The Receptor Binding Specificity of the Live Attenuated Influenza H2 and H6 Vaccine Viruses Contributes to Vaccine Immunogenicity and Protection in Ferrets

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To prepare for influenza pandemics that may be caused by the H2 and H6 subtype influenza viruses, live attenuated influenza virus (LAIV) H2 and H6 vaccines are being developed and evaluated. The H2 and H6 vaccine candidates with different receptor binding preferences specified by amino acid substitutions at residues 226 and 228 were generated and evaluated for their growth in embryonated chicken eggs and their immunogenicity and protection against wild-type virus challenge in the ferret model. The viruses containing Q226 and G228 in the hemagglutinin (HA) protein bound to the avian-like α 2,3-sialic acid (SA) receptor and replicated efficiently in chicken eggs. The viruses with L226 and G228 bound preferentially to the human-like α 2,6-SA receptor. The viruses containing L226 and S228 displayed dual binding to both α 2,3-SA and α 2,6-SA receptors and replicated efficiently in eggs. The strains containing L226/G228 or L226/S228 that preferentially bound to α 2,6-SA receptors replicated efficiently in the upper respiratory tract of ferrets, induced high levels of neutralizing antibody, and conferred a high level of protection against wild-type virus challenge infection compared to the strain with the Q226/G228 residues. Our data suggest that pandemic vaccines with receptor binding preference to both avian- and human-like receptors might be desired for efficient viral replication in eggs and for inducing protective immune responses in humans.

Influenza pandemics arise when a novel influenza virus with antigenically shifted hemagglutinin (HA) enters a population with little preexisting immunity and results in widespread infection and substantially high morbidity and mortality compared with annual seasonal influenza epidemics (42). In the 20th century, novel influenza pandemic strains emerged either from interspecies transmission of the avian reservoir viruses (the 1918 H1N1 pandemic) or from the reassortments between circulating human and avian influenza viruses (1957 H2N2 and 1968 H3N2 influenza pandemics) (17). The recent 2009 pandemic emerged from a swine-origin H1N1 virus with a novel combination of gene segments (36). The highly pathogenic H5 and H7 avian viruses which occasionally cause human infection with high mortality have been considered for pandemic preparedness. However, other influenza subtypes such as H2 and H6 viruses should also be considered because of their high probability to cause pandemics.

The influenza H2 viruses caused a pandemic in 1957 and disappeared from circulation in humans in 1968. Thus, people born after 1968 are predicted to be susceptible to H2 virus infection. The 1957 H2N2 pandemic virus was a reassortant virus that derived the HA, NA, and PB1 gene segments from an avian influenza virus and the remaining gene segments from a previously circulating human H1N1 influenza virus (21, 34). The continued circulation of the H2 subtype viruses in avian reservoirs worldwide and the recent isolation of H2 viruses from pigs signal its pandemic potential (22, 24, 25, 27). Therefore, the development of an H2 influenza vaccine candidate should be considered a priority in pandemic influenza preparedness planning. Although natural human infection with H6 viruses has not been reported, some of the H6 viruses can replicate efficiently in mice and ferrets without adaptation (14). The ability of H6 viruses to cause mild clinical symptoms with virus shedding in humans following experimental infection and the existence of anti-H6 antibodies in some veterinarians suggested that human infection with H6 viruses could

occur (5, 31). Moreover, the high sequence similarity of the six internal protein gene segments and the NA gene segment of H6N1 A/teal/Hong Kong (HK)/W312/97-like viruses to those of the human H5N1 viruses, and the prevalence and frequent reassortment of H6 viruses in birds raise a concern of the possible emergence of a pandemic H6 virus (12, 16, 29).

Vaccination is the most effective method for prevention of influenza. Live attenuated influenza vaccines (LAIVs) licensed in the United States since 2003 are 6:2 reassortant viruses bearing the six internal protein gene segments from the cold-adapted A/Ann Arbor/6/60 (H2N2) virus and the HA and NA protein gene segments from the circulating wild-type (wt) viruses (30). Seasonal LAIVs offer the advantage of providing protection against antigenically drifted strains in naive hosts (2, 6, 7). This is particularly important in pandemic preparedness as a pandemic LAIV may provide greater protection against newly emerged antigenic variant viruses of the same subtype. To prepare H2 vaccines, we evaluated a number of H2 influenza viruses and identified three candidate strains that induced a broadly cross-reactive antibody response to various human and avian H2 viruses: a human H2N2 strain, A/Japan/305/57 (A/Jap/57); an avian H2N2 strain, A/mallard/New York/6750/78 (A/mal/NY/78); and a swine H2N3 strain, A/swine/Missouri/4296424/2006 (A/sw/MO/06) (8). The H2N3 A/sw/MO/06 was selected for evaluation in a phase I clinical study. Similarly, three H6 vaccine candidates were also identified and A/teal/Hong Kong/W312/97 (H6N1, tl/HK/97) was further evaluated in a clin-

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ical trial. We found that both H2 and H6 vaccine candidates elicited relatively low antibody responses in animal models and in clinical trials (9, 38).

