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Short Communication

Role of host-specific amino acids in the pathogenicity of avian H5N1 influenza viruses in mice

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Recent large-scale sequence analyses revealed 'signature' amino acids at specific positions in viral proteins that distinguish human influenza viruses from avian viruses. To determine the role of these host lineage-specific amino acids in the pathogenicity of H5N1 avian influenza viruses, we generated mutant viruses possessing signature amino acids in the PB2, PA and NP proteins of human influenza isolates ('human-like amino acids') in the genetic background of an avian H5N1 virus, and tested their pathogenicity in mice. We found that some of these mutants exhibited enhanced pathogenicity in mice, suggesting the involvement of these host lineage-specific amino acids in the pathogenicity of H5N1 avian influenza viruses in mammals.

H5N1 influenza outbreaks in poultry have affected many countries in Asia, Europe and Africa (Cooper *et al.*, 1999; Deng *et al.*, 2006; Gabriel *et al.*, 2005; Itoh *et al.*, 2009; Maines *et al.*, 2005; Shaw *et al.*, 2002; Sorrell *et al.*, 2009). Continued circulation of H5N1 viruses in birds provides ample opportunity for them to be transmitted to humans. Indeed, H5N1 viruses have overcome host species barriers and infected humans sporadically in several countries, with 436 confirmed human cases of H5N1 infection and 262 fatalities as of 1 July 2009 (WHO, 2009). Nonetheless, H5N1 viruses have not yet caused a pandemic in humans because of their inefficient human-to-human transmission (Maines *et al.*, 2006; Yen *et al.*, 2007). In contrast, the recently emerged swine-origin H1N1 2009 influenza virus has transmitted very efficiently among humans, causing the first influenza pandemic of the 21st century (Dawood *et al.*, 2009; Itoh *et al.*, 2009; Munster *et al.*, 2009).

Recently, large-scale sequence analyses revealed 'signature' amino acids at specific positions in viral proteins that distinguish human influenza viruses from avian influenza viruses (Chen *et al.*, 2006; Finkelstein *et al.*, 2007; Shaw *et al.*, 2002). These host lineage-specific amino acids were mainly found in components of the viral RNA polymerase complex, such as PB2, PA and NP, essential for viral genome replication (Deng *et al.*, 2006; Klumpp *et al.*, 1997). It is likely that these amino acids contribute to the host-range restriction of influenza viruses (Gabriel *et al.*, 2005; Scholtissek *et al.*, 1985); however, their biological significance remains to be established, with the exception of the amino acids at positions 627 and 701 of PB2, whose importance in virulence has been demonstrated in a rodent model (Hatta *et al.*, 2001; Li *et al.*, 2005; Shinya *et al.*, 2004; Steel *et al.*, 2009). Therefore, to understand the biological role of the host-specific amino acids of the viral RNA polymerase complex of avian H5N1 viruses in a mammalian host, we generated a series of mutant viruses possessing human-like amino acids in PB2, PA and/or NP in the genetic background of an avian H5N1 virus and examined their pathogenicity in a mouse model. We found that some of these amino acid changes conferred enhanced pathogenicity in mice, suggesting a potential role for these host lineage-specific amino acids in pathogenicity in mammals.

To identify host lineage-specific amino acids that distinguish human influenza isolates from avian isolates, we first compared the sequences of avian and human viruses of

A supplementary table showing a comparison of avian- and human-like amino acids in viral proteins of influenza A viruses is available with the online version of this paper.

various subtypes. Sequence data were obtained from the Influenza Sequence Database (ISD) operated by Los Alamos National Laboratory (https://flu.lanl.gov/) (Macken *et al.*, 2001). Based on our analysis and other data (Chen *et al.*, 2006; Finkelstein *et al.*, 2007; Shaw *et al.*, 2002), we identified 75 amino acids that are conserved in either human or avian influenza viruses, which we designated 'human-like' or 'avian-like' amino acids, respectively (see Supplementary Table S1, available in JGV Online).

