JOURNAL OF VIROLOGY, May 2009, p. 4704–4708 Vol. 83, No. 9
0022-538X/09/$08.00+0 doi:10.1128/JVI.01987-08

Neuraminidase Stalk Length and Additional Glycosylation of the Hemagglutinin Influence the Virulence of Influenza H5N1 Viruses for Mice▽

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Received 19 September 2008/Accepted 3 February 2009

Following circulation of avian influenza H5 and H7 viruses in poultry, the hemagglutinin (HA) can acquire additional glycosylation sites, and the neuraminidase (NA) stalk becomes shorter. We investigated whether these features play a role in the pathogenesis of infection in mammalian hosts. From 1996 to 2007, H5N1 viruses with a short NA stalk have become widespread in several avian species. Compared to viruses with a long-stalk NA, viruses with a short-stalk NA showed a decreased capacity to elute from red blood cells and an increased virulence in mice, but not in chickens. The presence of additional HA glycosylation sites had less of an effect on virulence than did NA stalk length. The short-stalk NA of H5N1 viruses circulating in Asia may contribute to virulence in humans.

Sustained circulation of subtype H5 or H7 influenza viruses in terrestrial poultry often selects for viruses whose hemagglutinin (HA) has acquired additional glycosylation sites and whose neuraminidase (NA) stalks become shorter (2–4). These changes affect multicycle growth of viruses in cultured mammalian and avian cells (2, 22). Replication efficiency of influenza viruses in embryonating eggs and mice correlated with NA stalk length (5). These changes in the surface glycoproteins can, but do not always, correlate with virulence in chickens (3, 4, 16). Nearly all of the human H5N1 influenza virus infections have been acquired from infected domestic poultry, and the viruses isolated from the two species are very closely related (24). Therefore, human H5N1 viruses have glycosylated HAs and a shortened NA stalk.

We sought to determine the significance of these changes for nonaquatic bird hosts and their effect on virulence in mammalian hosts; we examined their frequency among H5N1 viruses isolated from different species between 1996 and 2007 and evaluated the virulence and infectivity in mice of reverse genetics-derived H5N1 viruses engineered with 0 to 3 glycosylation sites on the HA paired with a short- or long-stalk NA. We also examined the stability of these motifs on passage in mice.

Sequences for NA genes of H5N1 viruses isolated between 1996 and 2007 from avian species, including gallinaceous poultry, such as chickens and related land-based species, domestic waterfowl, such as ducks and geese, other bird species commonly traded in poultry markets, and wild birds, were retrieved from the National Center for Biotechnology Information database, and the frequency of NA genes with a short stalk was calculated for each year. A majority (12 out of 18) of the sequences from 1996 to 1999 were from viruses isolated in mainland China or Hong Kong in 1997; the Hong Kong viruses had a short NA stalk, whereas mainland China had a mix of both viruses. The percentage of viruses with a short NA stalk dramatically increased in 2002, and by 2007, all avian isolates, including those from wild birds, had short NA stalks (Table 1). It is possible that viruses with a long NA stalk have a selective advantage in aquatic birds, but once the short NA stalk is selected in H5N1 viruses in terrestrial poultry, it can be stably maintained after transmission into farmed aquatic birds, suggesting that while a short-stalk NA is advantageous in poultry, it is neutral in wild birds.

Among 162 NA sequences of human H5N1 isolates from 1996 to 2007, only two viruses isolated in Hong Kong in 2003 contained a long-stalk NA. Three of seven isolates from pigs contained a short-stalk NA. The H5N1 viruses isolated from other mammalian hosts, including cats, leopards, tigers, and dogs, had short NA stalks, likely because they were acquired from infected poultry (11).

A reverse genetics system for the influenza A/Hong Kong/486/97 (H5N1) virus was established (6, 12), and a 19-amino-acid (aa) sequence from the NA stalk of A/goose/Guangdong/1/96 (H5N1) was inserted into the 486NA plasmid. Three potential N-linked glycosylation sites (at asparagine residues at aa 131, aa 158 and aa 169, using H3 numbering) are frequently present within or near the receptor binding site of the H5 HA and were selected to assess their effect on viral pathogenesis

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▽ Published ahead of print on 18 February 2009.

