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Role of fusion protein cleavage site in the virulence of Newcastle disease virus

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Abstract

Newcastle disease virus (NDV) causes a highly contagious and economically important disease in poultry. Viral determinants of NDV virulence are not completely understood. The amino acid sequence at the protease cleavage site of the fusion (F) protein has been postulated as a major determinant of NDV virulence. In this study, we have examined the role of F protein cleavage site sequence in NDV virulence using reverse genetics technology. The sequence G-R-Q-G-R present at the cleavage site of the F protein of avirulent strain LaSota was mutated to R-R-Q-K-R, which is present in the F cleavage site of neurovirulent strain Beaudette C (BC). The resultant mutated LaSota V.F. virus did not require exogenous protease for infectivity in cell culture, indicating that the F protein was cleaved by intracellular proteases. The virulence of the mutant and parental viruses was evaluated in vivo by intracerebral pathogenicity index (ICPI) and intravenous pathogenicity index (IVPI) tests in chickens. Our results showed that the modification of the F protein cleavage site resulted in a dramatic increase in virulence from an ICPI value of 0.00 for LaSota to a value of 1.12 for LaSota V.F. However, the ICPI value of LaSota V.F. was lower than that of BC, which had a value of 1.58. Interestingly, the IVPI tests showed values of 0.00 for both LaSota and LaSota V.F. viruses, compared to the IVPI value of 1.45 of BC. In vitro characteristics of the viruses were also studied. Our results demonstrate that the efficiency of cleavage of the F protein plays an important role if the NDV is delivered directly into the brains of chicks, but there could be other viral factors that probably affect peripheral replication, viremia, or entry into the central nervous system.
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Keywords: Newcastle disease virus; Fusion protein cleavage site; Reverse genetics

1. Introduction

Newcastle disease virus (NDV) is a member of the family *Paramyxoviridae*, and has been assigned to the genus *Avulavirus* in the subfamily *Paramyxovirinae* [1]. It causes a serious respiratory and neurological disease in all species of birds and is an economically important infectious agent, causing substantial losses to the poultry industry. Newcastle disease varies in the degree of severity, ranging from an inapparent infection to severe disease causing 100% mortality. NDV strains cause a continuous spectrum of disease and can be categorized into three main pathotypes depending on the severity of disease produced in chickens. Strains of low virulence, known as the lentogenic strains, cause mild or inapparent disease in birds. Mesogenic strains are viruses with intermediate virulence, which cause respiratory and/or neurological signs. Highly virulent NDV strains causing high mortality in birds are called velogenic. Velogenic strains can be further sub-divided into two categories: viscerotropic velogenic strains that produce lethal infections in which hemorrhagic lesions are prominent; and neurotropic velogenic strains that produce high mortality preceded by neurological signs, with the absence of hemorrhagic lesions [2].

The genome of NDV is a single stranded negative-sense RNA consisting of 15,186 nucleotides [3]. The genomic RNA contains six genes that encode at least seven proteins [4,5]. The envelope of NDV contains two glycoproteins, the hemagglutinin-neuraminidase (HN) and fusion (F) proteins. The HN glycoprotein is involved in attachment and release of virus and the F glycoprotein mediates fusion of the viral envelope with cellular membranes [6]. Mutational analysis has shown that the fusion peptide and the adjacent heptad repeat region play a role in the fusion activity of F protein [7]. This protein is synthesized as a precursor, F_0, which

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must be proteolytically cleaved to F_1 and F_2 to activate fusion [6,8]. Cleavage of the F protein is known to be required for initiation of infection and is considered to be a major determinant of NDV virulence. Cleavage of the F protein of virulent NDV strains occurs by ubiquitous subtilisin-like proteases such as furin, PC6 and PACE 4; whereas, cleavage in avirulent strains occurs by trypsin-like enzymes found in limited tissues [9].

The amino acid sequence at the F protein cleavage site is different among most lentogenic, mesogenic and velogenic NDV strains [10]. The F protein of all lentogenic strains has a monobasic cleavage site, which is cleaved by extracellular proteases restricted to specific tissues; whereas, the F protein of all mesogenic and velogenic NDV strains has a multibasic cleavage site, which is cleaved by ubiquitous intracellular proteases [11]. Since the F protein cleavage efficiency has long been considered to be a major determinant of NDV virulence, we examined its role in neurovirulence.

Using reverse genetics techniques, we altered the F protein cleavage site of lentogenic and avirulent NDV strain LaSota to be identical to the F protein cleavage site of mesogenic and neurovirulent NDV strain Beaudette C (BC). The resultant mutated LaSota virus (LaSota V.F.) did not require exogenous trypsin for infectivity in cell culture, indicating that the F protein was cleaved by intracellular proteases. Intracerebral inoculation of this mutated recombinant virus to 1-day-old chicks showed some gain in neurovirulence; however, intranasal inoculation into three and 6-week-old chickens did not cause any neurological disease. These observations suggested that the cleavage efficiency of the F protein might contribute to neurovirulence if the virus is delivered artificially into the brain, but determinants in other regions of the NDV genome may be required for neurovirulence via natural route of infection.

2. Results

2.1. Recovery of recombinant LaSota virus with mutated F cleavage site

Recombinant LaSota V.F. (rLaSota V.F.) virus was recovered entirely from a cDNA clone of rLaSota using reverse genetics procedures [12]. Sequencing of the RT-PCR product containing the F cleavage site of rLaSota V.F. confirmed the presence of the desired mutations. Thus, the sequence G-R-Q-G-R present at the F cleavage site of avirulent NDV strain LaSota was altered to R-R-Q-K-R, to be analogous to the F cleavage site of neurovirulent strain BC (Fig. 1). It was also found that rLaSota V.F. did not require exogenous protease for propagation in cell culture, indicating that the F protein was cleaved by intracellular proteases.