Host lineage-specific amino acids were mostly found in the viral RNA polymerase complex components PB2, PA and NP (Supplementary Table S1), which is consistent with the findings of others (Chen *et al.*, 2006; Finkelstein *et al.*, 2007; Shaw *et al.*, 2002). We then examined the residues at these 'signature' amino acid positions in sequences of the PB2, PA and NP proteins of avian viruses that had been isolated from humans, including H5N1 viruses isolated from humans, A/Hong Kong/1774/99 (H3N2) virus, which is an avian-like swine virus isolated from a human (Gregory *et al.*, 2001), and A/Wisconsin/10/98 (H1N1) virus, which is a triple-reassortant virus isolated from a human whose PA and PB2 genes are of avian origin, whereas the PB1 gene is of human origin and the five remaining genes are of a classical swine virus (Cooper *et al.*, 1999; Olsen, 2002). We also included A/Brevig Misson/1/18 (H1N1; the 1918 pandemic virus), which is known to share many amino acids with avian viruses (Taubenberger *et al.*, 1997, 2005) in this analysis. For these viruses, the presence of 'human-like' amino acids at any of the 'signature' positions would suggest the acquisition of that human-like amino acid during replication in the new host. For example, two human H5N1 isolates, A/Hong Kong/482/97 and A/Hong Kong/483/97, encode Asn at position 9 of PB2 instead of Asp, which is conserved in avian viruses at this position (Table 1), suggesting a role for this residue in avian virus adaptation to humans.

For position 142 of PA and position 100 of NP, we also tested Glu and Ile, respectively, in the genetic background of an avian virus, as we found that two H5N1 human isolates (A/Hong Kong/156/97 and A/Vietnam/1203/2004) encode Glu at position 142 of PA and that the 1918 pandemic virus encodes Ile at position 100 of NP, suggesting roles for these residues in virus adaptation to humans. Based on this approach, we chose 12 of the 75 human-like amino acids (four in PB2, three in PA and five in NP; Table 1) to test their contribution to avian virus replication in a mammalian host. In this study, we used a mouse model because the biological significance of the amino acids at positions 627 and 701 of PB2 has been established in this model (Hatta *et al.*, 2001; Li *et al.*, 2005; Shinya *et al.*, 2004).

To examine the role of host lineage-specific amino acids in the pathogenicity of avian H5N1 viruses, we generated a series of mutant viruses possessing single human-like

Table 1. Comparison between avian- and human-like amino acids in the PB2, PA and NP proteins of influenza A viruses

We analysed protein sequences in the ISD and identified 75 host-specific amino acids conserved in human and avian influenza viruses. For this study, we selected 12 amino acids, as described in the text.

Protein	Amino acid			Isolates*
	Position	Avian-like	Human-like	
PB2	9	D	N	A/Hong Kong/482/97 (H5N1), A/Hong Kong/483/97 (H5N1)
	199	A	S	A/Brevig Misson/1/18 (H1N1), several Hong Kong isolates in 1997 (H5N1)
	368	R	K	A/Hong Kong/1774/99 (H3N2)
	627	E	K	Many H5N1 isolates (i.e. A/Hong Kong/483/97, A/Hong Kong/213/03, A/Vietnam/1203/04)
PA	142	K	N, E†	A/Hong Kong/156/97 (H5N1)‡, A/Vietnam/1203/04 (H5N1)
	421	S	I	A/Vietnam/1203/04 (H5N1), A/Vietnam/1204/04 (H5N1)
NP	33	V	I	A/Brevig Misson/1/18 (H1N1), A/Hong Kong/1774/99 (H3N2), A/Wisconsin/10/98 (H1N1), many 2006 Eurasian isolates (H5N1)
	100	R	V	A/Wisconsin/10/98 (H1N1)
			I§	A/Brevig Misson/1/18 (H1N1)
	283	L	P	A/Brevig Misson/1/18 (H1N1), A/Hong Kong/156/97 (H5N1)‡
	357	Q	K	A/Brevig Misson/1/18 (H1N1), A/Wisconsin/10/98 (H1N1), A/Thailand/5(KK494)/04 (H5N1)

*Human isolates originated from avian viruses possessing a human-like amino acid or those possessing the corresponding gene segment from an avian virus.

†Two H5N1 human isolates (A/Hong Kong/156/97 and A/Vietnam/1203/2004) encode Glu at position 142 of PA, whereas most human influenza viruses possess Asn at this position.

‡A mixed population of avian- and human-like amino acids was observed at positions 142 of PA and 283 of NP for A/HK/156/97 virus in the ISD.