It is well known that avian viruses preferentially bind sialic acid (SA) with an α 2,3-linkage to the penultimate galactose (α 2,3-SA), while human viruses preferentially bind to α 2,6-linked SA receptors (α 2,6-SA). Most human H2 isolates contain L226 and S228 (H3 numbering) and preferentially bind to α 2,6-SA, while avian H2 viruses contain Q226 and G228 and exhibit α 2,3-SA receptor binding specificity. Several H2 viruses isolated from pigs also contained leucine at 226 (13, 25, 28). The switch of receptor binding specificity from α 2,3-SA to α 2,6-SA is considered a prerequisite for avian viruses to infect humans. The influenza pandemics in the 19th century and the 2009 H1N1 pandemic were all caused by viruses with receptor binding preference for α 2,6-SA. In this regard, pandemic LAIVs should have the ability to bind to α 2,6-SA receptors in order to replicate sufficiently well in the upper respiratory tract of humans to induce protective immune responses.

The H2 and H6 candidate vaccines that we produced recently all contain Q226 and G228 and preferentially bind to α 2,3-SA. To evaluate whether changing the receptor binding specificity of the H2 and H6 vaccine viruses would improve virus immunogenicity, we modified the HA receptor binding specificity by engineering amino acid substitutions at HA residues 226 and 228. The effect of receptor binding change on vaccine virus replication in the host, vaccine virus antigenicity, immunogenicity, and protection against wild-type virus replication was evaluated in ferrets. Our data indicate that vaccine viruses with dual receptor binding are able to grow efficiently in embryonated chicken eggs and elicit better immune responses in ferrets than viruses with α 2,3-SA binding specificity.

MATERIALS AND METHODS

Generation of reassortant vaccine viruses. The wild-type (wt) H2 viruses A/Japan/305/57 (H2N2), A/swine/Missouri/4296424/2006 (H2N3), and A/mallard/New York/6750/78 (H2N2) and the H6 viruses A/teal/Hong Kong/W312/97 (H6N1), A/duck/Hong Kong/182/77 (H6N9), and A/mallard/Alberta/89/85 (H2N2) used in this study were described previously (8, 14). The HA and NA cDNAs were amplified by reverse transcriptase PCR (RT-PCR) using viral RNA of wt H2 or H6 viruses and cloned into the pAD3000 vector (15). To introduce specific amino acid changes into the HA, mutagenesis of the HA plasmid was performed by using the QuikChange site-directed mutagenesis kit (Agilent, La Jolla, CA). Recombinant 6:2 reassortant vaccine viruses were obtained by transfection of cocultured 293T and Madin-Darby canine kidney (MDCK) cells with eight plasmids encoding the HA and NA and the 6 internal protein gene segments of cold-adapted A/Ann Arbor/6/60 (AA ca), the master donor virus for influenza A vaccine strains of the commercial LAIV (18). To generate recombinant wt (rWt) A/mallard/New York/6750/78 (H2N2) viruses, the 6 internal protein gene segments were amplified by RT-PCR and cloned into the pAD3000 vector. Recombinant wt A/mallard/New York/6750/78 (H2N2) variants were then generated by transfection of the six plasmids encoding the wt internal protein segments and the two plasmids encoding the corresponding HA and NA segments. Viruses rescued from the transfected cells were propagated in the allantoic cavities of 10- to 11-day-old embryonated chicken eggs (Charles River SPAFAS, Franklin, CT) or MDCK cells. Virus titers were determined in MDCK cells and expressed as 50% tissue culture infective dose (TCID₅₀). The genomic sequences of the recombinant viruses were verified by cDNA sequencing.

The HA amino acid position numbering in this study follows the H3 numbering. The HA 226 and 228 positions correspond to residues 221

and 223 in the H2 subtype viruses and to residues 223 and 225 in the H6 subtype viruses.

