig. 3. Effects of NDV LaSota F gene mutations on (A) ICPI scores (maximum 2.0) and (B) viral distribution in 4-week-old chickens (numbers of positive tissues by IHC or ISH; total 21 tissues). (a) An up direction of the arrow indicates a change that increased virulence. (b) Fusion protein cleavage site converted to the consensus sequence of virulent NDV strain.

remained virulent (Fig. 4A), but the disease severity was markedly decreased compared to rBC in the pathogenesis assessment. Chickens infected with rBC LaSoHN showed no overt clinical signs of disease, and virus was distributed in multiple tissues but in fewer tissues than in those birds infected with rBC (Fig. 4B). Interestingly, in birds infected with rBC LaSoHN, mild encephalitis was observed histologically, but no virus was detected in brain by either IHC or ISH. The opposite chimera, substitution of virulent HN into a low virulent virus to generate rLaSo BCHN, also caused decreases in pathogenicity and disease severity, but only slightly compared to the parent virus rLaSo (Figs. 4A and B). In contrast, addition of the virulent HN to rLaSoVF to generate rLaSoVF BCHN retained high pathogenicity and increased the severity of disease (Figs. 3A, B and Figs. 4A, B). These results suggest that both the virulent F cleavage motif and the HN of a virulent virus are required within either a low virulent or virulent backbone to maximize both virulence and disease severity of these infectious clones. The importance of the F and HN interaction has been reported



Fig. 4. Effects of NDV HN gene substitutions or mutations on (A) ICPI scores (maximum 2.0) and (B) viral distribution in 4-week-old chickens (numbers of positive tissues by IHC or ISH; total 21 tissues). (a) An up direction of the arrow indicates a change that increased virulence, a down direction indicates a decreased virulence, and an even arrow indicates no change in virulence. (b) HN gene from lentogenic strain (rLaSo). (c) HN gene with glycosylation site 4 mutation. (d) HN gene from velogenic strain (rBC).

previously, an interaction that is optimal when the F and HN originate with the same virus (Gravel and Morrison, 2003; Takimoto et al., 2002).

The loss of HN glycosylation site 4 within a virulent virus, rBCG4, moderately decreased pathogenicity compared to the parent virus, rBC (Fig. 4A). However, the virus induced no overt clinicopathologic disease in 4-week-old chickens, and viral nucleoprotein/mRNA was not detected in any tissues by either IHC or ISH (Fig. 4B). Similar reduced pathogenicity had been reported previously by Panda et al. (2004a) and an even greater reduction in virulence of this virus was evident in the natural route infections in the pathogenesis component of the present study. The previously observed slow replication with reduced neuraminidase activity and fusogenicity of the virus (Panda et al., 2004a) may have greater influence on a natural route infection than one initiated by systemic inoculation in the pathogenicity tests.

Fig. 2. Histopathology, immunohistochemistry (IHC) for viral nucleoprotein, and in situ hybridization (ISH) for viral mRNA on tissues from 4-week-old chickens infected with wild-type NDV strains and their infectious clones. Scale bar=30 µm. (A) Spleen; chicken infected 9 days previously with wtBC. Viral mRNA was detected in macrophages and lymphocytes. ISH, Mayer's hematoxylin counterstain. (B) Cerebellum; chicken infected 14 days previously with rBC. Viral antigen was detected in cells within molecular layer. IHC, Mayer's hematoxylin counterstain. (C) Air sac; chicken infected 5 days previously with rLaSoVF. Viral antigen was detected in epithelial cells. IHC, Mayer's hematoxylin counterstain. (D) Pancreas; chicken infected 5 days previously with rBC LaSoHN. Non-suppurative encephalitis with Purkinje cells degeneration. Hematoxylin and eosin stain. (F) Bursa; chicken infected 5 days previously with rBC LaSoHN. Viral mRNA was detected in macrophages and lymphocytes, mainly within interfollicular areas. ISH, Mayer's hematoxylin counterstain. (G) Heart; chicken infected 5 days previously with rBC LaSoHN. Viral mRNA was detected in macrophages and lymphocytes, mainly within interfollicular areas. ISH, Mayer's hematoxylin counterstain. (H) Harderian gland; chicken infected 2 days previously with rBC/V-Stop. Viral antigen was detected in glandular epithelial cells and infiltrating macrophages and lymphocytes. IHC, Mayer's hematoxylin counterstain.

