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# The interferon antagonistic activities of the V proteins from two strains of Newcastle disease virus correlate with their known virulence properties

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## Summary

Newcastle disease virus (NDV) is an avian paramyxovirus that exists as hundreds of strains with widely different virulence properties. The NDV V protein exhibits interferon (IFN) antagonistic activity, which contributes to the virulence of the virus. The IFN antagonistic activities of the V proteins from the avirulent strain La Sota and the moderately virulent strain Beaudette C (BC) were compared in an assay for the rescue of a recombinant NDV expressing the green fluorescent protein (NDV-GFP). Consistent with the virulence properties of the two viruses, the BC V protein exhibits a 4-fold greater ability to rescue replication of NDV-GFP than the La Sota V protein. Four amino acid differences in the C-terminal region of V, as well as the N-terminal region, contribute to the difference in IFN antagonistic activity between the two V proteins.

Newcastle disease virus (NDV) is a member of the *Paramyxoviridae* family of enveloped negative-stranded RNA viruses, which also includes Sendai virus, the various parainfluenza viruses, measles virus, respiratory syncytial virus and the henipaviruses. The NDV genome contains six genes, which encode six major proteins: nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN) and the large (L) polymerase (Lamb and Parks, 2007). NDV also produces the V and W proteins by RNA editing during P gene transcription. The P gene mRNA is edited by insertion of one or two additional G residues into a run of G's within the conserved editing site, thus generating the V- and W-encoding mRNAs, respectively (Steward *et al.*, 1993).

NDV causes respiratory, neurological or enteric disease in birds. Strains are classified into three pathotypes. Avirulent (lentogenic) strains cause mild or asymptomatic infections,

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whereas virulent (velogenic) strains cause high mortality. Strains of intermediate virulence are called mesogenic (Alexander, 1997). NDV is also being used as a vaccine vector (Huang *et al.*, 2003a; Dinapoli *et al.*, 2009) and oncolytic agent due to its ability to kill tumor cells (Elankumaran *et al.*, 2006; Freeman *et al.*, 2006).

Cleavage of the F protein precursor ($F_0$) produces the active fusion protein (Scheid and Choppin, 1974) and is the primary determinant of virulence as determined by the number of basic residues in the cleavage site (Glickman *et al.*, 1988; Nagai *et al.*, 1976; Toyoda *et al.*, 1989). However, other viral proteins contribute to virulence (Panda *et al.*, 2004; Peeters *et al.*, 1999). Recombinant viruses lacking V have impaired growth in cell culture and embryonated chicken eggs and are highly attenuated in young chickens (Huang *et al.*, 2003b; Mebatsion *et al.*, 2001). These mutant viruses also exhibit increased sensitivity to exogenous interferon (IFN) (Elankumaran *et al.*, 2006; Huang *et al.*, 2003b). Using an IFN-sensitive NDV-GFP-based assay, it was demonstrated that the NDV V protein possesses IFN antagonistic activity, defined by the C-terminal region of the protein (Park *et al.*, 2003). This is consistent with the IFN-antagonistic activity of the NDV V protein contributing to virulence. However, the role of V in the differential virulence patterns exhibited by NDV pathotypes has not been examined.

Here, the NDV-GFP-based assay (Park *et al.*, 2003) was used to compare the relative IFN antagonistic activities of the V proteins from mesogenic strain Beaudette C (BC) and lentogenic strain LaSota. DF1 cells (chicken embryo fibroblast cell line) (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 4 U/ml penicillin and 4 μg/ml streptomycin. The enhanced GFP gene was inserted between the P and M genes of the BC cDNA and the virus was rescued from cDNA (Elankumaran *et al.*, 2006). It was demonstrated that the NDV-BC-GFP virus is susceptible to IFN by inhibition of growth following treatment with 1000 U/ml of chicken IFN-α (AbD serotec, Kidlington, Oxford, UK) prior to infection (data not shown).

