Inhibition of host innate immune responses and pathogenicity of recombinant Newcastle disease viruses expressing NS1 genes of influenza A viruses

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The NS1 protein has been associated with the virulence of influenza A viruses. To evaluate the role of the NS1 protein in pathogenicity of pandemic H5N1 avian influenza and H1N1 2009 influenza viruses, recombinant Newcastle disease viruses (rNDVs) expressing NS1 proteins were generated. Expression of the NS1 proteins resulted in inhibition of host innate immune responses (beta interferon and protein kinase R production). In addition, the NS1 proteins were localized predominantly in the nucleus of virus-infected cells. Consequently, expression of the NS1 protein contributed to an increase in pathogenicity of rNDV in chickens. In particular, mutational analysis of H5N1 NS1 protein indicated that both the RNA-binding and effector domains affect virus pathogenicity synergistically. Our study also demonstrated that expression of H1N1/09 NS1 resulted in enhanced replication of rNDV in human cells, indicating that function of the NS1 proteins can be host-species-specific.

Influenza A viruses are associated with significant morbidity and mortality, causing worldwide epidemics yearly and pandemics sporadically (Li et al., 2004). The genome of influenza A virus consists of eight RNA segments that encode nine structural proteins and two non-structural proteins, NS1 and PB1-F2 (Palese & Shaw, 2007). The NS1 protein has been identified as a determinant of virulence of influenza A viruses (Bergmann et al., 2000; Donelan et al., 2003). A major function of the NS1 protein is to antagonize host innate immune responses. However, the mechanisms and targets for the NS1 protein vary among influenza A viruses (Kochs et al., 2007). The length of the NS1 protein is strain-specific. The NS1 protein is divided into two distinct functional domains: an N-terminal RNA-binding domain and a C-terminal effector domain for interactions with host cellular proteins. In particular, the RNA-binding domain is highly conserved among influenza A viruses, and three residues (Arg-35, Arg-38 and Lys-41) in the domain are associated with binding double-stranded RNA (dsRNA). The C-terminal 4 aa of NS1 has been identified as a potential PDZ domain-binding motif that might influence the activity of PDZ domain-containing proteins, which are often involved in cellular signal-transduction pathways (Jackson et al., 2008).

Newcastle disease virus (NDV) is a member of the family Paramyxoviridae and has a non-segmented, negative-sense RNA genome consisting of six transcriptional units (3’-NP-P-M-F-HN-L-5’) (Lamb & Parks, 2007). NDV causes a highly contagious respiratory and neurological disease in chickens (Alexander, 1989). NDV strains are categorized into three pathotypes in chickens: lentogenic (avirulent), mesogenic (moderately virulent) and velogenic (highly virulent) (Panda et al., 2004). In this study, we have used a recombinant mesogenic NDV strain to evaluate the role of pandemic influenza A virus NS1 protein in preventing innate host defences and pathogenicity in chickens. The NS1 genes of H5N1 highly pathogenic avian influenza and pandemic H1N1 2009 influenza (H1N1/09) viruses were inserted individually between the P and M genes of NDV mesogenic strain Beaudette C (BC) (Fig. 1a). As the functional role of the specific amino acid residues of the NS1 proteins has been primarily studied with human influenza virus strains, we attempted to identify critical amino acids of the H5N1 NS1 protein responsible for virus pathogenicity. The open reading frame (ORF) of the NS1 gene was altered by a single amino acid change, according to previously published studies (Donelan et al., 2003; Jiao et al., 2008). In addition, the C-terminal 4 aa (ESEV) of H5N1 NS1 was replaced with that of H3N2 virus (RSKV) to evaluate the effect of this potential PDZ domain-binding motif on the function of the NS1 protein. We also generated NS1 protein- and effector domain-deleted viruses by introducing a stop codon at the N-terminal region and the effector domain of the H5N1 NS1 protein, respectively. Recombinant BCs (rBCs) were recovered by using our standard protocol (Krishnamurthy et al., 2000) and the expression of NS1 proteins by recovered viruses was confirmed by Western blot analysis. Multicycle growth...
kinetics of rBCs indicated that the expression of NS1 protein did not affect in vitro replication of rBCs in DF-1 cells [a chicken embryo fibroblast (CEF) cell line] (Fig. 1b). Interestingly, in HeLa cells, rBC expressing H1N1/09 NS1 grew to 1 log_{10} higher titres than rBC expressing H5N1 NS1 up to 24 h post-infection, indicating that H1N1/09 NS1 enhanced replication of rBC in human cells (Fig. 1c).