§A/Brevig Misson/1/18 (1918 pandemic) virus encodes Ile at position 100 of NP, whereas most human isolates possess Val at this position.

amino acids in their PB2, PA or NP proteins in the genetic background of the avian H5N1 virus A/chicken/Vietnam/NCVD5/2003 (H5N1; VD5), which was kindly provided by the Centers for Disease Control and Prevention (CDC). We selected this virus because of its low pathogenicity in mice, despite possessing a virulent-type HA (Hatta *et al.*, 2007). All experiments with live viruses and with transfectants generated by reverse genetics were performed in a biosafety level 3 containment laboratory approved for such use by the CDC and by the US Department of Agriculture.

After transfection of 293T cells (kindly provided by Dr Tadashi Matsuda) with plasmids for generating influenza virus (Neumann *et al.*, 1999), virus in the supernatant was harvested 18 h post-transfection to minimize virus replication in mammalian cells, and then amplified in 10-day-old embryonated eggs to make virus stock. Most mutants were rescued successfully and grew well in eggs, although three NP mutant viruses, VD5-NP-100I, VD5-NP-100V and VD5-NP-283P, were not viable (Table 2).

To determine the effect of introducing a single human-like amino acid in PA, PB2 or NP into an avian H5N1 virus on its pathogenicity in mice, we examined the dose lethal to 50 % of mice (MLD_{50}). Based on the MLD_{50} values, we categorized the mutant viruses into three groups (Table 2). The first group included four mutants, PB2-368K, PA-142E, NP-33I and NP-357K, whose MLD_{50} values were $>10^6$ p.f.u. The second group comprised PB2-9N, PB2-199S, PA-142N and PA-421I, which showed intermediate pathogenicity (MLD_{50} values $>10^5$, but $<10^6$ p.f.u.). The third group contained PB2-627K, whose MLD_{50} was 41 p.f.u., representing a significant increase in pathogenicity in mice, as has been reported previously (Fornek *et al.*, 2009; Hatta *et al.*, 2001, 2007; Maines *et al.*, 2005). Thus, the following substitutions of avian-like amino acids with the respective human-like amino acids increased the pathogenicity of the VD5 virus in mice: PB2-9N, PB2-199S, PA-142N, PA-421I and PB2-627K.

To examine whether additional mutations were introduced into the mutant viruses during replication in mice, we collected lungs from 16 dead mice infected with the mutant viruses and attempted to isolate virus with Madin–Darby canine kidney (MDCK) cells (kindly provided by Dr Robert G. Webster) for sequencing. We successfully isolated and

Table 2. Biological properties of mutant VD5 viruses with human-like amino acids in their PB2, PA or NP genes

Titres of stock virus grown in eggs (p.f.u. ml^{-1}) are shown. Mutant viruses possessing human-like amino acids in the genetic background of VD5 were generated by reverse genetics as described previously (Neumann *et al.*, 1999). Rescued viruses were amplified in eggs and their virus titres were determined by plaque assays in MDCK cells. To determine the MLD_{50}, we infected 6-week-old female BALB/c mice intranasally with 50 µl of serial 10-fold dilutions of virus and observed them for 2 weeks. –, Virus was not rescued; ND, not determined.

Virus	Mutations in:			Titre (p.f.u. ml^{-1})	MLD_{50} (p.f.u.)
	PB2	PA	NP		
VD5				2.6×10^7	$>10^6$
VD5-PB2-9N	D9N			1.8×10^8	8.9×10^5
VD5-PB2-199S	A199S			6.6×10^7	3.3×10^5
VD5-PB2-368K	R368K			1.9×10^8	$>10^6$
VD5K	E627K			2.6×10^8	41
VD5-PA-142N		K142N		1.6×10^8	2.5×10^5
VD5-PA-142E		K142E		3.3×10^8	$>10^6$
VD5-PA-421I		S421I		4.9×10^7	5.7×10^5
VD5-NP-33I			V33I	9.1×10^7	$>10^6$
VD5-NP-100V			R100V	–	ND
VD5-NP-100I			R100I	–	ND
VD5-NP-283P			L283P	–	ND
VD5-NP-357K			Q357K	3.0×10^8	$>10^6$
VD5K-PB2-9N	E627K/D9N			3.9×10^8	11
VD5K-PB2-199S	E627K/A199S			4.3×10^8	6.8
VD5K-PB2-368K	E627K/R368K			3.7×10^8	1.0
VD5K-PA-142N	E627K	K142N		6.0×10^8	1.7
VD5K-PA-142E	E627K	K142E		3.4×10^8	5.4
VD5K-PA-421I	E627K	S421I		2.6×10^8	7.3
VD5K-NP-33I	E627K		V33I	2.5×10^8	41
VD5K-NP-100V	E627K		R100V	5.8×10^8	$>10^6$
VD5K-NP-100I	E627K		R100I	4.1×10^8	$>10^6$
VD5K-NP-283P	E627K		L283P	5.0×10^8	4.5×10^3
VD5K-NP-357K	E627K		Q357K	2.8×10^8	0.8