The P genes of the La Sota and BC viruses were cloned into pBluescript SK(+) (pBSK) (Stratagene, La Jolla, CA) within a few egg passages of the original stock (Veterinary Services Laboratory, Ames, IA). Each V gene was generated from the respective P gene by insertion of a G nucleotide into the editing site as described previously (Corey and Iorio, 2007). Mutated V genes were prepared using the same protocol. The presence of all mutations was confirmed by DNA sequencing. The wild type (wt) or mutated V genes were subcloned into pCAGGS by blunt-end ligation.

The IFN antagonistic activities of the V proteins were tested by their ability to rescue growth of NDV-GFP virus. DF1 cells were seeded in 6-well plates and transfected in triplicate at 80% confluence using Lipofectamine 2000 (Invitrogen). After 24 h, the cells were washed with PBS and infected with NDV-GFP (moi of 0.001). Virus growth was monitored at 24 h post-infection (Fig. 1A) and quantitated by counting fluorescent cells in 3-5 fields (approximately 3000 cells) (Fig. 1B). It should be noted that, although the NDV-GFP virus is a BC virus and has an intact V open reading frame, the IFN-induced inhibition of NDV-GFP growth occurs prior to infection. Thus, within the time frame of the assay, an antiviral state has already been established before the V protein is expressed from the virus rendering it a non-factor.

Although the virus grows well in mock-transfected cells, transfection with the empty pCAGGS vector inhibits growth by inducing IFN and the subsequent antiviral state, as shown previously by Park *et al.* (2003). Transfection with empty vector results in a significant decrease (211 ± 15 infected cells) in virus growth as compared to mock (1522 ± 70 infected cells) (Fig. 1B). Transcription from a plasmid may lead to generation of double-stranded RNA or plasmid DNA

may be recognized by a cytosolic DNA sensor, triggering IFN production. Most importantly, expression of either V protein rescues virus growth (Fig. 1A), but with BC V (1753 ± 58 infected cells) doing so four times more efficiently than La Sota V (394 ± 29 infected cells) (Fig. 1B). This correlates with the difference in virulence between the two strains of the virus.

One possibility is that the difference in IFN antagonistic activity of the two V proteins is due merely to differences in expression. Western blots were performed at 24 h post-transfection to quantitate V protein expression. Briefly, the cells were scraped into cold phosphate buffered saline (PBS), pelleted for 5 min at 16,000×g and lysed for 30 minutes using 50 μl NP-40 cell lysis buffer (Biosource International, Inc., Camarillo, CA) supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO). Twenty μl of the cleared lysate were electrophoresed on a NuPAGE 4-12% Bis-Tris gel (Invitrogen) under reducing conditions. Proteins were transferred onto an Immobilon-P membrane (Millipore, Bedford, MA) and blocked overnight at 4°C with Detector Block (Kirkegaard and Perry Laboratories, Gaithersburg, MD). A polyclonal antibody (V18) raised in rabbits against the C-terminal 18 amino acids of the BC V protein was used followed by horseradish peroxidase-conjugated goat anti-rabbit antibody (Kirkegaard and Perry Laboratories). Proteins were visualized using the Amersham ECL Western Blotting Analysis System (GE Healthcare, Buckinghamshire, UK). The membrane was stripped using Re-Blot Plus Strong Solution (Chemicon International, Temecula, CA), blocked overnight and re-probed with anti-actin (Sigma).

Both V proteins are expressed, although the BC V protein is detected 1.4-fold more efficiently than LaSota V (Fig. 1C). There are two possible explanations for this result. One is that the BC protein is actually expressed more efficiently. Alternatively, the V18 antiserum may recognize La Sota V less efficiently than it does BC V. The antiserum is directed against the C-terminal sequence of BC V and there is a difference between La Sota (aspartic acid) and BC (asparagine) at amino acid 234 in this region.

To determine whether this amino acid difference modulates antiserum reactivity, both genes were mutated at position 234 to the amino acid in the other protein. D234N-mutated La Sota V is recognized by the V18 antiserum at a level similar to wt BC V and N234D-mutated BC V is recognized at a reduced level similar to wt La Sota V (Fig. 1C). Hence, the amino acid difference at position 234 appears to account for the differential recognition of the two V proteins by the V18 antiserum. Still, we cannot definitively rule out the possibility that the residue at position 234 modulates expression except to note that, if it does completely account for the observed difference in IFN antagonism between the two V proteins, then a 40% difference in expression would have to account for a four-fold difference in IFN antagonism.