TABLE 1. Distribution of H5N1 viruses with NA sequences with shortened stalks isolated from different species from 1996 to 2007[a]

Year(s)	% H5N1 viruses isolated from[b]:			
	Gallinaceous poultry (n = 466)	Domestic waterfowl (n = 328)	Wild birds (n = 172)	Humans (n = 162)
1996 to 1999	75	50	NA	100
2000	NA	16	NA	NA
2001	42	45	NA	NA
2002	89	67	67	NA
2003	95	94	71	33
2004	97	71	84	100
2005	100	100	90	100
2006	100	94	98	100
2007	100	100	100	100
1996 to 2007	92	81	90	99

[a] Sequence data was obtained from National Center for Biotechnology Information (NCBI).
[b] Each number is shown as a percentage of H5N1 viruses isolated in the indicated year that had a short NA stalk. NA, no sequence data available.

and host adaptation (13). Consensus sequons for glycosylation at aa 131 were noted in H5N9, H5N3, and H5N2, but not in H5N1 viruses. Glycosylation at aa 158 was reported to influence the antigenicity of viruses isolated in Hong Kong in 1997 (13), and approximately 50% of the HAs of H5N1 viruses derived from the A/goose/Guangdong/96 progenitor are glycosylated at this site. Glycosylation at aa 169 was highly conserved and more than 95% of H5 viruses contain this glycosylation site. Viruses lacking all three glycosylation sites, with only one or two of the three, or with all sites present were constructed in combination with either a short-stalk NA or a reconstructed long-stalk NA. Viruses recovered by reverse genetics were amplified in the allantoic cavities of 10- to 11-day-old embryonating hen's eggs and were designated based on the glycosylation patterns of HA and the length of NA stalk (Table 2). The mutation to add glycosylation at aa 131 entailed a D131N change because it was the only option to add a potential glycosylation motif at this site. To add a potential glycosylation motif at aa 158, there were two options—to modify aa 160 to T or to S. Among the viruses with glycosylation at this position, T and S were almost equally distributed, and we selected the A160T change. The potential glycosylation at aa 169 is highly conserved among H5N1 viruses, and we selected an N169S substitution with a small neutral aa to eliminate this glycosylation motif.

TABLE 2. Generation of A/Hong Kong/486/97 (H5N1) mutant viruses

Virus	HA glycosylation site at aa:			NA stalk	
	131	158	169	Long[b]	Short
486-none-L				+	
486-131-L	+			+	
486-158-L		+		+	
486-169-L			+	+	
486-131/169-L	+		+	+	
486-158/169-L		+	+	+	
486-131/158/169-L	+	+	+	+	
486-none-S					+
486-131-S	+				+
486-158-S		+			+
486-169-S[a]			+		+
486-131/169-S	+		+		+
486-158/169-S		+	+		+
486-131/158/169-S	+	+	+		+

[a] 486-169-S represents the wt A/Hong Kong/486/97 (H5N1) virus.
[b] Long NA stalk contains a 19-aa insertion in the stalk of the NA.

TABLE 3. Enzymatic parameters measured for the short- and long-stalk NA

A/Hong Kong/486/97 NA type	K_m (μM)[a]	V_{max} (fluo AU/s)[a,b]	V_{max} ratio[c]
Short stalk	59 ± 7	0.76 ± 0.12	5.8
Long stalk	50 ± 11	0.13 ± 0.02	5.8

[a] The results are given as the mean ± standard deviation from two (short-stalk NA) or three (long-stalk NA) independent determinations, on duplicate samples using two independent plasmid clones for NA expression.
[b] The difference in V_{max} was found to be significant ($P < 0.001$) using Student's t test. fluo AU, fluorescence units.
[c] V_{max} ratio of short-stalk NA to long-stalk NA.

NA stalk length. The function of the reconstructed long-stalk NA was examined. A fluorogenic enzymatic assay was performed, using the small MUNANA [2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid] substrate on cells transiently expressing the NA, as described previously (17). The Michaelis-Menten constants (K_m) that reflect the affinity for the substrate were very similar (59 ± 7 and 50 ± 11 μM) for the short- and long-stalk NAs, respectively, and were in the range reported for the N1 NA of influenza A viruses (25 to 100 μM) (10, 23). The maximal velocity of reaction (V_{max}), which is determined by both the specific activity and the amount of enzyme in the reaction, was about fivefold higher for the short-stalk NA than for the long-stalk NA ($P < 0.001$) (Table 3). To establish the surface expression of NA, fluorescence-activated cell sorter analysis was performed using a rabbit polyclonal antibody raised against total A/New Caledonia/20/99 (H1N1) virions diluted 1:150. This dilution corresponded to saturating levels, as similar signals were observed when dilutions at 1:400 and 1:150 were compared in preliminary experiments. Similar levels of the short- and long-stalk NAs were detected at the surface of transfected cells (data not shown). However, an underestimation of the levels of short-stalk NA due to a lower accessibility of the NA head to the anti-NA antibodies used for fluorescence-activated cell sorter analysis cannot be excluded. Earlier studies showed that a similar deletion in an N2 NA did not affect recognition of the NA head by a series of monoclonal antibodies, or the efficiency of small substrate catalysis (8), suggesting possible subtype or strain differences in the functional consequences of the stalk deletion.