Phosphoprotein gene (V and W proteins)

The pathogenicity indices of both clones with P gene mutations, rBC/V-Stop and rBC/Edit, were lower than those of rBC as parent virus (Fig. 5A) but much higher than those of rLaSo. However, the disease severity and viral distribution in tissues of both clones were markedly decreased compared to those of the parent virus (rBC) (Fig. 5B) and were similar to those of rLaSo. The pathogenesis results were due in part to the observation that rBC/Edit clone was found to be poorly infectious for chickens inoculated by the natural route. These results suggest that not only the V protein but also the possible W protein have a role in virus virulence and an even greater role in virus pathogenesis. The results from the natural route infections may be consistent with the role of the V protein as an interferon antagonist (Huang et al., 2003; Park et al., 2003b).

Our findings are summarized as follows: (i) the presence of a virulent F cleavage activation site within a low virulent backbone increased pathogenicity indices as well as severity of clinicopathologic disease and viral distribution in chickens; (ii) the presence of a low virulent HN within a virulent backbone mildly decreased viral pathogenicity and resulted in no clinical disease; (iii) the presence of a virulent HN within a low virulent backbone did not change either pathogenicity indices or severity of clinicopathologic disease in chickens; (iv) the loss of glycosylation site 4 from the HN within a virulent virus moderately decreased viral pathogenicity and resulted in no clinicopathologic disease; and (v) the modifying the P gene within a virulent virus caused mildly decreased viral pathogenicity indices and the viruses caused no clinical disease with markedly decreased viral distribution.



Fig. 5. Effects of NDV P gene mutations on (A) ICPI scores (maximum 2.0) and (B) viral distribution in 4-week-old chickens (numbers of positive tissues by IHC or ISH; total 21 tissues). (a) A down direction of the arrow indicates a change that decreased virulence. (b) Lack expression of V protein. (c) Lack expression of V and W proteins.

The data provide further evidence consistent with the hypothesis that the pathogenicity of NDV is multigenic (de Leeuw et al., 2005; Huang et al., 2004) and at least the F, HN, and P genes influence viral pathogenicity as well as clinicopathological disease in chickens. Although several of the infectious clones attained virulence similar to wtBC, none of them resulted in the extensive virus distribution in tissues similar to that parent virus.

Materials and methods

Viruses

Two reference strains of NDV, a virulent strain Beaudette C and a low virulent strain LaSota, their recombinants, and recombinants with mutations or chimeras were utilized in this study. Recombinant viruses of Beaudette C (rBC) and LaSota (rLaSo) were constructed as described previously by Krishnamurthy et al. (2000) and by Huang et al. (2001), respectively. Recombinant LaSota virulent F (rLaSoVF) had genetically modified fusion cleavage site from ¹¹²GRQGRL¹¹⁷ to ¹¹²RRQKRF¹¹⁷, a typical virulent motif, the same as that of Beaudette C. Hemagglutinin-neuraminidase (HN) gene chimeras, rBC with HN gene from rLaSota (rBC LaSoHN) and rLaSota with HN gene from rBC (rLaSo BCHN), were constructed as previously described by Huang et al. (2004). In addition, the fusion cleavage site of rLaSo BCHN was modified from avirulent to virulent motif ¹¹²RROKRF¹¹⁷ (rLaSoVF BCHN). Recombinant BC with a mutation of the Nlinked glycosylation site 4, at residue 481 (rBCG4), resulting in elimination of the site for glycosylation, was constructed as previously described by Panda et al. (2004a). Two infectious clones with P gene mutations that lacked expression of the V protein (rBC/V-Stop) or both the V and possible W proteins (rBC/Edit) were constructed as described previously by Huang et al. (2003). For comparison to the infectious clones, wild types of Beaudette C (wtBC) and LaSota (wtLaSo) were also utilized. Viruses were propagated in embryonating eggs at the Southeast Poultry Research Laboratory (SEPRL), and the amnioallantoic fluid harvested from those infected eggs was used as inoculum after proper dilution.