Previously, the C-terminal region of NDV V was demonstrated to be necessary and sufficient for its IFN antagonistic activity (Park *et al.* 2003). The C-terminal regions of the two proteins have four amino acid differences at positions 144, 153, 161 and 234. These residues are S, E, S and D, respectively, in La Sota V and P, K, P and N, respectively, in BC V (Fig. 2). To test the contribution of these residues to IFN antagonistic activity, both proteins were mutated at each position to the amino acid in the other protein and the IFN antagonistic activities of the mutated proteins were determined (Fig. 3A). All of the La Sota V proteins carrying single mutations have an increased ability to rescue growth of the NDV-GFP virus, ranging from between 512 ± 13 and 761 ± 27 compared to 394 ± 29 for La Sota V. Conversely, all of the individually mutated BC V proteins have decreased ability to rescue growth compared to wt ranging from 569 ± 40 to 1329 ± 138, relative to BC V (1753 ± 58) (Fig. 3B). Thus, each residue contributes to IFN antagonism.

To determine the possible contribution of differences in expression to the differences in IFN antagonism, the plasmid constructs were transfected into DF1 cells and Western blots were performed using the V18 antibody. With the exception of the D234N-mutated La Sota V protein, the increased IFN antagonistic activities (filled bars) of the individually mutated proteins cannot be accounted for by increased expression (open bars) (Fig. 3C). This exception is consistent with the results in Fig. 1C. Conversely, none of the individually mutated BC V proteins exhibits an expression level reduced enough to account for its reduced IFN antagonism (Fig. 3D).

To test whether the mutations in the C-terminal region of V have an additive effect, double, triple and quadruple mutants of both wt V proteins were evaluated. The double mutated proteins of La Sota V are better than the single mutated proteins in rescue of viral growth (Fig. 3A). The number of fluorescent cells for La Sota V S144P/E153K and S161P/D234N mutated proteins was 1144 ± 17 and 897 ± 28, respectively. However, the triple and quadruple mutated proteins of La Sota V are not significantly more effective than the double mutated proteins.

Fig. 3B shows that the BC V P144S/K153E double mutated protein (645 ± 47 fluorescent cells) has a decreased ability to rescue the virus as compared to the proteins carrying individual P144S and K153E mutations. The BC V P161S/N234D double mutated protein rescues viral growth at almost the same level as the P161S and N234D mutated proteins. Surprisingly, the triple and quadruple mutated proteins of BC V are better at rescuing the NDV-GFP virus than the double mutated proteins. In no case can increased IFN antagonistic activity be accounted for by increased expression (Fig. 3D). For the BC V protein carrying multiple mutations, the results are less clear. For the two proteins carrying N234D mutations (P161S/N234D and the quadruple mutated protein), reduced rescue can be accounted for by reduced expression. This correlates with the presence of the mutation at position 234, though the single mutant does not exhibit this problem.

Overall, the ability of the mutated V proteins to rescue growth of NDV-GFP does not correlate with the expression levels. For example, even though the V18 antibody detects the BC V quadruple mutated protein at only 20% of BC V, it rescues growth of the virus at a greater level than that of other mutated proteins that are recognized to a greater extent by the antibody (i.e., BC V P161S-mutated protein which is recognized at 100% of BC V). Most importantly, neither the quadruple-mutated LaSota V nor BC V protein exhibits rescue activity commensurate with the other wt V protein. Thus, other differences between the two proteins located in the N-terminal region of V contribute to their differences in IFN antagonistic activity.

The V proteins of paramyxoviruses show 44% overall homology, with the region of highest homology being the C-terminus, which contains seven conserved cysteines capable of binding two zinc atoms (Paterson *et al.*, 1995; Steward *et al.*, 1995). Several studies point to the importance of the C-terminus of the paramyxovirus V protein to IFN antagonism and virulence (Fukuhara *et al.*, 2002; He *et al.*, 2002; Huang *et al.*, 2000; Kawano *et al.*, 2001; Nishio *et al.*, 2001; Park *et al.*, 2003). Our results indicate that residues 144, 153, 161 and 234 in the C-terminal region of the NDV V protein all contribute to IFN antagonistic activity.