2.2. Analysis of virus growth in vitro and in vivo

The kinetics and the magnitude of replication of rBC, rLaSota and rLaSota V.F. were very similar in DF1 cells (Fig. 2A). The plaque sizes and morphologies of all three viruses were also very similar (Fig. 2B). The replication of rBC, rLaSota, and rLaSota V.F. viruses in the brains of 1-day-old chicks after intracerebral inoculation was measured (Fig. 3). The rBC and rLaSota V.F. viruses initiated new virus formation after one day of infection. However, the yields of rBC virus were 100-fold higher than those of rLaSota V.F. virus. By day 4, all birds inoculated with rBC were dead. The replication kinetics of rLaSota virus was delayed and the virus yields were lower than those of rBC and rLaSota V.F. viruses. These results suggested that intracellular cleavage of the F protein is important for replication of NDV in the brains of chicks, but, probably, other viral factors are necessary for efficient replication.

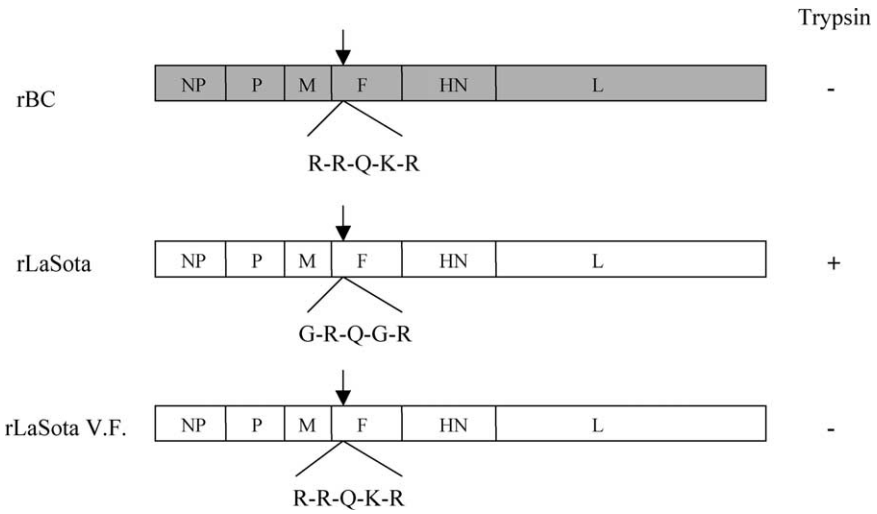

Fig. 1. Genomes of rBC, rLaSota and rLaSota V.F. viruses are shown schematically. Amino acid sequence at the F protein cleavage site of rLaSota was modified by mutagenesis to that of rBC to obtain rLaSota V.F. virus. The trypsin requirement for in vitro propagation shown on the side.

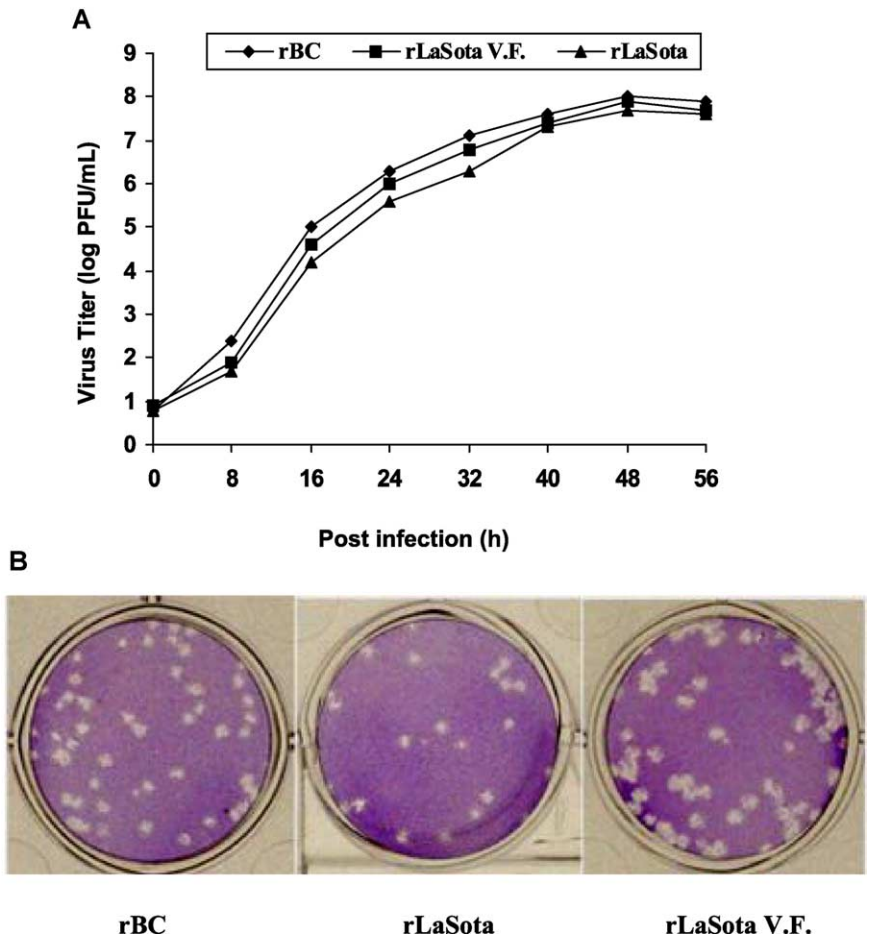

Fig. 2. (A) Multistep growth curve of rBC, rLaSota and rLaSota V.F. viruses in DF1 cells. Cell monolayers in 25 cm^2 flask were infected with 0.05 PFU/cell with three replicate flasks per virus. Supernatant samples were taken every 8 h for 56 h. The virus in the supernatant was titrated by plaque assay. The medium of cells infected with rLaSota contained 1 μg/ml of acetyl-trypsin. (B) Plaque size and morphology of rBC, rLaSota and rLaSota V.F. The viruses were serially diluted and 100 μl of each serial dilution was added per well to confluent DF1 cells in 12-well plates. Duplicate wells were used for each serial dilution of every virus sample. After 60 min adsorption, cells were overlaid with DMEM (containing 2% fetal bovine serum and 0.9% methyl cellulose) and incubated at 37 °C for 3–4 days. The cells were then fixed with ethanol and stained with crystal violet for observation of plaques.