NS1 has been implicated in inhibition of the host antiviral defence mediated by interferon (IFN) (Donelan et al.,
Point mutation, truncation or deletions of the NS1 gene of influenza A viruses resulted in enhanced IFN-inducing capacity of the virus in vivo and in vitro (Donelan et al., 2003; Ferko et al., 2004; Jiao et al., 2008; Z. Li et al., 2006). In contrast, NDV has been known as a good IFN-β inducer in infected cells (Poole et al., 2002) and the NDV V protein can prevent IFN responses only in avian cells, thus playing a role in host-range restriction (Park et al., 2003). Therefore, we first determined whether rBCs expressing NS1 proteins of avian and human strains affected the levels of IFN-β mRNA in avian and human cells differently (Fig. 2a). Our results showed that the H5N1 NS1 protein inhibited IFN-β production efficiently in both CEF and HeLa cells, whereas H1N1/09 NS1 protein antagonized IFN-β synthesis more efficiently in HeLa cells than in CEF cells, probably due to its interaction with

Fig. 2. RT-PCR analysis of IFN-β mRNA levels in virus-infected CEF and HeLa cells and PKR inhibition by NS1 protein in virus-infected HeLa cells. (a) CEF and HeLa cells were infected at an m.o.i. of 2. Total RNAs were extracted at 24 h post-infection and RT-PCR was carried out using primers specific for IFN-β. The levels of β-actin and GAPDH mRNAs in CEF and HeLa cells, respectively, were measured as loading controls. (b) Activated PKR in virus-infected HeLa cells was analysed by Western blotting. HeLa cells in six-well plates were infected with rBCs at an m.o.i. of 1. Total proteins were collected from virus-infected cells at 24 h post-infection, electrophoresed, transferred to a nitrocellulose membrane and immunostained using polyclonal antibodies against phospho-PKR (top panel) and NS1 (middle panel) or a monoclonal antibody against β-actin as a loading control (bottom panel). Levels of activated PKR were quantified from three independent analyses; error bars indicate SEM. (c) Localization of wild-type and mutated NS1 proteins was evaluated in virus-infected HeLa cells infected with rBCs at an m.o.i. of 1. Twenty-four hours post-infection, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, stained with a monoclonal antibody against the NS1 protein (N-terminal domain) followed by anti-Alexa Fluor 488 conjugate and DAPI (4',6-diamidino-2-phenylindole), and analysed by confocal microscopy.
unique sequences of specific host species (Meléndez et al., 2007; Park et al., 2003). Furthermore, point mutations in RNA-binding domains of H5N1 NS1 resulted in alteration of IFN-β mRNA levels in CEF and HeLa cells. The basic amino acids R38 and K41 are known to be associated directly with RNA binding of NS1 (Geiss et al., 2002; Talon et al., 2000). However, in our study, rBC/NS1 R38A induced higher levels of IFN-β mRNA than did rBC/NS1 K41A, because mutation of R38A would be more effective on complete abrogation of binding affinity of NS1 than mutation of K41A (Wang et al., 1999). In influenza A/WSN/33, mutations of NS1 protein (R38AK41AS42G) resulted in increased IFN-β production and virus attenuation in mice, indicating that IFN-antagonist properties of the NS1 protein can also be modulated by other amino acid residues, such as S42, without direct association with RNA binding (Donelan et al., 2003). Similarly, our study suggested that, in the H5N1 NS1 protein, a certain amino acid at position 42 would modulate IFN production in virus-infected cells. In addition, point (E92A) and deletion mutations in the effector domain of H5N1 NS1 resulted in enhanced levels of IFN-β mRNA synthesis in virus-infected cells, indicating that the effector domain also plays an important role in antagonizing IFN-β synthesis. Indeed, elimination of the effector domain has been shown to alter the dsRNA-binding affinity of NS1 significantly (Li et al., 2004). The effector domain is also known to bind to the cellular proteins that are essential for the processing of cellular pre-mRNA, including IFN-β mRNA (Noah et al., 2003).

The NS1 protein can prevent activation of the IFN-inducible dsRNA-dependent protein kinase R (PKR), leading to stimulation of host protein synthesis in infected cells (Donelan et al., 2003). Therefore, we further characterized the function of the NS1 protein in inhibition of PKR activation in virus-infected HeLa cells by Western blotting (Fig. 2b). In general, levels of the mutated NS1 proteins were similar to that of the NS1 protein, indicating that mutation of the NS1 protein did not affect levels of protein expression. However, levels of activated PKR were correlated with those of IFN-β mRNA in infected cells with rBC and rBCs expressing various NS1 proteins. H5N1 and H1N1/09 NS1 proteins inhibited PKR activation in infected HeLa cells. In contrast, point mutations of R38A and S42G and deletion of the effector domain in the H5N1 NS1 protein resulted in a slight increase in PKR activation in infected HeLa cells. In A/Udorn/72, certain amino acid residues in the effector domain of NS1 have been shown to bind to a linker region in PKR, thus preventing a conformational change required for release of PKR auto-inhibition (S. Li et al., 2006; Min et al., 2007). Therefore, it is possible that H5N1 NS1 inhibits PKR activation by dsRNA binding- and direct binding-mediated mechanisms.