Receptor binding assay. Receptor binding preference of reassortant viruses was analyzed by hemagglutination of resialylated receptor-specific red blood cells (RBCs) by the method described previously (10, 43). Hemagglutination assays were performed in V-bottom microtiter plates by incubating equal volumes (50 μ l) of 2-fold serially diluted virus and chicken red blood cells (cRBCs) that were resialylated with either α 2,3-SA or α 2,6-SA for 1 h at room temperature. The hemagglutination titer was defined as the reciprocal of highest virus dilution that hemagglutinated RBCs.

Ferret studies. Eight- to 10-week-old male and female ferrets ($n = 3$ /group) from Simonsen Laboratories (Gilroy, CA) were used in the studies. The animal protocol was approved by MedImmune's Animal Care and Use Committee. To evaluate vaccine viruses for their attenuated phenotype, ferrets were inoculated intranasally with 7.0 log₁₀ TCID₅₀ of virus per 0.2-ml dose. Three days after infection, ferrets were euthanized, and their lungs and nasal turbinates (NTs) were harvested for virus titration. Virus titers in the lungs and nasal turbinates were determined in eggs and expressed as 50% egg infective dose (EID₅₀). For immunogenicity and protection studies, ferrets were vaccinated intranasally with vaccine virus at 10⁷ TCID₅₀ per 0.2-ml dose and were bled on days 14 and 28 postinfection. Virus-specific antibody titers were determined by microneutralization (MN) assays (8, 11). On day 28 postvaccination, ferrets were infected with the indicated virus at a dose of 7.0 log₁₀ TCID₅₀ in 0.5 ml and sacrificed 3 days postchallenge. Virus titers in the nasal turbinate and lungs were determined by the EID₅₀ assay. All animal studies were conducted in accordance with the Institutional Animal Care and Use Committee-approved protocols.

RESULTS

Replication and receptor binding specificity of H2 vaccine viruses. Sequence variations at amino acid residues 226 and 228 (H3 numbering, corresponding to the H2 HA residues 221 and 223) of the H2 HA of influenza viruses have previously been found to affect receptor binding preference and host range (13, 28, 44). Amino acid residues Q226-G228 (Q-G) are present in all avian strains while human strains typically contain L226-S228 (L-S). L226-G228 (L-G) were found in some early human H2 strains and the recent swine strains isolated in 2006 (25). However, egg-amplified human influenza A/Japan/305/57 (H2N2, Jap/57), avian influenza A/mallard/New York/6750/78 (H2N2, mal/NY/78), and swine influenza A/swine/Missouri/2006 (H2N3, sw/MO/06) viruses all contain Q-G. To evaluate the impact of sequence variations at HA 226 and 228 on receptor binding specificity and replication of the L-G and L-S vaccine viruses, L-G and L-S were introduced into the HA cDNAs of each of the three viruses. Q-G, L-G, and L-S variants of the 6:2 LAIV reassortants were generated by plasmid rescue and expanded in MDCK cells and embryonated chicken eggs. Virus titers were determined in MDCK cells (Table 1). Jap/57 and mal/NY/78 viruses with L-G replicated less efficiently than those with the Q-G and L-S and were unstable in eggs. Jap/57 with L-G rapidly mutated to L-S, and mal/NY/78 with L-G changed to Q-G during virus amplification in eggs. In contrast, the viruses containing the Q-G and L-S residues replicated efficiently in eggs, reaching titers of >10⁸ TCID₅₀. All variants replicated efficiently in MDCK cells without any sequence changes in the HA and NA genes. Egg-grown vaccine variants were used in the following studies, except for the L-G variants of Jap/57 and mal/NY/78, which were grown in MDCK cells to maintain their HA sequence stability.

Each of the H2 variants was examined for its receptor binding

TABLE 1 Virus titer and receptor binding specificity of LAIV H2 variants

Virus	HA 226-228	Virus titer ^a (log ₁₀ TCID ₅₀ /ml)		Receptor binding specificity ^d		
		Egg	MDCK	α2,6 + α2,3	α2,3	α2,6
Jap/57	Q-G	9.0	7.2	256	512	4
	L-G ^b	7.9	6.7	64	<2	64
	L-S	8.7	6.7	1,024	64	128
mal/NY78	Q-G	9.0	7.2	128	128	2
	L-G ^c	8.2	7.0	64	<2	32
	L-S	8.7	6.0	4,096	64	128
sw/MO06	Q-G	8.6	7.5	128	64	<2
	L-G	8.2	7.6	256	<2	128
	L-S	8.4	7.0	256	64	128

^a The titers are the mean titers of two independent experiments with titer difference less than 0.5.