The genetic stability of the mutations introduced into the genome of LaSota V.F. virus was determined by sequence analysis of the RT-PCR fragment that covered the region of the F protein cleavage site of the mutant virus that was passed five times in 9-day-old chicken embryos, and also from the mutant virus that was passaged once in chicken brains. All the introduced mutations were found to be stable.

2.3. Effect of temperature on the growth and biological activities of recombinant viruses

To examine the role of growth temperature in replication and biological activities of rBC and rLaSota V.F. viruses, the viruses were grown in primary chicken neuronal cells at two different temperatures. It was found that the rBC virus grew faster and induced a more rapid cytopathic effect at 41.5 °C (body temperature of chickens) than at 37 °C (Fig. 4A). To prove that the growth temperature does play a role in replication kinetics, we tested the growth of

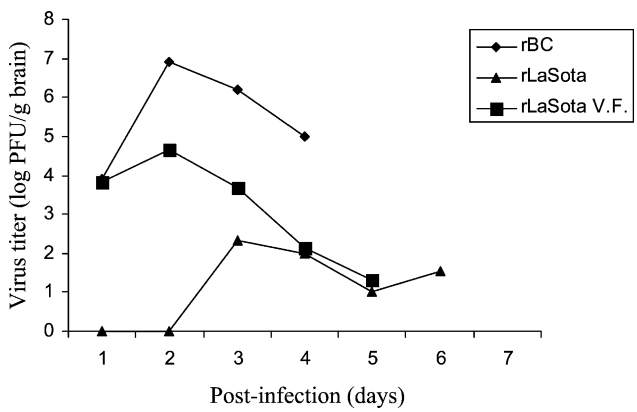

Fig. 3. Growth rate of NDV strains rBC, rLaSota, and LaSota V.F. in the brain of 1-day-old chicks inoculated intracerebrally with 10^3 PFU of virus. Brains from live birds were collected daily, homogenized and plaque assayed for virus content.

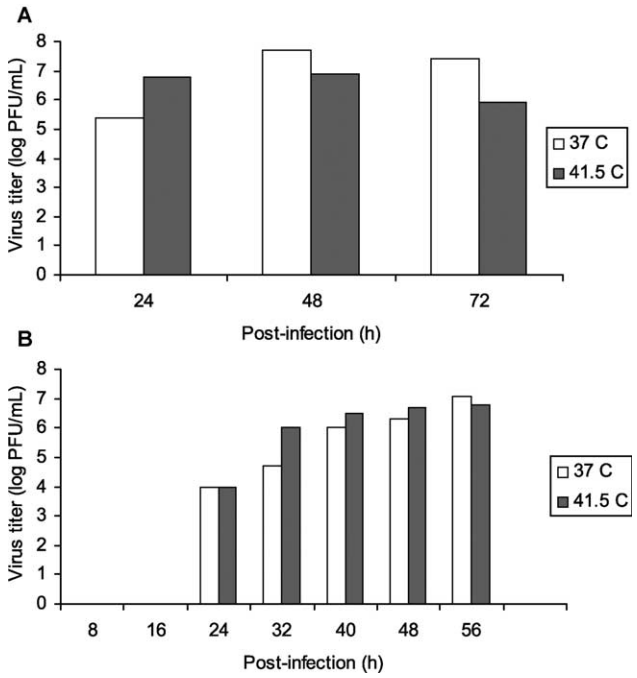

Fig. 4. (A) Multi-step growth curve of rBC in chicken neuronal cells at 37 °C and 41.5 °C. The virus was inoculated into chicken neuronal cells at a m.o.i. of 0.05 and maintained at 37 and 41.5 °C. The virus titers at 24, 48, and 72 h were determined by a plaque assay on DF 1 cells. (B) Multi-step growth curve of rBC in Vero cells at 37 and 41.5 °C. The viruses were inoculated into Vero cells at a m.o.i. of 0.05 and maintained at 37 and 41.5 °C. Supernatant was collected at 8 h interval until 56 h P.I. The virus titers were determined by a plaque assay on DF 1 cells.

neurovirulent rBC virus by a multi-step growth curve in Vero cells at 37 and 41.5 °C. The results showed that the rBC virus replicated to higher titers at 41.5 °C than at 37 °C (Fig. 4B). The rBC virus also produced more cytopathic effects in chicken neuronal cells at 41.5 °C than at 37 °C, as evidenced by the destruction of the cell monolayers (Fig. 5A). Our results showed that chicken neuronal cells are more susceptible to rBC than Vero cells. At 41.5 °C, rBC in chicken neuronal cells grew to higher titers at 24 h post-infection. At 48 h and 56 h post-infection the rBC titer in chicken neuronal cells was lower at 41.5 °C, because most of the cells were destroyed at this temperature and at these time points. Hemadsorption (HAd) studies revealed higher HAd of rBC and rLaSota V.F. viruses at 41.5 °C than at 37 °C. HAd activity of rBC at 41.5 °C was 128% when compared to its activity at 37 °C (100% HAd activity), while rLaSota V.F. had a HAd activity of 14% at 37 °C and 70% at 41.5 °C. Virus-binding studies with purified biotinylated virus in a streptavidin-colloidal gold amplification system indicated that the binding of chicken neuronal cells by rBC was significantly higher than rLaSota V.F. (Fig. 5B).