Eggs and chickens

The source of embryonating chicken eggs and chickens was the SEPRL SPF White Leghorn flock (King, 1993). Birds were housed in negative-pressure isolators under BSL-3 agriculture conditions at SEPRL and provided feed and water *ad libitum*. Embryonating eggs were used for viral propagation, isolation, titration, and the MDT test. Chickens were utilized for the ICPI, IVPI, and intracloacal inoculation tests as well as in the clinicopathologic assessment experiment.

Pathogenicity index tests

To characterize the virus, four standard pathogenicity assays were performed (Alexander, 1998). These included the MDT in

9- to 10-day-old embryonating chicken eggs. Chickens were inoculated for the ICPI at one-day of age and the IVPI and intracloacal inoculation at 6 weeks of age (Alexander, 1998).

Clinicopathologic assessment in chickens

Each of eleven groups of ten 4-week-old chickens was inoculated via instillation bilaterally in the conjunctival sac with a total of 0.1 ml of one of the viruses or PBS as a non-infected control: wild-type viruses (wtBC and wtLaSo), their clones (rBC and rLaSo), rLaSo with a virulent F motif (rLaSoVF), their HN chimeras (rLaSo BCHN and rBC LaSoHN), rLaSo BCHN with virulent F motif (rLaSoVF BCHN), rBC with G4 deletion of HN (rBCG4), or rBC with P gene modifications (rBC/V-Stop and rBC/Edit). Each bird received approximately $10^{5.0}$ 50% embryo infectious doses (EID₅₀) of viral inoculum based on a titration in embryonated eggs to confirm the administered dose.

The birds were monitored clinically every day, and two birds of each group were euthanized after collecting oral and cloacal swabs at 2, 5, 10, and 14 dpi. Earlier sampling than scheduled occurred in birds that were euthanized when they exhibited severe clinical signs. Tissues (eyelid, spleen, thymus, bursa, Harderian gland, proventriculus, small intestine, cecal tonsils, large intestine, air sac, trachea, lung, heart, esophagus, pharynx, crop, brain, liver, pancreas, kidney, comb, head of left femur including bone marrow, and turbinate) were collected and fixed by immersion in 10% neutral buffered formalin for approximately 52 h. Blood samples for serology were also collected at 10 and 14 dpi. The sections of femur and turbinates were decalcified in 5% formic acid for 3-4 h. All sampled tissues were routinely processed into paraffin, and 3-µm sections were sectioned for hematoxylin and eosin staining, immunohistochemistry, and in situ hybridization.

Immunohistochemistry (IHC)

All sampled tissues were examined by IHC to detect viral nucleoprotein. Briefly, the protocol was as follows. After deparaffinization, tissue sections were subjected to antigen retrieval by microwaving for 10 min at full power in Vector antigen unmasking solution (Vector Laboratories, Burlingame, CA) followed by blocking with universal blocking reagent (Biogenex, San Ramon, CA) as recommended by the manufacturer. The primary antibody, made in rabbits, was anti-peptide (nucleoprotein), used at a 1:8000 dilution (Kommers et al., 2001). Detection system was an avidin–biotin–alkaline phosphatase (Vector Laboratories) with a Vector Red substrate (Vector Laboratories). Sections were counterstained lightly with hematoxylin and coverslipped for a permanent record.