^b Changed to L-S in all egg isolates.

^c Changed to Q-G in one batch of egg isolates.

^d Egg-grown viruses were used except for the L-G variants of Jap/57 and mal/NY78, which were grown in MDCK cells.

specificity by hemagglutinating α2,3-SA- or α2,6-SA-specific red blood cells (RBCs). cRBCs expressing both α2,3-SA and α2,6-SA receptors were completely desialylated and then resialylated with either α2,3-SA or α2,6-SA for the hemagglutination (HA) assay (Table 1). The viruses with Q-G preferentially bound to α2,3-SA with minimal binding to α2,6-SA. In contrast, sw/MO/06 with L-G bound to α2,6-SA with an HA titer of 128 but had no detectable binding to α2,3-SA. The MDCK-grown L-G variants of Jap/57 and mal/NY78 also preferentially bound to α2,6-SA. The viruses with L-S bound to both α2,3-SA and α2,6-SA. Thus, both the 226 and 228 residues determine virus receptor binding specificity.

Evaluation of H2 vaccine viruses for their replication, immunogenicity, and induction of protective antibody response in ferrets. The Q-G and L-S variants of Jap/57 and mal/NY78, and the Q-G, L-G, and L-S variants of sw/MO/06 that maintained their genetic sequence after replication in eggs were examined for their ability to replicate in the respiratory tract of ferrets. Ferrets were intranasally inoculated with 7.0 log₁₀ TCID₅₀ of vaccine virus. After 3 days, nasal turbinates (NT) and lung tissues were harvested

for virus titration (Table 2). No virus was detected in the lungs of any of the infected animals, confirming the attenuation phenotype of the AA *ca* reassortant vaccine viruses. In the NT, the Q-G variants with receptor binding preference to α2,3-SA replicated at a titer of at least 10-fold lower than the viruses with the L226 residue (L-G- or L-S-containing viruses).

The immunogenicity of each variant virus was evaluated in ferrets. Fourteen days after a single dose of vaccination, serum samples were collected and tested by a microneutralization (MN) assay with different virus variants (Table 2). The neutralizing antibody titers of Jap57 Q-G (50) and mal/NY78 Q-G (80) against the homologous viruses were 20-fold and 8-fold lower than their corresponding L-S variants, Jap57 L-S (1016) and mal/NY78 L-S (640), respectively. For the three variants of sw/MO/06, both L-G and L-S variants induced levels of neutralizing antibody 16- to 20-fold higher than the sw/MO/06 Q-G virus. This result indicated that the vaccine viruses with α2,6-SA receptor binding ability had better replication efficiency and higher immunogenicity in ferrets than those with exclusively α2,3-SA receptor binding preference.

The changes at the HA 226 and 228 residues also affected virus antigenicity. The antibodies induced by the L-S variants of Jap/57, mal/NY78, and sw/MO/06 cross-reacted well with heterologous viruses bearing the L-S residues (neutralizing antibody titers were within 2-fold), but the sera did not react well with either the homologous or heterologous Q-G viruses (neutralizing antibody titers were 3- to 8-fold lower). The L-G variant of sw/MO/06 induced antibodies that cross-reacted well with the L-S viruses but not with the Q-G viruses. Thus, the amino acid at HA residue 226 played an important role in virus antigenicity. Interestingly, although the Q-G-containing viruses were less immunogenic, the antibody they induced cross-reacted well with all the Q-G, L-S, and L-G viruses.

The H2 vaccine variants were further evaluated for their ability to protect against wt mal/NY78 virus challenge infection in ferrets in groups of three animals. The wt mal/NY78 virus contains Q-G in the HA and did not replicate well in the lungs of ferrets (Fig. 1A). An average titer of 10^{2.2} EID₅₀ was detected in the lungs of ferrets 3 days postchallenge. All immunized ferrets were fully protected from wt mal/NY78 virus replication in the lungs and displayed significant reduction in challenge virus replication in