2.4. Pathogenicity studies

The virulence of rBC, rLaSota, and rLaSota V.F. viruses was evaluated by intracerebral pathogenicity index (ICPI) tests in 1-day-old chicks, and by intravenous pathogenicity index (IVPI) tests in 6-week-old chickens (Table 1). The ICPI results showed that the modification of the F protein cleavage site resulted in a dramatic increase in virulence from ICPI value of 0.00 for rLaSota to ICPI value of 1.12 (out of a maximum possible value of 2.0) for rLaSota V.F. However, the virulence of rLaSota V.F. was lower than that of rBC virus (ICPI value of 1.58). Surprisingly, the results obtained by IVPI tests showed a different pattern. The rBC virus had an IVPI value of 1.45 (out of a maximum possible value of 3.00), but the IVPI values of rLaSota V.F. and rLaSota viruses were 0.00. These results demonstrated that the efficiency of cleavage of the F protein plays an important

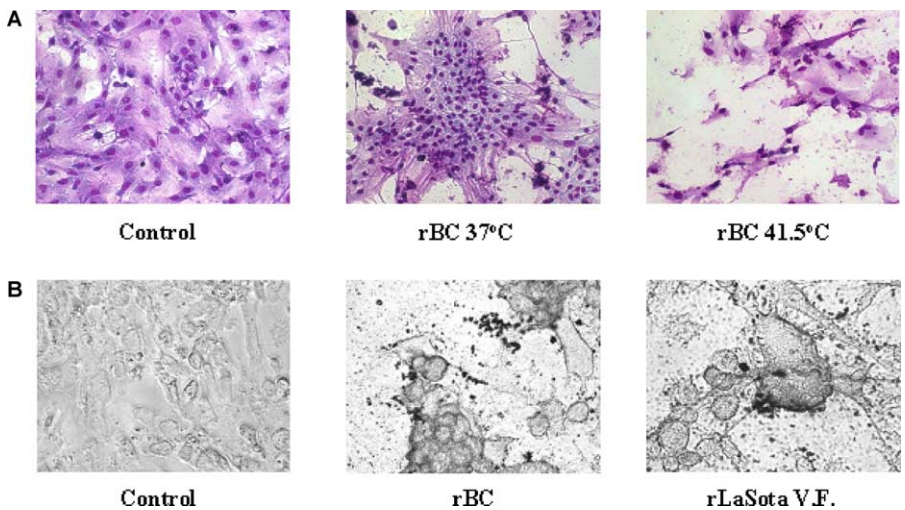

Fig. 5. (A) Chicken neuronal cells were prepared from 10-day-old embryonated chicken eggs. Ara C was added in the medium to inhibit growth of non-neuronal cells. The identity of the neuronal cells was confirmed by immunostaining with an antibody to human neurofilament 200 kDa (Research Diagnostic, Inc., NJ). Chicken neuronal cells were inoculated with 0.01 m.o.i. of virus. At 72 h post-infection, the cells were fixed and stained with Hematoxylin-eosin. Magnification × 20. (B) Binding of purified biotinylated virus to a monolayer of chicken neuronal cells at 4 °C was examined with streptavidin-colloidal gold. The specificity of binding was determined by using a competition assay for binding sites with unlabelled NDV. Magnification × 20.

