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

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SUMMARY

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 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 (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2009_07_01/en/index.html). Nonetheless, H5N1 viruses have not caused a pandemic in humans yet because of their inefficient human-to-human transmission (Maines *et al*., 2006; Yen *et al*., 2007). By

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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, which is 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 have 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 a 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.

Identification of host lineage-specific amino acids that distinguish human from avian virus isolates

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 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 as 'human-like' or 'avian-like' amino acids, respectively (Supplementary table 1).

Host lineage-specific amino acids were mostly found in the viral RNA polymerase complex components, PB2, PA and NP (Supplementary table 1), 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 the 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, while PB1 gene is of human origin and the 5 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; Taubenberger *et al*., 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 in 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, since we found that two H5N1 human isolates (A/Hong Kong/156/97 and A/Veitnam/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 viral adaptation in humans. Based on this approach, we chose 12 out of the 75 human-like amino acids (4 in PB2, 3 in PA and 5 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).

Generation of mutant viruses possessing a single human-like amino acid in their PA, PB2, or NP proteins in the genetic background of an avian H5N1 virus

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 the test human-like amino acids in their PB2, PA or NP 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. 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 Centers for Disease Control and Prevention and by the U.S. Department of Agriculture.

After transfection of 293T cells with plasmids for generating influenza virus (Neumann *et al*., 1999), virus in the supernatant was harvested at 18 hours 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 successfully rescued 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).

Pathogenicity of mutant VD5 viruses possessing a single human-like amino acid in PA, PB2 or NP in mice

To determine the effect of introducing a human-like amino acid 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}s, 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}s were more than 10^6 PFU. The second group comprised PB2-9N, PB2-199S, PA-142N and PA-421I, which showed intermediate pathogenicity (MLD_{50}s > 10^5 PFU, but < 10^6 PFU). The third group contained PB2-627K whose MLD_{50} was 41 PFU, representing a significant increase in pathogenicity in mice, as has been previously reported (Fornek *et al*., 2009;Hatta *et al*., 2001;Hatta *et al*., 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.

Generation of VD5 viruses possessing a second human-like amino acid mutation in addition to 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 MDCK cells for sequencing. We successfully isolated and 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 6 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}s 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 as 'VD5K'). All of the mutant viruses, including three NP mutants that were not rescued without the PB2-627K mutation, were successfully rescued and replicated well in eggs. Titers of all stock viruses with double mutations were greater than 10^8 PFU (Table 2), suggesting that these host-specific signature amino acids are compatible in the genetic background of an avian H5N1 virus.

Pathogenicity in mice of VD5 possessing a second human-like amino acid mutation in addition to PB2-627K

To examine whether the combination of a human-like amino acid and PB2-627K contributes to pathogenicity in mice, we calculated MLD_{50}s of viruses possessing such mutations (Table 2). Two NP mutants, VD5K-NP-100I and VD5K-NP-100V, were significantly attenuated and did not kill any mice, suggesting that the human-like amino acids of NP at position 100 have a negative effect on the 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 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 approximately 41, 24, 7.6 and 51 times, respectively (Table 2). Two human-like amino acids, PB2-368K and NP-357K, showed no change in pathogenicity when they were introduced into VD5 alone, suggesting that these two human-like amino acids affect viral pathogenicity in mice only when they co-exist with PB2-627K.

Growth in mouse organs of VD5 mutant viruses with human-like amino acids

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 PFU 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 titers were comparable to those of the VD5K-infected mice (Table 3). Interestingly, lung titers 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.

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 a 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/Veitnam/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 (Table 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 the 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, which 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

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

Protein	Amino acid position	Avian-like amino acid	Human-like amino acid	Human isolates originated from avian viruses possessing a human-like amino acid or those possessing the corresponding gene segment from an avian virus
PB2	9	D	N	A/Hong Kong/482/97 (H5N1), A/Hong Kong/483/97 (H5N1)
	199	A	S	A/Brevig Misson/1/1918 (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/Vietman/1204/04 (H5N1)
NP	33	V	I	A/Brevig Misson/1/1918 (H1N1), A/Hong Kong/1774/99 (H3N2), A/Wisconsin/10/98 (H1N1), many 2006 European isolates (H5N1)
	100	R	V I‡	A/Wisconsin/10/98 (H1N1) A/Brevig Misson/1/1918 (H1N1)
	283	L	P	A/Brevig Misson/1/1918 (H1N1), A/Hong Kong/156/97 (H5N1)§
	357	Q	K	A/Brevig Misson/1/1918 (H1N1), A/Wisconsin/10/98 (H1N1), A/Thailand/5(KK494)/04 (H5N1)

*We analyzed protein sequences in The Influenza Sequence Database 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.

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

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

§A mixed population of avian- and human-like amino acids was observed at position 142 of PA and 283 of NP for A/HK/156/97 virus in the Influenza Sequence Database.

Table 2

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

Virus	Mutations			Titers of stock virus grown in eggs (PFU/ml)*	MLD_{50}(PFU)†
	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

*Mutant viruses possessing human-like amino acids in the genetic background of VD5 generated by reverse genetics as described previously (Neumann *et al*., 1999). Rescued viruses were amplified in eggs and their virus titers were determined by plaque assays in MDCK cells. -, virus was not rescued.

† To determine the dose lethal to 50% of mice (MLD_{50}), we infected 6-week-old female Balb/c mice intranasally with 50μl of serial 10-fold dilutions of virus and observed them for 2 weeks. ND, not determined.

Table 3

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

Virus	Days post-infection	Virus Titer (mean log_{10} PFU ± SD/g) 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	-

*Mice (3 per group) were infected intranasally with 100 PFU of virus. Organs were collected from infected mice on days 1 and 3 post-infection for virus titration. Individual titers were recorded when virus was not recovered from all three animals.

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

-, Virus was not recovered (detection limit for lung and nasal turbinates is < 1.6 log_{10} PFU/g and for brain is <1.1 log_{10} PFU/g).