TABLE 2 The replication and immunogenicity of H2 LAIV variants in ferrets^a

Immunizing virus	HA 226-228	Virus titer (log ₁₀ EID ₅₀ /g ± SE)		GMT ^b of MN antibody in ferrets immunized with:					
		NT	Lung	Jap/57		mal/NY78		sw/MO/06	
				Q-G	L-S	Q-G	L-S	Q-G	L-S
Jap/57	Q-G	<2.5	<1.5	50	320	101	<u>202</u>	80	<u>254</u>
	L-S	3.4 ± 0.1	<1.5	40	1,016	101	1,016	63	806
mal/NY78	Q-G	3.0 ± 0.1	<1.5	16	<u>127</u>	80	<u>202</u>	50	<u>320</u>
	L-S	4.9 ± 0.3	<1.5	13	640	63	640	50	806
sw/MO/06	Q-G	4.5 ± 0.3	<1.5	16	<u>127</u>	80	<u>160</u>	101	<u>320</u>
	L-G	5.6 ± 0.1	<1.5	16	508	127	806	101	1,613
	L-S	5.9 ± 0.3	<1.5	13	508	80	806	63	2,032

^a Groups of three ferrets in were vaccinated with 10⁷ TCID₅₀ of the indicated LAIVs with sequence variations at HA 226 and 228. At 3 days postinfection, virus titers in nasal turbinates (NTs) and lungs were measured by 50% egg infective dose and expressed as log₁₀ EID₅₀/g tissue. At 14 days postinfection, sera were collected, and antibody titers were determined by a microneutralization (MN) assay. Homologous antibody titers are in bold. The titers that are ≥4-fold different from the homologous titers are underlined.

^b GMT, geometric mean titer.

FIG 1 Groups of three ferrets were vaccinated intranasally with 10^7 TCID₅₀ of the indicated LAIV viruses. After 28 days, ferrets were infected intranasally with rWt NY78 with Q226-G228 (A) or L226-S228 (B) in the HA. Virus titers in the NT and lung tissues at 3 days postinfection were measured by 50% egg infective dose and expressed as log₁₀ EID₅₀/g tissue. Dashed lines: limit of detection. All the vaccinated groups are significantly different from the mock group ($P < 0.05$ by one-way analysis of variance [ANOVA]). *, $P < 0.05$ compared to the Q-G variant of the same virus.

the upper respiratory tract (NT), except for the group that was immunized with Jap/57 Q-G, in which the NT titer was reduced by only approximately 6-fold after wt mal/NY/78 virus challenge. Since wt mal/NY/78 virus did not replicate efficiently in the lower respiratory tract of ferrets, the L-S residues were introduced into the HA of mal/NY/78; the L-S variant of wt mal/NY/78 replicated to $10^{6.5}$ EID₅₀ in the lungs (Fig. 1B), which was approximately 10,000-fold higher than the Q-G virus. These data confirmed our previous report that viruses with the ability to bind to $\alpha 2,6$ -SA receptors replicated more efficiently in the lungs of ferrets than those with binding preference to $\alpha 2,3$ -SA (43). All the ferrets vaccinated with the three L-S variants and sw/MO/06 L-G were completely protected against wt mal/NY/78 L-S challenge virus infection in the lungs; no challenge virus was detected in the lungs of these ferrets. In contrast, all the vaccine viruses with Q-G offered partial protection against mal/NY/78 L-S virus challenge infection in the lungs. Viruses were detected in one, two, or three ferrets vaccinated with the Q-G variants of sw/MO/06, mal/NY/78, and Jap/57, respectively. Consistent with the lung protection result, virus was not detected in the NT of the ferrets vaccinated with the L-S or L-G variants. However, the ferrets that were vaccinated with the Q-G variants were not completely protected from wt challenge virus replication in the NT. Although all the vaccinated

FIG 2 Groups of five ferrets were vaccinated intranasally with 10^7 TCID₅₀ of the indicated LAIV viruses (L-G variants were derived from MDCK cells). After 28 days, ferrets were infected intranasally with rWt NY78 with L226-S228 in the HA. Virus titers in the NT and lung tissues at 3 days postinfection were measured by 50% egg infective dose and expressed as log₁₀ EID₅₀/g tissue. Dashed lines: limit of detection. All the vaccinated groups are significantly different from the mock group ($P < 0.05$ by one-way ANOVA). *, $P < 0.05$ compared to the Q-G variant of the same virus.

animals had statistically significant reduction in wt challenge virus replication in both the NT and lungs compared to the mock group, only Jap/57 Q-G offered protection that was significantly lower than Jap/57 L-S. The level of challenge virus titer reduction in the respiratory tract of ferrets vaccinated with Jap/57 Q-G correlated well with their lower level of neutralizing antibody titers (Table 2). Thus, vaccine viruses with L-S or L-G that preferentially bind to $\alpha 2,6$ -SA receptor are more immunogenic than the ones with receptor binding preference to $\alpha 2,3$ -SA receptor and offered complete protection against wt H2 virus challenge.