In situ hybridization (ISH)

All sections of eyelid, spleen, thymus, bursa, and Harderian gland, as well as other selected tissue sections, were probed with a negative sense digoxigenin-labeled 850-base riboprobe representing the 5' end of the matrix gene of B1 as described previously (Brown et al., 1999; Kommers et al., 2001, 2002, 2003). Briefly, tissues sections were deparaffinized, rehydrated, and digested with 30 μ g/ml proteinase K for 15 min at 37 °C. Hybridization was conducted overnight at 42 °C with approximately 20 ng of probe in the prehybridization solution. After stringent washes, anti-digoxigenin alkaline phosphate was added to the sections. The development was with chromogen/substrate nitroblue tetrazolium (NBT)/5-bromo, 4-chloro, 3-indolylphosphate (BCIP). Tissues were counterstained lightly with hematoxylin and coverslipped with Permount.

Virus isolation from swabs

For those chickens used in the clinicopathologic assessment experiment, immediately prior to euthanasia, oral and cloacal swabs were obtained from each bird. Swabs were collected at 2, 5, 10, and 14 dpi, except birds inoculated with wtBC (2, 5, 6, 9, and 10 dpi), with rLaSoVF (2, 4, 5, 8, 9, 10, and 14 dpi), and with rLaSoVF BCHN (2, 4, 5, 8, 10, and 14 dpi). Each swab was placed in a tube containing 1.5 ml of brain-heat infusion broth (BHI) with antibiotics (2000 U/ml penicillin G, 200 µg/ml gentamicin sulfate, and 4 µg/ml amphotericin B; Sigma Chemical Co., St. Louis, MO) (Alexander, 1998; King, 1993). The swab tubes were centrifuged at $1000 \times g$ for 20 min and supernatant fluids were removed for inoculation of 9- to 10-dayold SPF embryonating chicken eggs. Newcastle disease virusinfected dead or surviving embryos were identified by hemagglutination (HA) activity in amnioallantoic fluid harvested from chilled eggs. Newcastle disease virus was confirmed in HA-positive samples by a hemagglutinationinhibition (HI) test with NDV-specific antiserum (Alexander, 1998).

Serology

The HA and HI assays were conducted by conventional microtiter methods (King, 1993) with serum separated from the blood samples taken at 10 and 14 dpi. An exception to that sampling schedule involved birds inoculated with wtBC which were collected at 9 and 10 dpi due to the early severe disease onset and subsequent euthanasia. Four HA units of beta propiolactone (BPL) inactivated NDV LaSota was used as test antigen in completing the HI tests.

Nucleotide and predicted amino acid sequence analysis

Newcastle disease virus was isolated from oral swabs taken at 5 dpi. The isolates were replicated in embryonating eggs, and RNA was extracted directly from amnioallantoic fluid. Oligonucleotide reverse transcription polymerase chain reaction (RT-PCR) primers were designed to amplify regions of the fusion protein gene, including the fusion protein cleavage site and the matrix protein gene region encoding the nuclear localization signal of the matrix protein (Seal et al., 1995). Primers amplifying the HN gene were also utilized (Panda et al.,

2004a; Sakaguchi et al., 1989). A single tube RT-PCR for genomic NDV RNA was completed with Superscript[™] (Life Technologies, Gaithersburg, MD) and Amplitag[™] (PE Biosystems, Foster City, CA) polymerase. Amplification products were separated by gel electrophoresis in 1.0% agarose with Tris-borate buffer, and stained with ethidium bromide. Amplification products were purified with Microcon[™] (Amicon, Belford, MA) spin filters and spectrophotometrically quantified. Additionally, amplification products were cloned with the TA cloning systemTM according to the methods described by the manufacturer (Invitrogen, San Diego, CA). Direct double-stranded nucleotide sequencing was completed with Taq polymerase (Applied Biosystems, Inc., Foster City, CA) with the oligonucleotide primers used for RT-PCR, fluorescent-labeled dideoxynucleotides, and an automated nucleic acid sequencer (Seal et al., 1995).

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