Elutions of the A/Hong Kong/486/97 (H5N1) virus with a glycosylation site at aa 169 (486-169) and the A/Hong Kong/486/97 (H5N1) virus with glycosylation sites at aa 131, 158, and 169 (486-131/158/169) (Table 2) with a short- or long-stalk NA from erythrocytes were also compared. Viruses were adsorbed on chicken erythrocytes at 4°C for 1 h, and the release at 37°C was monitored for 6 h (Fig. 1). Elution of the 486-131/158/169 and 486-169 long-stalk viruses was complete after only 1 and 3 h, respectively. In contrast, the short-stalk viruses were not even partially eluted after a 6-h incubation period at 37°C. In

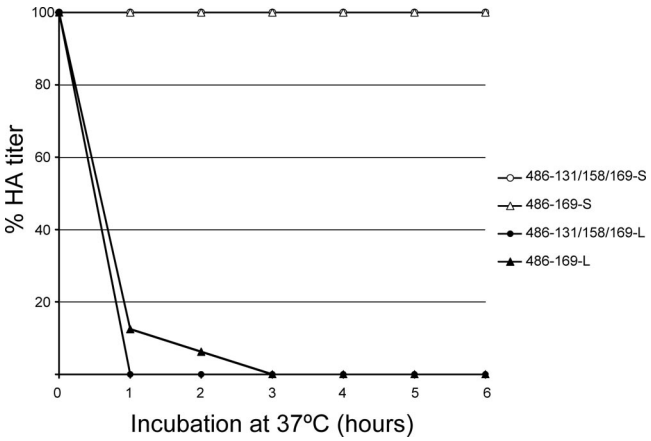

FIG. 1. Decreased elution from chicken erythrocytes of viruses with a short-stalk NA compared to that from chicken erythrocytes of viruses with a long-stalk NA. The 486-131/158/169 (circles) and 486-169 (triangles) viruses with a short- (open symbols) or long-stalk (closed symbols) NA were adsorbed to a 1% suspension of chicken erythrocytes at 4°C for 30 min, and the HA titer at 37°C representing virus elution from chicken erythrocytes was monitored each hour for 6 h (*x* axis). The HA titer following incubation at 37°C is expressed as a percentage of the HA titer at time zero at 4°C (*y* axis).

control elution experiments performed in parallel at 4°C and in the absence of calcium, conditions under which the NA is inactive, none of the viruses were eluted. These data, taken together with the observation that the activity of the short-stalk NA on the small MUNANA substrate is not reduced, suggest that the accessibility of the active site of the short-stalk NA to the sialic acid present at the cell surface is markedly reduced.

The effects of the NA stalk length and presence of potential HA glycosylation sites on virulence for 6- to 8-week-old female BALB/c mice were evaluated by determining the 50% mouse lethal dose (MLD_{50}) and the 50% mouse infectious dose (MID_{50}) and by monitoring the body weights of the mice for 14 days after intranasal virus inoculation as described previously (6, 7, 18). The short-NA-stalk viruses with glycosylation sites at aa 131, aa 169, or both were about 10-fold more virulent than were the corresponding viruses with long NA stalks (Table 4). Remarkably, the short-NA-stalk viruses with no HA glycosylation were 10,000-fold more virulent than were the long-stalk-NA counterparts. All viruses with long-stalk NAs had MLD_{50} values of $>10^{7.0}$ 50% egg infectious doses (EID_{50}) (Table 4). Although most groups of mice exhibited up to 25% weight loss between days 6 and 12 postinfection at high inoculum doses (10^6 to 10^7 EID_{50}), there was no significant difference in the pattern or degree of weight change between viruses with long and short NA stalks (data not shown). Except for the virus with no HA glycosylation, MID_{50} values revealed no apparent effect of NA stalk length on infectivity for mice, and there was no significant difference in virus replication and recovery of infectious virus from different organs (data not shown).