An additional ferret study was conducted that increased the number of animals from 3 to 5 ferrets per group to compare the protective capacity of Q-G, L-G, and L-S variants of Jap/57 and mal/NY/78 against wt mal/NY/78 (L-S) virus challenge (Fig. 2). Since the L-G variant was not stable in eggs, the L-G variants grown in MDCK cells were used in this study. All the vaccinated animals were protected from wt virus challenge infection in both the NT and lung tissues. Only one ferret vaccinated with Jap/57 Q-G had the virus detected in the lung. The L-G and L-S variants of mal/NY/78 provided complete protection in the replication of wt virus replication in the NT, which was significantly different from the Q-G variant. In this study, Jap/57 Q-G offered greater protection against wt challenge virus replication in the lungs.

Generation of H6 vaccine strains with different 226 and 228 residues. Avian H6 viruses contain Q226 and G228 (H3 numbering, corresponding to H6 numbers 223 and 225) residues in their HA molecules. To evaluate whether the introduction of Q226L and G228S mutations affects H6 virus receptor binding specificity, Q226L and/or G228S was introduced into the HA of two H6 viruses, A/duck/Hong Kong/182/77 (H6N9, dk/HK/77) and A/teal/Hong Kong/W312/97 (H6N1, tl/HK/97), by site-directed mutagenesis. Recombinant reassortant vaccine viruses with variations of Q-G, L-G, and L-S at these two residues were generated by plasmid rescue and expanded in both MDCK cells and eggs (Table 3). The amino acids at HA residues 226 and 228 did not significantly affect virus growth in eggs. The titers of all the vaccine variants exceeded $10^{8.5}$ TCID₅₀/ml. Replication of dk/

TABLE 3 Virus titer and receptor binding specificity of the LAIV H6 variants

Virus	HA residue	Virus titer ^b		Receptor binding		
		(log ₁₀ TCID ₅₀ /ml)				
		Egg	MDCK	α2,6 + α2,3	α2,3	α2,6
dk/HK/77	Q-G	9.2	7.7	256	512	8
	L-G	8.9 ^a	8.0	128	<2	128
	L-S	9.3	8.0	256	32	256
tl/HK/97	Q-G	8.5	6.0	256	256	<2
	L-G	8.5	7.2	256	<2	32
	L-S	9.0	6.0	1,024	512	1,024

^a The NA had the S367N change in one virus stock which was not used in the study.
^b The titers are the mean titers of two independent experiments with titer difference of less than 0.5.

HK/77 variants in MDCK cells was higher than that of tl/HK/97. dk/HK/77 with the L-G residue had an S367N (N2 numbering) mutation in the NA gene. The S367N change corresponds to residue 368 of the N2 NA and was found to be an escape mutation selected from an N9 monoclonal antibody (1). Residue 367 is located on the rim of the enzymatic active-site crater. Our results indicated that the S367N change may have affected functional balance between the HA and NA proteins. Similar to the H2 viruses, the H6 viruses with the Q-G residues preferentially bound α2,3-SA, L-G preferred α2,6-SA, and L-S exhibited receptor binding to both α2,3-SA and α2,6-SA receptors. Thus, the changes at positions 226 and 228 also altered receptor binding preference of the H6 viruses.

Replication, immunogenicity, and protection of H6 vaccine variants in ferrets. The HA 226 and 228 variants of dk/HK/77 and tl/HK/97 were evaluated in ferrets for replication, antibody response, and protection against wt tl/HK/97 virus challenge. The dk/HK/77 L-G vaccine was not evaluated in ferrets because of the NA S368N change that had occurred during virus growth in eggs. As shown in Table 4, the Q-G-containing viruses replicated in the NT to a level approximately 100-fold lower than that of the viruses with L-S or L-G. None of the viruses replicated in the lungs, confirming that the change in receptor binding specificity did not affect the attenuation phenotype of the vaccine viruses. The dk/

TABLE 4 Evaluation of H6 variants for replication, immunogenicity, and protection against wt virus challenge infection in ferrets^a