sequenced the entire genomes of 12 viruses. We confirmed that all of these viruses possessed the human-like amino acid that was originally introduced. Interestingly, we found that six of the 12 viruses had the PB2-627K mutation in addition to the original human-like amino acid (data not shown). These data suggest that avian H5N1 viruses with a human-like amino acid may require the PB2-627K mutation to replicate efficiently in mice and that the MLD_{50} values of the mutant viruses described above represent the sum of the effect of the intended human-like amino acids and the PB2-627K substitution that was introduced during replication in mice.

To examine the combined effect of introducing selected human-like amino acids and PB2-627K on pathogenicity in mice, we generated viruses possessing these two mutations (Table 2; VD5 with the PB2-627K mutation was designated VD5K). All of the mutant viruses, including three NP mutants that were not rescued without the PB2-627K mutation, were rescued successfully and replicated well in eggs. Titres of all stock viruses with double mutations were $>10^8$ p.f.u. (Table 2), suggesting that these host-specific signature amino acids are compatible in the genetic background of an avian H5N1 virus.

To examine whether the combination of a human-like amino acid and PB2-627K contributes to pathogenicity in mice, we calculated MLD_{50} values of viruses possessing such mutations (Table 2). Two NP mutants, VD5K-NP-100I and VD5K-NP-100V, were attenuated significantly and did not kill any mice, suggesting that the human-like amino acids at position 100 in NP have a negative effect on pathogenicity in mice, even though the viruses could replicate well in eggs. VD5K-NP-283P was also attenuated and its MLD_{50} value was 100-fold higher than that of VD5K, whereas the pathogenicity of VD5K-NP-33I was comparable to that of VD5K in mice. In contrast, all of the PA and PB2 mutants, as well as the NP-357K mutant, had increased pathogenicity in mice: the MLD_{50} values of VD5K-PB2-368K, VD5K-PA-142N, VD5K-PA-142E and VD5K-NP-357K were reduced by approximately 41, 24, 7.6 and 51 times, respectively (Table 2). Three human-like amino acids, PB2-368K, PA-142E and NP-357K, showed no change in pathogenicity when they were introduced into VD5 alone, suggesting that these three human-like amino acids affect virus pathogenicity in mice only when they co-exist with PB2-627K.

To examine the effect of these mutations on virus growth in mice, we collected organs from infected mice on days 1 and 3 post-infection for virus titration. No virus was recovered from mice infected with 100 p.f.u. of a single human-like amino acid mutation (data not shown). In contrast, all of the VD5K mutant viruses that possessed PB2-627K and an additional human-like amino acid replicated well in mouse lungs and their lung titres were comparable to those of the VD5K-infected mice (Table 3). Interestingly, lung titres of mice infected with VD5K-PA-142E virus were significantly higher on day 1 post-infection than those of mice infected with other VD5K mutants. These results suggest a potential role for Glu at position 142 of PA, which occurs in two H5N1 human isolates from 1997 and 2004, in efficient replication and high virulence in mice in combination with PB2-627K.

Table 3. Growth properties in mice of mutant VD5 viruses possessing human-like amino acids

Mice (three per group) were infected intranasally with 100 p.f.u. virus. Organs were collected from infected mice on days 1 and 3 post-infection (p.i.) for virus titration. Individual titres were recorded when virus was not recovered from all three animals. –, Virus was not recovered (detection limit for lung and nasal turbinates was <1.6 $\log_{10}$ p.f.u. g^{-1} and for brain was <1.1 $\log_{10}$ p.f.u. g^{-1}).