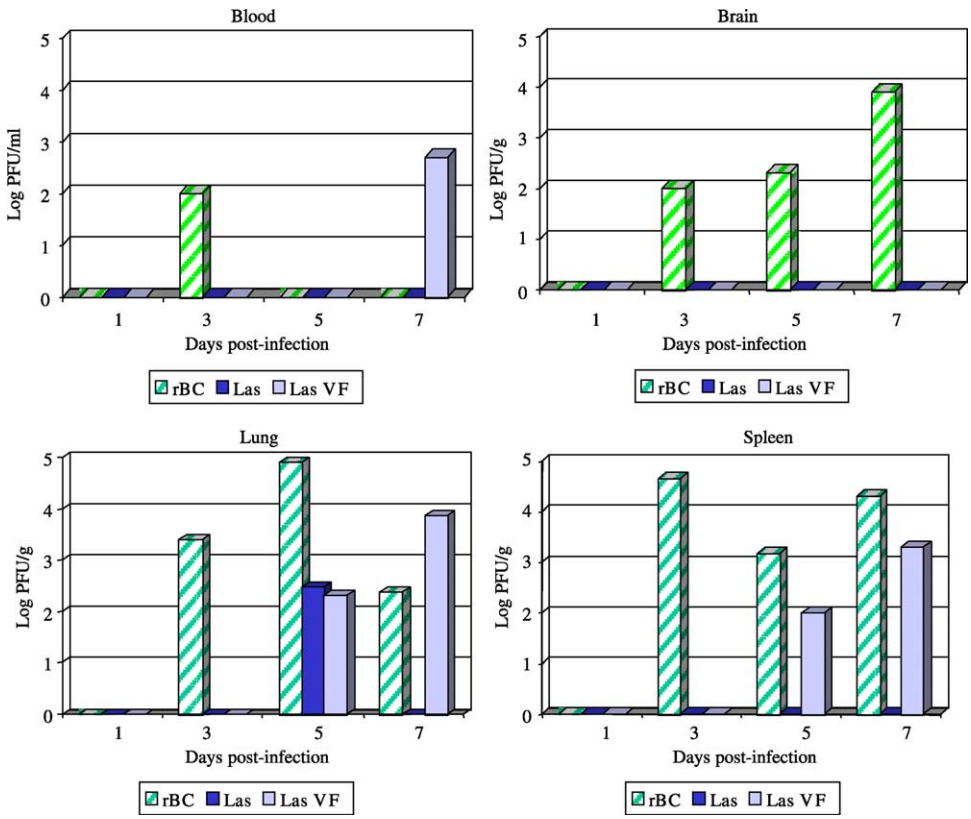

Fig. 6. Virus titers (PFU/g tissue at PFU/mL blood) in representative organs. Three-week-old chickens were infected intranasally with rBC, rLaSota or rLaSota V.F. virus (10^3PFU/chicken). Chickens were sacrificed at 1, 3, 5 and 7 days post-infection, tissues were removed, homogenized and titrated by plaque assay.

role if the NDV is delivered directly into the brains of chicks, but there could be other viral factors that probably affect peripheral replication, viremia, or entry into the central nervous system. The efficiency of attachment may, in part, determine the virulence, as indicated by our virus binding studies. To compare the pathogenesis of rBC, rLaSota and rLaSota V.F. viruses, 3-week-old chickens were infected intranasally with 10^3 plaque forming units (PFU) of virus. The virus distribution in different tissues of the inoculated birds are presented in Fig. 6. The rBC virus first appeared by day 3 in the blood, lungs, spleen and brain, and by day 5, the virus was undetectable in the blood. In the lungs, rBC grew to higher titers (500-fold) on day 5, but the titer was significantly reduced on day 7. It is possible that the humoral antibody IgA, which appears on day 5 post-infection and plays a major role in virus clearance in the respiratory tract, may be responsible for the decrease in virus titer seen on day 7. The rLaSota V.F. virus appeared by day 5 in the lungs and spleen, and in the blood by day 7, but was never detected in the brain. The rLaSota virus was detected only in the lungs by day 5. Sections of brain were also evaluated for the presence of viral RNA by in situ hybridization, using an antisense NP-specific ^{35}S-labeled riboprobe (Fig. 7A). We found that only rBC virus exhibited focal areas of viral infection in the brain by days 5 and 7. These areas were mostly found in the cerebellum. Immunohistochemical studies using a cocktail of monoclonal antibodies to the HN protein corroborated the results of hybridization (Fig. 7B). These results indicated that viral factors other than the cleavage efficiency of the F protein may determine the neurovirulence of NDV isolates, and that entry into the brain is an important factor.

3. Discussion

Viral determinants of NDV neurovirulence are not completely understood. The amino acid sequence at the F protein cleavage site has been postulated as a major determinant of NDV virulence [10]. Fewer basic amino acids are present in the F protein cleavage site of lentogenic NDV strains than in the proteins of either mesogenic or velogenic NDV strains, which have a similar multi-basic cleavage site sequence [11,13]. Recently, using reverse genetics techniques, others have also observed that changing the F protein cleavage site of an avirulent NDV strain to the consensus F protein cleavage site of virulent strains only slightly increased the virulence [14]. These results indicated that cleavage efficiency is not the sole determinant governing NDV neurovirulence. The idea that ‘cleavability’ of the viral penetration protein is not necessarily the major determinant of virulence is illustrated by the recent