However, the quadruple mutated La Sota and BC V proteins fail to convert rescue to the level of the other protein. This indicates that residues in the N-terminal region of V also contribute to IFN antagonistic activity. This is consistent with earlier results obtained with other paramyxoviruses (Chatziandreou *et al.*, 2002; Fontana *et al.*, 2008). It is conceivable that N-terminal changes could affect the structure of the protein making the C-terminus less accessible to interacting proteins. There are five amino acid differences between La Sota and BC in the N-terminal region at residues 30, 41, 46, 65 and 82. It will be interesting to determine how the

identity of the residues at these positions affects IFN antagonistic activity. This is the first evidence that the N-terminus of NDV V contributes to its IFN antagonistic activity.

Though we unable to examine the IFN antagonistic activity of a V protein from a velogenic strain due to the requirement for BSL-3 containment for these viruses, we have compared the sequence of velogenic strains (Locke *et al.*, 2000) to that of La Sota. Surprisingly, lentogenic strains are more similar to velogenic strains than they are to mesogenic strains at positions 144, 153 and 234 and there is no pattern for residue 161 for these strains. In addition, there are no amino acids anywhere in the V protein that are specific for any of the three pathotypes (data not shown).

There are hundreds of NDV isolates, which exhibit widely different virulence patterns. The attenuation of virulence in a recombinant NDV that fails to express the V protein has been attributed to its inability to antagonize IFN-α (Huang *et al.*, 2003b). To better understand the role of the V protein in virulence, we compared the IFN antagonistic activities of the V proteins from a lentogenic and a mesogenic strain. We showed that the V protein from a mesogenic strain has a four-fold greater ability to rescue the growth of an NDV-GFP virus than that from an avirulent, lentogenic strain. This is consistent with the two proteins having significantly different IFN antagonistic activities. These differences correlate with their virulence and suggests that the IFN antagonistic activity of the NDV V protein may have an important role in the virulence of NDV. This possibility can be tested by evaluation of recombinant viruses in which the two V proteins have been exchanged.

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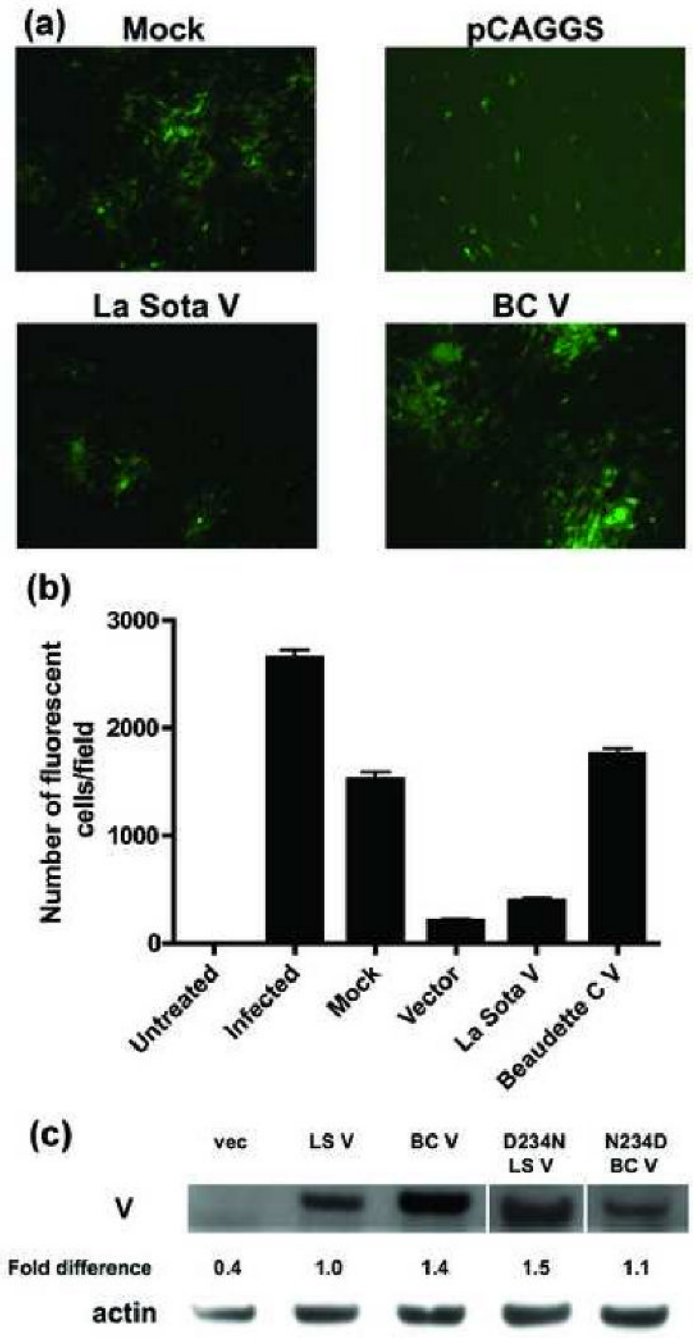