Virus	Day p.i.	Virus titre (mean ± SD $\log_{10}$ p.f.u. g^{-1}) in:		
		Lung	Nasal turbinates	Brain
VD5	1	–	–	–
	3	–	–	–
VD5K	1	4.7 ± 0.9	–	–
	3	7.0 ± 0.4	3.0 ± 0.7	–
VD5K-PB2-9N	1	4.9 ± 0.5	–	–
	3	7.0 ± 0.2	2.9, 6.0	–
VD5K-PB2-199S	1	4.8 ± 0.3	–	–
	3	7.1 ± 0.3	2.2	–
VD5K-PB2-368K	1	3.9 ± 0.3	–	–
	3	6.9 ± 0.6	3.9	–
VD5K-PA-142E	1	7.0 ± 0.3*	2.3	–
	3	7.5 ± 0.1	3.7, 4.4	1.4
VD5K-PA-142N	1	5.7 ± 0.5	–	–
	3	7.2 ± 0.4	5.7, 5.2	–
VD5K-PA-421I	1	4.7 ± 0.4	–	–
	3	6.8 ± 0.1	–	–
VD5K-NP-33I	1	4.0 ± 0.3	–	–
	3	6.3 ± 0.1	4.8	–
VD5K-NP-357K	1	4.6 ± 0.1	–	–
	3	6.2 ± 0.2	2.2	–

*The virus titre in lungs of mice infected with VD5K-PA-142E was significantly higher than that of VD5K-infected mice on day 1 p.i., as determined by Student's *t*-test ($P<0.02$).

In this study, to examine the role of host lineage-specific amino acids in the pathogenicity of H5N1 avian influenza viruses in a mammalian host, we generated mutant viruses that possessed conserved amino acids in the PB2, PA and/or NP of human influenza isolates ('human-like amino acids') in the genetic background of an avian H5N1 virus of low pathogenicity in mice, and tested their pathogenicity in a mouse model. We found that some of the mutants possessing human-like amino acids acquired increased pathogenicity in mice (Table 2), suggesting roles for host lineage-specific amino acids, in addition to the amino acid at position 627 of PB2 (Hatta *et al.*, 2001), in the pathogenicity of H5N1 avian influenza viruses in mammals.

Our sequence analyses revealed that two H5N1 human isolates (A/Hong Kong/156/97 and A/Vietnam/1203/2004) encode Glu at position 142 of PA (Table 1), whereas the PA of most avian and human viruses possesses Lys and Asn at position 142, respectively (Macken *et al.*, 2001). We demonstrated that the Lys-to-Glu mutation at position 142 of PA resulted in enhanced replication and pathogenicity in mice when combined with the PB2-627K mutation (Tables 2 and 3), suggesting a potential role of PA-142E in adaptation and pathogenicity of H5N1 viruses in a mammalian host. The mechanism by which PA-142E supports increased replication in mice at an early time point (day 1; Table 3) is not clear. However, one possibility is that this substitution may cause conformational changes in the PA protein, affecting the structure of the N-terminal residues of the PA subunit, which were recently shown to be involved in promoter binding (Kashiwagi *et al.*, 2009) and contain the cap-snatching endonuclease active site (Dias *et al.*, 2009).

We were unable to rescue three of the NP mutants in the VD5 background (Table 2). However, in the presence of the PB2-627K mutation, viruses with these NP mutations were viable. We also showed that some of the human-like amino acid mutants (i.e. PB2-R368K, PA-K142E and NP-Q357K) enhanced pathogenicity in mice only in combination with PB2-627K. These results highlight the critical contribution of PB2-627K to avian virus replication in mammals, as well as the contribution of other human-like amino acids identified in this study.

An H5N1 pandemic in humans has not yet occurred, but we can assume that the gradual acquisition of a number of amino acid changes in viral proteins that are important for host restriction (i.e. HA, polymerase proteins and NP; Gabriel *et al.*, 2005; Harvey *et al.*, 2004; Matrosovich *et al.*, 2004; Scull *et al.*, 2009) would promote adaptation of H5N1 viruses to humans and make an H5N1 pandemic more likely. To be fully prepared for such an event, it is important to strengthen worldwide surveillance to keep track of these amino acid changes in H5N1 viruses.

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