Immunizing virus	HA 226-228	Virus titer (log ₁₀ EID ₅₀ /g ± SE)		GMT of MN antibody in ferrets immunized with:				
		NT	Lung	dk/HK/77		tl/HK/97		
				Q-G	L-S	Q-G	L-G	L-S
dk/HK/77	Q-G	3.2 ± 0.0	<1.5	160	<u>403</u>	<u>63</u>	<u>80</u>	<u>50</u>
	L-S	5.9 ± 0.1	<1.5	160	1,613	<u>40</u>	<u>101</u>	<u>63</u>
tl/HK/97	Q-G	3.3 ± 0.1	<1.5	<u>40</u>	<u>80</u>	320	403	806
	L-G	5.1 ± 0.3	<1.5	<u>40</u>	<u>80</u>	202	1,016	1,016
	L-S	5.8 ± 0.3	<1.5	<u>32</u>	<u>160</u>	127	640	806

^a Groups of three ferrets were vaccinated with 10⁷ TCID₅₀ of the indicated LAIVs with sequence variations at HA 226 and 228. At 3 days postinfection, virus titers in nasal turbinates (NTs) and lungs were determined in eggs and expressed as log₁₀ EID₅₀/g tissue. At 14 days postinfection, serum was collected and antibody titers were determined by a microneutralization (MN) assay. Homologous antibody titers are in bold. The titers that are ≥4-fold different from the homologous titers are underlined.

FIG 3 Groups of three ferrets were vaccinated intranasally with 10⁷ TCID₅₀ of the indicated LAIV viruses. After 28 days, ferrets were infected intranasally with wt A/teal/HK/W312/97. Virus titers in NT and lungs at 3 days postinfection were measured by 50% egg infective dose and expressed as log₁₀ EID₅₀/g tissue. Dashed lines: limit of detection. All the vaccinated groups are significantly different from the mock group (*P* < 0.05 by one-way ANOVA).

HK/77 vaccine virus with Q-G was less immunogenic than that with L-S, and the neutralizing antibody titers elicited by these two viruses differed by 10-fold (160 by Q-G versus 1,613 by L-S). The dk/HK/77 Q-G postinfection serum cross-reacted well with dk/HK/77 L-S, but the dk/HK/77 L-S postinfection ferret serum neutralized dk/HK/77 Q-G at a titer 4-fold lower than that of the homologous neutralizing titer. Thus, the changes at residues 226 and 228 also affected the antigenicity of dk/HK/77. Tl/HK/97 with Q-G was also less immunogenic; the homologous antibody titer (320) was lower than those induced by L-G (1,016) and L-S (806), but the cross-reactivity was not significantly different. dk/HK/77 was antigenically different from tl/HK/97, and postinfection sera did not cross-react well each other.

The H6 vaccine variants were further evaluated for their ability to protect against wt tl/HK/97 (226Q-228G) virus challenge. Ferrets receiving a single dose of vaccine were infected with wt tl/HK/97 virus at a dose of 10⁷ TCID₅₀, and viral replication in the NT and lungs at day 3 after challenge infection was determined (Fig. 3). Except for one dk/HK/77 Q-G-vaccinated ferret that had a nearly 1,000-fold reduction in viral lung titer compared to the control animals, all the remaining animals were completely protected from wt virus replication in the lungs at 3 days postchallenge. The protection offered by the tl/HK/97 Q-G, L-G, and L-S variants against wt A/tl/HK/97 virus replication in the lungs of ferrets could not be differentiated. All of these variants elicited neutralizing antibodies at titers from 40 to 806 (Table 4), which was sufficient in protecting against wt tl/HK/97 virus replication in the lungs. All the vaccinated animals were partially protected from wt virus replication in the NT, and the challenge virus titer reduction was significant compared to the control animals. The titer reduction was about 100-fold in heterologous dk/HK/77-vaccinated groups and greater than 1,000-fold in homologous tl/HK/97-vaccinated groups.

DISCUSSION

Influenza pandemics in the last century and the latest 2009 H1N1 pandemic were caused by influenza viruses that acquired receptor binding preference to α2,6-SA. In order to provide greater protection against future influenza pandemics, live attenuated pandemic influenza vaccine viruses should replicate well enough in the hu-

man upper respiratory tract to induce strong immune responses. Recently, we have demonstrated that the 2009 pandemic A/CA/7/09 vaccine virus with dual receptor binding to both α 2,3-SA and α 2,6-SA not only replicated well in embryonated chicken eggs but also induced high antibody responses in the ferret model (10).