**Fig 1.**

BC V exhibits a four-fold greater ability than La Sota V to rescue growth of NDV-GFP. (A) DF1 cells were mock-transfected, or transfected with empty pCAGGS vector, La Sota V or BC V. After 24 h, the cells were infected with NDV-GFP virus and examined for fluorescence 24 h later. (B) The growth of NDV-GFP virus was quantitated by counting the number of fluorescent cells from three different fields. The average values and standard deviations (error bars) are shown for one experiment out of a total of four. The absolute numbers of fluorescent cells vary from one experiment to another but the relative activities are consistent. (C) DF1 cells were transfected with wt or mutant V plasmids. After 24 h, lysates were prepared and Western blot was performed using V18 antiserum. V protein levels standardized to actin were

determined by densitometry and are expressed relative to wt (set at 100%). These data represent one out of at least two experiments.

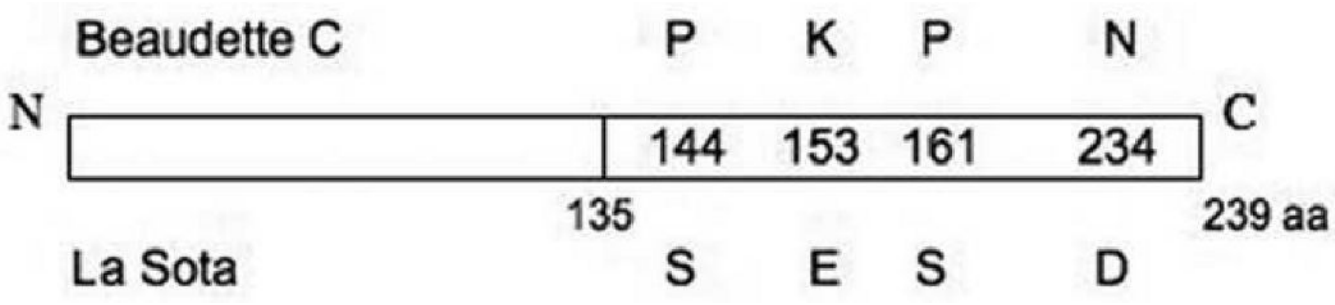


**Fig. 2.**
Schematic representation of the amino acid differences in the BC and LaSota V proteins. The figure shows the residues at the four positions in the C-terminal region that differ between the two proteins.