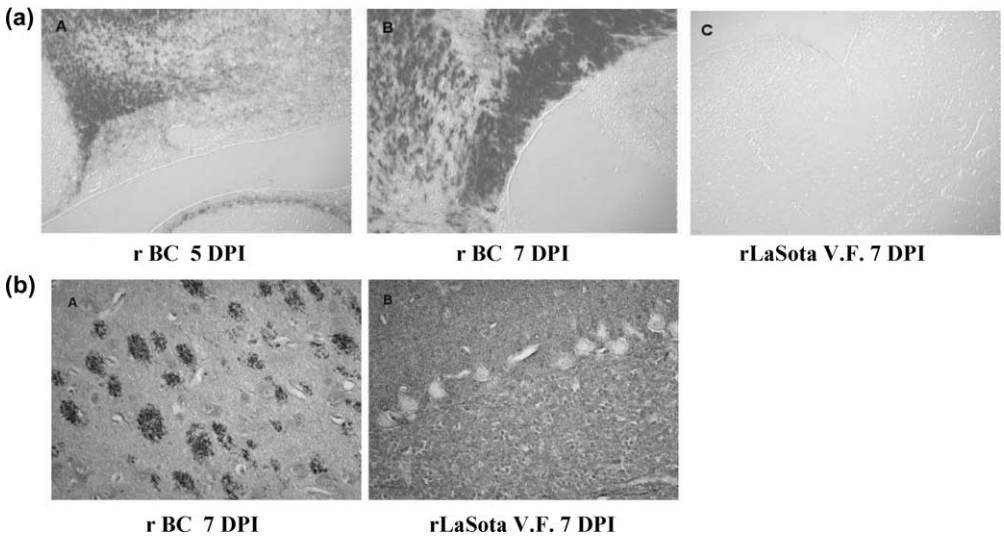

Fig. 7. (a) In situ hybridization to localize the sites of infection by rBC, rLaSota or rLaSota V.F. virus. Three-week-old chickens were infected intranasally with 10^3 PFU of virus. At 1, 3, 5 and 7 days post-infection, chickens were sacrificed and perfused with 4% paraformaldehyde. Brains were divided sagitally down the midline, embedded in paraffin, and sectioned at 5 μm/section. Sections were subjected to in situ hybridization with ^{35}S-labeled riboprobe specific for NP gene of NDV or P gene of bovine RSV. No specific signals were observed with either mock-infected chickens or with bovine RSV probe. Shown are representative sections of the cerebellum infected with rBC at 5 days post-infection (D P.I.) (A), and 7 D P.I. (B), and of cerebellum infected with LaSota V.F. virus at 7 D P.I. (C). (b) Immunohistochemistry of the brains of chicks infected with rBC, rLaSota or rLaSota V.F. virus. Three-week-old chickens were infected intranasally with 10^3 PFU of virus. At 1, 3, 5 and 7 days post-infection, chickens were sacrificed and perfused with 4% paraformaldehyde. Brains were divided sagitally down the midline, embedded in paraffin, and sectioned at 5 μm/section. Presence of NDV antigens was detected with a cocktail of monoclonal antibodies to NDV HN protein. (A) rBC virus infected brain shows plaque-like areas of viral antigen staining in the cerebellum at 7 D P.I. (B) rLaSota V.F. virus-infected brain shows no viral antigen in the cerebellum at 7 D P.I.

sequence analysis of the 1918 influenza pandemic virus, which showed that this highly virulent virus did not have a 'highly cleavable' HA protein and, hence, the determinants of virulence lay elsewhere and remain to be identified [15].

Using reverse genetics techniques, we altered the F protein cleavage site of lentogenic and avirulent NDV strain LaSota to be identical to the F protein cleavage site of mesogenic and neurovirulent NDV strain BC. The resultant mutated LaSota virus (rLaSota V.F.) did not require exogenous trypsin for infectivity in cell culture, indicating that the F protein was cleaved by intracellular proteases. In vitro growth kinetics of the mutant virus was very similar to that of rBC virus. These results demonstrated that the alteration of the F protein cleavage site of the rLaSota virus to that of the rBC virus resulted in a virus with growth characteristics that were similar to those of rBC in cell culture. The in vitro growth characteristics provided us with valuable insights into the pathogenesis and growth of the virus in vivo.

The in vivo growth kinetics of the mutant virus was intermediate between rBC and rLaSota. These results indicate that there are viral factors other than the F protein cleavage site that play a role in the growth and spread of the virus in vivo. Since rLaSota V.F. has the same F protein cleavage site as rBC, we speculate that other viral proteins, such as the hemagglutinin-neuraminidase (HN) protein of NDV, may play a role in entry of the virus into neuronal cells. It is also possible that the difference in growth seen in the cases of rLaSota V.F. and rBC in vivo, could be due to the difference in the antagonistic function of these two viruses to host factors like interferons.

Intracerebral inoculation of this mutated recombinant virus into 1-day-old chicks showed some gain in neurovirulence; however, intranasal inoculation into 3-week-old chickens did not cause any neurological disease. These observations suggested that the cleavage efficiency of the F protein may contribute to neurovirulence if the virus is delivered artificially into the brain, but determinants in other regions of the NDV genome are required for neurovirulence via natural route of infection.

In this study, we have shown that altering the F protein cleavage site alone of an avirulent strain to that of a neurovirulent strain of NDV did not convert the avirulent strain into a neurovirulent strain after a natural route of infection; however, multibasic amino acids at the F cleavage site offered a relative advantage when the virus was artificially delivered to the brain. Although the biological activities of the fusion protein and growth characteristics of the virus in vivo improved over the avirulent parental strain, the complete spectrum of virulence phenotype could not be achieved by modifying the F cleavage site. Our results demonstrate that the efficiency of cleavage of the F protein plays an important role if the NDV is delivered directly into the brains of chicks, but there are other viral factors that probably affect peripheral replication, viremia, or entry into the CNS.

There have been several reports of proteins, such as the HN, V, W and C proteins of NDV and other paramyxoviruses, being responsible for virus virulence [16–18]. Many studies showing the requirement of the HN protein in fusion along with the F protein suggest the importance of interaction of these two viral proteins in influencing viral infectivity [19]. The role of HN in affecting virus pathotype is seen in lentogenic and avirulent NDV strains, like Ulster, which have a longer reading frame consisting of 616 amino acids, as opposed to the neurovirulent, mesogenic strain BC, which has a shorter reading frame of 577 amino acids in the HN protein [10]. The hemagglutinin protein of measles virus has molecular determinants important for neurovirulence of the virus [20]. Therefore, it is possible that the HN protein might also contribute to the virulence of NDV.

Another protein influencing viral virulence is the V protein of NDV, which suppresses interferon-alpha/beta activity in host cells, thus helping in virus survivability and spread [18]. Therefore, the V protein may also be an important determinant of NDV virulence. In addition to the protein-coding genes, the *cis*-acting sequence element of negative-stranded RNA viruses can also alter the virulence, most probably by affecting the genome transcription and replication. For instance, mutations in the leader region of human parainfluenza virus 3 (hPIV3) vaccine candidates have been shown to specify attenuation phenotype [21]. Besides the envelope protein genes affecting neurovirulence, there have been reports of several viruses, like the human parainfluenza virus [22], polio virus [23], measles virus [24], corona virus [25], bunya virus [26] and Sindbis virus [27], in which alterations in the internal protein genes affect virus tropism and virulence.

Other factors, like apoptosis, or programmed cell death, may also play a role in viral pathogenesis [28]. Apoptosis may facilitate the spread of virus progeny to the neighboring cells and minimize the inflammatory response evoked by the host, thereby facilitating virus pathogenesis. Differential induction of apoptosis by virus stains of differing virulence has been observed in influenza virus [29] and Sindbis virus [30]. Our preliminary studies show that NDV can induce apoptosis in chicken embryo fibroblast cells, and there are significant differences in the extent of apoptosis-inducing ability between neurovirulent Beaudette C and avirulent LaSota V.F. viruses (data not shown). The highly neurovirulent NDV strain G.B. Texas has been found to induce apoptosis in chicken brain cells [31]. Thus, it may be interesting to explore the role of apoptosis in neurovirulence of NDV.