In this study, we showed that the HA 226 and 228 residues determined receptor binding specificity for both H2 and H6 viruses. We also confirmed that receptor binding specificity is an important factor for vaccine virus immunogenicity. Viruses containing Q226-G228 bound to the avian-like α 2,3-SA receptors allow the virus to replicate efficiently in avian hosts or chicken eggs but induced poor antibody responses in ferrets. Viruses with L226-G228 mainly bound α 2,6-SA receptors. Due to the lack of binding to α 2,3-SA, most of the viruses with L-G were not stable in eggs and rapidly mutated to L-S or Q-G in order to gain fitness in eggs. However, sw/MO/06 L-G was genetically stable in eggs; the parent swine strain originally contained L-G (22), but the virus isolate that we obtained contained Q226. It is possible that other residues in the HA and/or NA might contribute to the stability of the A/sw/MO/06 L-G virus in eggs. Viruses containing the L226 and S228 residues in the HA that displayed dual binding to both α 2,3-SA and α 2,6-SA receptors also replicated efficiently in eggs. The L-S variants of the three H2 viruses evaluated in this study and sw/MO/06 L-G induced higher levels of neutralizing antibody and conferred greater protection against wt virus challenge infection than did Q-G strains. In order for avian viruses to cause influenza pandemics, it is likely that they may need to acquire receptor binding changes during passage in an intermediate host such as pigs to transmit efficiently from person to person. Thus, for pandemic preparedness, L226-S228 that allows binding to both types of receptors might be desirable in both H2 and H6 vaccine strains.

The HA residues 226 and 228 are located in the 220 loop of the HA receptor binding site (23, 35, 44). The effect of the 226 residue on receptor binding preference has been studied extensively for various subtypes of influenza viruses. Similar to human H2 viruses, the early H3N2 human viruses acquired the L226-S228 residues and receptor binding preference to α 2,6-SA (13, 28, 39). In more contemporary strains, substitutions at the HA 226 residue were found to affect virus replication *in vitro* (11). The L226 residue was also found to specify HA binding to α 2,6-SA for H4, H5, and H9 viruses (3, 4, 26, 40, 41). Our study is the first to demonstrate that substitution of L226 in avian H6 viruses also confers receptor binding specificity to α 2,6-SA. In contrast, Q226 is conserved in seasonal human H1N1 viruses and the 2009 pandemic H1N1 viruses. The Q226R substitution of the H1 viruses from virus replication in eggs changed receptor binding preference from α 2,6-SA to α 2,3-SA (10, 43).

Several recent reports showed that residues 226–228 not only determined virus host range but also correlated with viral transmission efficiency or virus-induced innate immune responses (32, 33). We found that residues 226 and 228 also affect virus antigenicity of H2 and H6 viruses. The three L-S variants of different H2 viruses were antigenically similar to each other as analyzed by their cross-reactivity in a neutralization assay, regardless of HA sequence differences (Table 2). However, the two H6 vaccine variants did not display good cross-reactivity, indicating that the H6 viruses are more divergent and that dk/HK/77 and tl/HK/7 have distinct antigenicities (Table 4).

Ferrets have proven to be a good animal model for influenza virus infection and vaccine evaluation. They have receptor distri-

bution in the respiratory tract similar to that of humans (43). The low immunogenicity of H6 A/teal/Hong Kong/W312/97 virus with the Q-G residue in ferrets may explain their low immunogenicity in humans (38). The H9N2 A/chicken/HongKong/G9/97 candidate vaccine that contains L226 with preference for α 2,6-SA replicates efficiently in ferrets. Although replication of the H9N2 vaccine virus was not detected in the upper respiratory tract of humans, it elicited antibody response in seronegative healthy adults at a rate of 100% (19). Thus, we expect that the L-S variants with higher immunogenicity in the ferret model might be able to induce higher immune responses in humans. The ferret studies described in this report showed that the level of neutralizing antibodies correlates with protection. However, although the Q-G variants were less immunogenic than the L-G or L-S variants, they still provided great protection against wt virus challenge in the lungs, indicating a low threshold of protective antibodies required for the LAIV-mediated protection in ferrets. This result is consistent with the previous reports that the H5N1 vaccine candidates were not immunogenic in both ferrets and in the small clinical phase I human study, but they still provided protection against pulmonary replication of wt challenge virus in ferrets (20, 37). Further investigations are needed to understand the correlation of the immunogenicity and protective efficacy in ferrets and in humans.

In summary, our study underscores the importance of the residues at the influenza virus HA receptor binding sites in vaccine virus replication, immunogenicity, and protection against wt virus challenge in ferrets. A pandemic vaccine strain should have the following desired features: the ability to bind to both α 2,6-SA and α 2,3-SA receptors in order to achieve high yield in embryonated chicken eggs for manufacture and the ability to induce protective immune response. More investigations are needed to assess if these features are applicable to other pandemic influenza vaccines.

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