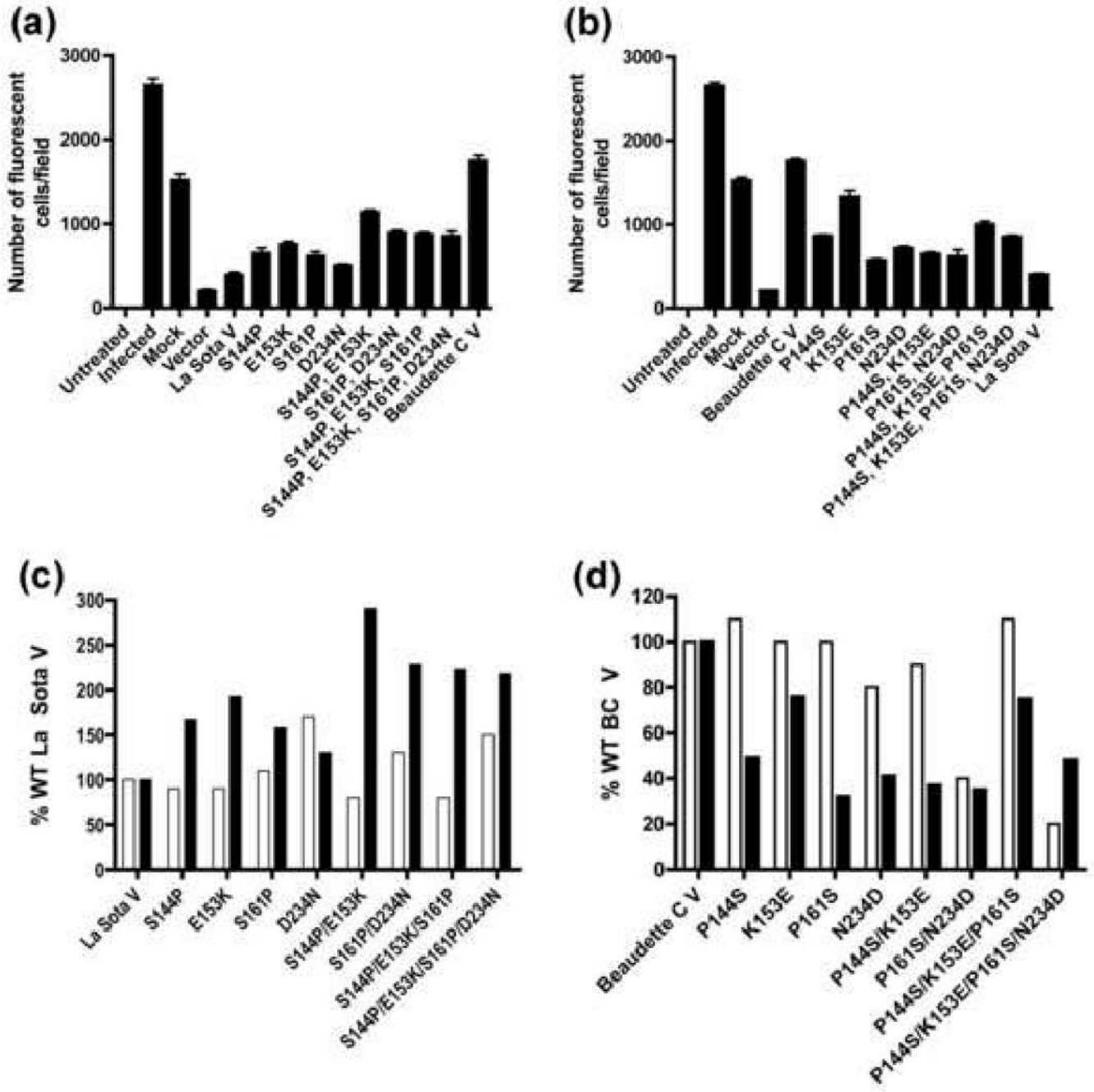


**Fig 3.**

Effect of wt and mutated V proteins on the growth of NDV-GFP virus. DF1 cells were mock-transfected (mock), or transfected with empty vector (vector), La Sota wt or mutated V plasmids (panel A) or BC wt or mutated V plasmids (panel B). After 24 h, the cells were infected with NDV-GFP at a moi of 0.001 and examined for fluorescence 24 h later. In panels C and D, the percent expression relative to wt (open bars) is shown for mutated LaSota (panel C) and BC V proteins (panel D), along with the percent of wt rescue of growth of NDV-GFP virus (filled bars), based on the data in panels A and B. Expression levels were determined as described in the legend to Fig. 1C. These data represent one experiment out of at least two.