In infected cells, NS1 protein sequesters viral dsRNAs in the nucleus, which leads to blocking the dsRNA activation of PKR in the cytoplasm (Bergmann et al., 2000; Meléndez et al., 2007). Consequently, we analysed the cellular localization of wild-type and mutated NS1 proteins in virus-infected HeLa cells by confocal microscopy. Both H5N1 and H1N1/09 NS1 expressed by rBCs were localized predominantly in the nucleus (Fig. 2c). However, mutations of R38A and the effector domain (E92A and deletion) resulted in localization of the NS1 protein in the cytoplasm of virus-infected cells, indicating that these amino acids are involved in the distribution of NS1 in the nucleus of infected cells. In H1N1 NS1, the location of the NS1 protein can be facilitated by two nuclear-localization signals (NLSs): NLS1 at residues 34–38 (Asp-Arg-Leu-Arg-Arg) and NLS2 at residues 216–221 (Pro-Lys-Gln-Lys-Arg-Lys) (Greenspan et al., 1988). Our study also suggests that the subcellular distribution of the H5N1 NS1 protein can be affected by alteration of NLSs located in both the RNA-binding and effector domains.

We then evaluated the contribution of the NS1 protein to pathogenicity of rBCs in embryonated chicken eggs and in chickens (Table 1). The pathogenicity of rBCs was determined by mean death time (MDT) test in 9-day-old embryonated specific-pathogen-free (SPF) chicken eggs and by intracerebral pathogenicity index (ICPI) test in 1-day-old SPF chicks (Alexander, 1989). The scale of the ICPI value in evaluating the virulence of NDV strains is from 0.00 (avirulent strains) to 2.00 (highly virulent NDV strains). In general, MDT and ICPI values were correlated in evaluating pathogenicity of rBCs expressing wild-type and mutated NS1 proteins. Expression of H5N1 and

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<th>Virus</th>
<th>MDT (h)*</th>
<th>ICPI†</th>
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<tr>
<td>rBC</td>
<td>57</td>
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<tr>
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<td>1.54</td>
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<tr>
<td>rBC/H1N1/09 NS1</td>
<td>45</td>
<td>1.73</td>
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*Mean time (h) for the minimum lethal dose of virus to kill all of the inoculated embryos. Five eggs for each diluent of allantoic fluid of infectious virus were tested. The criteria for classifying the virulence of NDV strains are: taking < 60 h to kill embryos for virulent strains; taking 60–90 h to kill embryos for moderately virulent strains; and taking > 90 h to kill embryos for avirulent strains.
†Pathogenicity of rBCs in 1-day-old SPF chicks (10 chicks for each virus) was evaluated by the ICPI test: virulent strains, 1.50–2.00; moderately virulent strains, 0.70–1.50; and avirulent strains, 0.00–0.70.
H1N1/09 NS1 proteins resulted in increased pathogenicity of rBCs (ICPI: 1.75 and 1.73, respectively) compared with those of rBC and rBC/ΔNS1 (ICPI: 1.51 and 1.48, respectively) \( (P<0.05) \). We also confirmed the contribution of the NS1 protein to increased virus replication in 2-week-old chickens. The rBC expressing H5N1 NS1 replicated to 2–3 \( \log_{10} \) higher titres than rBC in various tissues at 3 days post-infection. The titres of rBC/NS1 and rBC were 4.5 \( \pm \) 0.10 and 2.2 \( \pm \) 0.15 \( \log_{10}(\text{p.f.u. g}^{-1}) \), respectively, in brain; 7.2 \( \pm \) 0.33 and 5.4 \( \pm \) 1.04 \( \log_{10}(\text{p.f.u. g}^{-1}) \), respectively, in trachea; 6.2 \( \pm \) 0.54 and 3.6 \( \pm \) 0.11 \( \log_{10}(\text{p.f.u. g}^{-1}) \), respectively, in lung; and 5.5 \( \pm \) 0.15 and 3.7 \( \pm \) 0.44 \( \log_{10}(\text{p.f.u. g}^{-1}) \), respectively, in spleen. However, ICPI results indicated that expression of NS1 with point mutations in the RNA-binding domain (R38A and S42G) did not alter the pathogenicity of rBC \( (P>0.05) \). Deletion of the effector domain and alteration of the C-terminal 4 aa also did not affect the pathogenicity of rBC. Furthermore, our ICPI results suggest that the uniquely present glutamic acid residue at position 92 in H5N1 NS1 (Talon et al., 2000) plays an important role in virus pathogenicity \( (P<0.05) \; \text{ one-way ANOVA, ssps 13.0 for Windows) }\).

In summary, we have evaluated the function of the NS1 protein of H5N1 highly pathogenic avian influenza and pandemic H1N1 2009 influenza (H1N1/09) viruses in pathogenicity. Expression of H5N1 NS1 protein led to alteration of host innate immune response in avian and human cells and enhancement of the replication of rNDV in chickens. Mutational analysis of H5N1 NS1 suggests that amino acids R38 and S42 in the RNA-binding domain and effector domain synergistically affect inhibition of host innate immune system and, consequently, virus pathogenicity. Particularly, the expression of H1N1/09 NS1 enabled rNDV to circumvent host innate immune responses and replicate well in human cells, indicating that the function of NS1 proteins can be host-species-specific.

Acknowledgements

We thank Daniel Rockemann, Flavia Dias, Yunsheng Wang and our laboratory members for excellent technical assistance and LaShae Green for proofreading of the manuscript.

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