HA glycosylation. Among viruses with a short NA stalk, viruses with fewer glycosylation sites were more virulent (MLD_{50}) than viruses with a highly glycosylated HA. The wild-type (wt) 486-169 virus was less virulent than the A/Hong Kong/486/97 (H5N1) virus with no glycosylation sites (486-none) but more virulent than 486-158/169 and 486-131/158/169. Among viruses with the same number of glycosylation sites, those with a glycosylation site at aa 158 were relatively less virulent ($MLD_{50} = 10^{6.7} \sim 10^{8.37}$ EID_{50}) than those without this glycosylation site ($MLD_{50} = 10^{4.82} \sim 10^{6.2}$ EID_{50}) (Table 4). This pattern was not seen among viruses with a long-stalk NA. It was previously shown that loss of carbohydrate at aa 158 increased the binding affinity of the H5 HA (13) and reduced the dependence of an H7 or H1 HA expressed in CV-1 cells on treatment with *Vibrio cholerae* NA for hemadsorbing activity (14). Our observation that additional glycosylation sites on the HA protein resulted in decreased virulence in mice is consistent with a report that increased glycosylation of human H3N2 influenza viruses results in decreased virulence in mice, mediated by lung surfactant protein (21).

Virulence for poultry. The pathogenicity of four mutant viruses with either none or all three targeted glycosylation sites in the HA in combination with a short or long NA stalk was evaluated in 4-week-old specific-pathogen-free White Leghorn chickens. Unlike in mice, there was no significant difference in the virulence of these viruses for chickens; the viruses were uniformly lethal for chickens at a dose of 10^6 EID_{50}, presumably because a highly cleavable HA was present in the four mutant viruses and because this virulence determinant was sufficient for lethality. The mean chicken 50% lethal doses

TABLE 4. Effect of HA glycosylation and the length of NA stalk on infectivity and virulence in mice

Virus	MLD_{50} (log_{10})[a]			MID_{50} (log_{10})[b]		
	Short NA	Long NA	Difference (long NA − short NA)	Short NA	Long NA	Difference (long NA − short NA)
486-none	4.82[c]	9.2	4.38	0.2	3.7	3.5
486-131	6.2	7.32	1.12	1.46	1.45	−0.1
486-158	7.41	7.2	−0.21	1.96	1.46	−0.5
486-169	5.53	>7.0[d]	>1.47	2.2	1.05	−1.15
486-131/169	6.2	>7.7[d]	>1.5	0.46	0.96	0.5
486-158/169	6.7	7.12	0.42	1.2	1.66	−0.46
486-131/158/169	8.37	>9.0[d]	>0.63	3.26	2.26	−1.0

[a] MLD_{50} was determined by inoculating groups of five mice with serial 10-fold dilutions of virus stock.
[b] MID_{50} was determined by inoculating groups of five mice with serial 10-fold dilutions of virus stock and by determining the presence of virus in lung at 3 days postinfection.
[c] MID_{50} and MLD_{50} titers were calculated by the method of Reed and Muench (18) and were expressed as the EID_{50} value corresponding to 1 MID_{50} or MLD_{50}.
[d] Highest achievable dose for inoculation of mice due to reduced virus yields in culture.

TABLE 5. Effect of HA glycosylation and the length of NA stalk on infectivity and virulence in chickens

Virus	Mean death time (days)[a,b]	Virus detection in oropharyngeal swabs[b]	
		No. of positive results/total	Mean titer ($\log_{10}$ EID_{50}/ml)
wt A/Hong Kong/486/97 (H5N1)	2.8*	2/5	≤1.8*
486-none-S	3.3*	2/5	≤2.1*
486-none-L	6.4**	1/5	≤1.3*
486-131/158/169-S	3.1*	5/5	5.3**
486-131/158/169-L	3.0*	5/5	5.1**

[a] Mean death time and viral shedding by RT-PCR in samples collected 3 days postinfection in chickens intranasally inoculated with 10 50% chicken lethal doses of the indicated virus.