In this study, we show that the cleavage efficiency of the F protein of NDV alone may not determine the neurovirulence phenotype differences among NDV isolates, and that entry into the brain is necessary for neurovirulence. Identification of other viral factors and molecular mechanisms involved in NDV pathogenesis and neurovirulence will enhance the understanding of paramyxovirus pathogenesis in general. In addition, this natural host system will provide a setting for identifying new ways to produce attenuated viruses as candidate vaccines.

4. Materials and methods

4.1. Cells and viruses

DF1 (a chicken embryo fibroblast cell line) cells were maintained in DMEM, and Vero and HEp2 cells were maintained in EMEM media. Chicken neuronal cells were prepared from 10-day-old specific pathogen free (SPF) embryonated chicken eggs. Cytosine arabinoside (Ara C) was added to the medium to inhibit the growth of non-neuronal cells [32]. The identity of the neuronal cells was confirmed by immunostaining with an antibody to human neurofilament 200 kDa (Research Diagnostic, Inc. NJ).

NDV strains LaSota and BC were received from the National Veterinary Services Laboratory, Ames, IA. Each virus was propagated in the allantoic cavity of embryonated chicken eggs and the virus was purified from the allantoic fluid. Briefly, the allantoic fluid was harvested two days post-infection and clarified by low-speed centrifugation at 1800*g* for 30 min. Virus was pelleted by ultracentrifugation at 35,000*g* for 18 h and the pelleted virus was resuspended in 4 ml sterile PBS. The virus was then layered on top of a discontinuous sucrose gradient made with 3 ml of 55% and 5 ml of 20% sucrose in PBS, after which the gradient was ultracentrifuged at 43,000*g* for 1 h. The virus band at the interface of the 20 and 55% sucrose gradients was collected and pelleted at 35,000*g* for 18 h. The virus pellet was resuspended in 500 μl of PBS and stored at 4 °C.

4.2. Mutagenesis of recombinant LaSota virus

Construction of the full-length antigenomic cDNAs of NDV strains LaSota and BC, and recovery of the recombinant viruses are described previously [12,33]. In this study, the full-length antigenomic cDNAs of LaSota and BC were designated as pLaSota and pBC, respectively. The F protein cleavage site of lentogenic and avirulent NDV strain LaSota was altered and made identical to the F protein cleavage site of mesogenic and neurovirulent NDV strain BC. The amino acid sequence G-R-Q-G-R present at the cleavage site of the F protein of avirulent NDV strain LaSota was altered to R-R-Q-K-R, to be analogous to the cleavage site of the F protein of neurovirulent strain BC (Fig. 1). This was done by subcloning the *Sac* II-*Not* I fragment from pLaSota into a plasmid vector, pGEM-7Z (+) (Promega, Madison, WI). This subclone was designated *Sac* II-*Not* I pLaSota 7Z. The F cleavage site of pLasota was then made identical to that of pBC by site-directed mutagenesis [34], with phosphorylated forward primer ($5'$-^{4886}aaaCGCtTTATAGGCGCCATTATTGGCG^{4913}-$3'$)

and phosphorylated reverse primer (5′-^{4885}CTGTCTCCtC-CCTCCAGATGTAGT^{4862}-3′, lower case representing mutation), on *Sac* II-*Not* I pLaSota 7Z. The mutated fragment was digested with *Sac* II and *Not* I restriction enzymes and inserted back into pLaSota. The resultant mutated pLaSota was called pLaSota V.F. To confirm the presence of the desired mutations at the F cleavage site and the absence of any undesired mutations in pLaSota V.F., it was sequenced to its entirety. Using reverse genetics procedures [11,33], the recombinant virus rLaSota V.F. was recovered from its cDNA clone, pLaSota V.F.

4.3. RNA extraction and RT-PCR of recovered mutant viruses

The recovered recombinant viruses, rLaSota, rBC and rLaSota V.F., were grown in the allantoic cavity of 9-day-old SPF embryonated chicken eggs. After five passages, the allantoic fluid was harvested and clarified, and the virus was purified as described above. Viral RNA was extracted from the recovered viruses using TRIzol (Invitrogen, USA), according to the manufacturer's instructions. Reverse transcription was done with the extracted RNA, using the Thermoscript RT kit (Invitrogen, USA) to synthesize the first strand cDNA. RT-PCR was done to amplify the region that covered the F protein cleavage site and the resulting PCR product was sequenced to confirm the presence of the mutations made at the F cleavage site of rLaSota V.F. Similarly, the rLaSota V.F. virus was re-isolated from the brains of 1-day-old chicks and from 3-week-old chickens, and was subjected to RT-PCR and sequencing analysis to confirm the presence of the introduced mutations.

4.4. Growth characteristics of viruses

The growth kinetics of rBC, rLaSota and rLaSota V.F. were performed by multi-step growth curves in DF1 cells. The viruses were inoculated to confluent monolayers of DF1 cells at a m.o.i. of 0.05. The medium of cells infected with rLaSota contained 1 μg/mL of acetyl trypsin. Supernatant was collected at 8 h intervals until 56 h post infection (P.I.) The virus titer in the cell culture supernatant was assessed by plaque assay on DF1 cells.

To observe the growth of virus at different temperatures, the viruses were inoculated into chicken neuronal cells and Vero cells at a m.o.i. of 0.05, and maintained at 37 and 41.5 °C. The virus titers at 24, 48, and 72 h were determined by a plaque assay on DF 1 cells.