[b] Different number of asterisks indicate statistically significant differences between treatment groups ($P \leq 0.05$) after pairwise comparison using the t test or the Mann-Whitney rank sum test, depending on data normality.

were similar to those of the parent virus ($10^{2.4}$ EID_{50}): $10^{2.0}$, $10^{2.4}$, $10^{1.8}$, and $10^{1.9}$ EID_{50} for 486-none with a long-stalk NA (486-none-L), 486-none with a short-stalk NA (486-none-S), 486-131/158/169-L, and 486-131/158/169-S, respectively. Increased glycosylation of the HA protein did not affect the mean death time (Table 5) in association with short-stalk NA, but the mean death time was significantly longer for chickens infected with the virus that had an NA protein with a long stalk and an HA protein with no glycosylation at positions 131, 158, and 169. This suggests that, in addition to a multibasic HA cleavage site, the presence of a truncated NA protein may contribute to a shorter death time. To determine the effect of HA glycosylation and NA stalk length on virus replication in the respiratory tract of chickens, nucleic acid was extracted from oropharyngeal swabs obtained 2 days postinoculation, and viral M gene RNA copies were quantified by real-time reverse transcription-PCR (20). In general, the virus shedding data suggested a trend toward increased viral replication when the HA protein had numerous glycosylation sites, irrespective of the NA protein stalk length (Table 5). The virus with a long-stalk NA and an HA protein with no glycosylation sites was seldom detected in oropharyngeal swabs.

Stability of mutant HA and NA upon passage in mice. The stability of the engineered HA and NA genes was studied following multiple cycles of replication in mice; the HA and NA genes from 115 samples of lung, nasal turbinate, brain, and spleen from mice infected 4, 7, or 10 days earlier with different viruses were amplified and sequenced. With only one exception, the sequences of the HA and NA genes were unchanged; a sample from the lungs of a mouse infected with the 486-131/169-S virus had a coding change at residue 158 of the HA from aspartic acid (D) to asparagine (N), resulting in acquisition of a potential glycosylation site at aa 158. The deletion in the NA stalk was stably maintained. Thus, these changes are stably maintained in mice.

The functional basis for the selective advantage of the variants with a shorter NA stalk in poultry is still unclear, although in our study, a long-stalk NA in combination with a lack of glycosylation sites on the HA lengthened mean death times and reduced virus shedding in chickens. In vitro, a long-stalk NA was reported to be the optimal combination for an H7 HA lacking glycosylation, and a short-stalk NA was optimal for an HA with glycan at aa 158 (2). In contrast, in our study with the A/Hong Kong/486/97 (H5N1) virus, the increase in infectivity and virulence in mice conferred by a short NA stalk was most apparent when the HA had no glycosylation sites at aa 131, aa 158, and aa 169. Overall, the data indicate that while the short-stalk NA contributes to the virulence of the H5N1 viruses in mice, multiple parameters are involved in the functional balance between HA receptor affinity and NA sialidase activity; this is achieved differently in different viruses, and optimal balance in vitro is not necessarily optimal in vivo. Glycosylation of the HA can block recognition of the protein by antibodies (1, 19) and can also lead to a reduced receptor affinity of the HA (15). Reduced binding of avian sialic acids by HA may require a compensatory reduction in NA activity (2, 22). In agreement with previous studies (2, 8, 9), we found that shortening of the NA stalk was associated with reduced enzyme activity, as measured by the rate of virus elution from chicken erythrocytes. This reduction may be attributed to reduced accessibility of the NA active site to sialic acids present at the cell surface, because it was not observed when a small soluble MUNANA substrate of the NA was used. Whether the fivefold higher activity we measured for the short-stalk NA using MUNANA results from a higher specific activity and/or level of expression of the enzyme remains to be ascertained.

In summary, the deletion in the NA stalk and the acquisition of additional glycosylation sites on the HA are now widespread in H5N1 viruses isolated from several avian species. These features were stably maintained in experimentally infected mice, suggesting that they are not detrimental. Short-stalk-NA viruses were more virulent than long-stalk-NA viruses in mice. Glycosylation of the HA had less of an impact on virulence than did NA stalk length in mice, but such an increase in glycosylation was associated with increased quantity of virus shed from the respiratory tract of infected chickens. As H5N1 viruses have replicated in poultry and acquired glycans on the HA, the resulting HA receptor affinity was likely balanced by the shortening of the NA stalk. We conclude that the short-stalk NA of currently circulating H5N1 viruses may contribute to virulence in humans.

C.W. and M.A. were supported by Oakridge Institute for Science and Education (ORISE) fellowships. M.-A.R.-W. was supported by a fellowship from the Institut Pasteur.

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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