4.5. Virus binding studies

The ability of NDV to bind chicken neuronal cells was determined using biotinylated virus. Briefly, purified NDV was labeled with biotin by using a protein biotinylation kit (Amersham). The virus pellet was resuspended in biotinylation buffer (40 mM Na_2CO3, pH 8.6) at a protein concentration of 1 mg/mL. After brief sonication, 40 μL biotin reagent was added per mg of NDV protein. The mixture was shaken for 1 h at 4 °C and the reaction was terminated by addition of Tris–HCl (pH 8.5) to a final concentration of 50 mM. Biotinylated virus was collected after purification on a Sephadex G-25 column and diluted in PBS at a concentration of 0.2 mg/mL. Biotinylated virions were stored at 4 °C.

Direct virus binding studies were carried out with biotinylated virus. The chicken neuronal cells were washed three times with cold PBS containing 0.2% BSA (PBSA) and incubated over ice for 10 min. Different concentrations of biotinylated NDV in PBSA were mixed with 2×10^5 cells per well and incubated on ice for 60 min. The cells were then washed three times with cold PBSA and incubated on ice with 1:10 diluted streptavidin-colloidal gold conjugate (CytImmune Sciences, MD, USA) for 60 min. The cells were washed once and fixed with 1% formaldehyde in PBS at room temperature for 1 min. The cells were then washed once with PBS plus 2% fetal calf serum. The mean number of colloidal gold particles bound to each cell was then counted using a bright field microscope. Specificity of binding was demonstrated in a competition experiment, in which 2×10^5 chicken neuronal cells were incubated with different amounts of unlabeled NDV before performing the virus binding assay as described above.

4.6. HAd assay

Virus was inoculated to confluent monolayers of Vero cells in six-well plates at a m.o.i. of 10. After 18–24 h P.I., the media was decanted and the cells were overlaid with guinea pig RBCs in PBS at a concentration of 1×10^8 cells/ml. The plates were kept at 4 °C for 15 min. The unbound RBCs were removed by washing twice with PBS. The RBCs bound to the virus-infected cells were lysed with 0.05 M ammonium chloride and the released hemoglobin was measured at 549 nm in a spectrophotometer.

4.7. Pathogenicity studies

To test the pathogenicity of the recovered viruses in vivo, ICPI and IVPI tests were performed in accord with standard procedures [2]. For ICPI, 0.05 ml of 1:10 dilution of fresh infective allantoic fluid of each virus was inoculated into groups of 10 1-day-old SPF chicks via the intracerebral (i.c.) route. The inoculation was done using a 27-guage needle, attached to a 1 ml stepper syringe dispenser that was set to dispense 0.05 ml of inoculum per inoculation. The birds were inoculated by inserting the needle up to the hub into the right or left rear quadrant of the cranium. The birds were observed for clinical symptoms and mortality once in every 12 h for a period of 8 days. For IVPI, 0.1 ml 1:10 dilution of fresh infective allantoic fluid of each virus was inoculated into groups of 10 6-week old SPF chickens intranasally

Table 1
Pathogenicity of rBC, rLaSota, and rLaSota V.F. viruses in vivo

Virus	ICPI	IVPI
rBC	1.58	1.45
rLaSota	0.00	0.00
rLaSota V.F	1.12	0.00

The virulence of the viruses was evaluated by ICPI in day-old-chicks and IVPI in 6-week old chickens. ICPI was determined by inoculating 0.05 ml of a 1:10 dilution of fresh infective allantoic fluid of each virus into the brains of 10 1-day-old chicks. The birds were observed daily for 8 days and at each observation, scored 0 if normal, 1 if sick, and 2 if dead. The ICPI value is the mean score per bird per observation. Highly virulent viruses give values approaching two and avirulent viruses give values approaching 0. For IVPI, 0.1 ml of a 1:10 dilution of fresh infective allantoic fluid of wild type and mutant viruses, were inoculated into 6-week old chickens intra-nasally and intra-ocularly. The birds were observed for clinical symptoms and mortality, every 24 h for a period of 10 days and scored 0 if normal, 1 if sick, 2 if paralyzed and 3 if dead. The IVPI value is the mean score per bird per observation. Highly virulent viruses give values approaching three and avirulent viruses give values approaching 0.

(i.n.). We modified the route of inoculation of the virus from intravenous (which is the typical route of inoculation for the IVPI test) to intranasal in order to follow the natural route of infection of NDV in the birds. The birds were observed for clinical symptoms and mortality once every 12 h for a period of 10 days. Equal numbers of chickens were used in each experiment. Each experiment had mock-inoculated controls that received a similar volume of sterile PBS by the respective routes. The ICPI and IVPI values were calculated as described by Alexander [2] (Table 1).

To study the growth kinetics and tissue distribution in vivo, groups of 1-day-old and 3-week-old SPF chickens were inoculated with 10^3 PFU of virus/chicken by the i.c. and i.n. routes, respectively. Brains from the 1-day-old chicks were collected daily for 7 days. Tissues, such as brain, spleen and lung were collected from inoculated 3-week-old chickens on days 1, 3, 5 and 7 P.I. and snap frozen immediately. Blood was also collected from the birds on these same days and assayed for viremia. The tissues were homogenized, and the virus titers in tissues and blood were estimated by plaque assay on DF1 cells. To determine the sites of localization of these recombinant viruses in the brain, one-half of the brains collected from the 3-week-old chickens were embedded in paraffin and sectioned at 5 μm/section. These sections were subject to either an immunohistochemical assay [35] using a cocktail of NDV monoclonal antibodies [36] or an in situ hybridization assay [35] employing a ^{35}S-labeled riboprobe specific for NP gene of NDV.

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