Short Communication

Composition of Hemagglutinin and Neuraminidase Affects the Antigen Yield of Influenza A(H1N1)pdm09 Candidate Vaccine Viruses

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SUMMARY: To improve the hemagglutinin (HA) antigen yield of influenza A(H1N1)pdm09 candidate vaccine viruses, we generated 7:1, 6:2, and 5:3 genetic reassortant viruses between wild-type (H1N1)pdm09 (A/California/7/2009) (Cal7) and a high-yielding master virus, A/Puerto Rico/8/34 (PR8). These viruses contained the HA; HA and neuraminidase (NA); and HA, NA, and M genes, respectively, derived from Cal7, on a PR8 backbone. The influence of the amino acid residue at position 223 in Cal7 HA on virus growth and HA antigen yield differed between these reassortant viruses. NIIDRG-7, a 7:1 virus possessing arginine at position 223, exhibited a 10-fold higher 50% egg infectious dose (EID₅₀) (10.0 log₁₀EID₅₀/ml) than the 5:3 and 6:2 viruses. It also had 1.5- to 3-fold higher protein (13.8 µg/ml of allantoic fluids) and HA antigen (4.1 µg/ml of allantoic fluids) yields than the 5:3 and 6:2 viruses, which possessed identical Cal7 HA proteins. However, the HA antigen yield of the other 7:1 virus, which possessed glutamine at position 223 was 60% of that of NIIDRG-7. In addition, a novel 6:2 virus possessing Cal7 HA and the NA of A/Wisconsin/10/98 (a triple reassortant swine-like H1N1 virus), produced 107% of the HA yield of NIIDRG-7. In this study, we showed that the balance between HA and NA in the influenza A(H1N1)pdm09 virus affects its protein and antigen yield.

In April 2009, a novel influenza A(H1N1)pdm09 virus was identified in the United States. Within 2 months, this virus had spread globally, causing the first pandemic of the 21st century (1). The A(H1N1)pdm09 virus is a novel reassortant virus that contains 6 gene segments from the North American triple-reassortant swine-influenza viruses. It also encompasses neuraminidase (NA) and M gene segments from the Eurasian swine influenza viruses (1,2). Due to the origin of the HA gene, the A(H1N1)pdm09 virus is antigenically different from the previous seasonal H1N1 viruses that circulated globally until April 2009 (3). To prevent the spread of the viruses through the population, the World Health Organization (WHO) Global Influenza Surveillance and Response System initiated the development of candidate vaccine viruses (CVVs) (4). Currently, influenza vaccine viruses are propagated in embryonated chicken eggs. However, human influenza isolates, in most cases, grow poorly in chicken eggs; and A(H1N1)pdm09 virus is not an exception. There have been efforts to develop high-growth reassortant viruses between A/California/7/2009 (Cal7) and a high-yielding master virus (A/Puerto Rico/8/34 [PR8] or its derivative) using the classical reassortment method (5,6) or by reverse genetics using plasmid DNAs (7–9). One such reassortant virus, X-179A, exhibited higher growth in eggs (hemagglutination [HA] titer, 2048) than the parental virus, Cal7 (HA titer, 64) (4). Despite the high HA titer of X-179A, the total protein and HA antigen yield was lower than that for the previous seasonal hu-

man H1N1 vaccine viruses (4). To overcome this disadvantage, we attempted to develop vaccine viruses with higher growth in eggs and higher protein yield. We used 12 plasmid-based reverse genetics system and the high-growth PR8 virus (UW strain) as the backbone virus (Table 1) (8). For pandemic vaccine viruses, the WHO recommends that the reassortant virus possesses at least the HA and NA genes of the pandemic virus with the remaining genes originating from PR8 (6:2) (10). However, an improvement in virus growth or antigen yield, relative to 6:2 viruses, was observed with H5N1 reassortant viruses possessing the HA (7:1), HA/NA/PB1 (5:3), or HA/NA/M (5:3) from H5 viruses (11). In addition, studies have shown that there are different residues (D/Q, G/Q, or D/R) at the receptor-binding site of Cal7 HA at positions 222/223 (H1HA numbering) (4,12). The residues at these positions reportedly affect the receptor binding specificity of H1N1, H2N2, and H3N2 influenza viruses (13–17). In this study, we examined the effect of gene constellations and amino acid residues at 222/223 in Cal7 HA on virus growth and antigen yield.

We first developed three 6:2 reassortant viruses with D/Q, G/Q, and D/R at positions 222/223 using the appropriate cloned Cal7 HA cDNAs (Table 1). The HA titer of the virus with G/Q was 64. Passaging the viruses 4 times in embryonated chicken eggs did not improve the HA titer. However, the HA titers of the D/Q and D/R viruses increased to 128 and 512, respectively (Table 1). Next, to assess the impact of D/Q and D/R at positions 222/223 and the impact of the gene constellations on virus growth and infectivity, we developed 5:3 (NIIDRG-3, -6) and 7:1 (NIIDRG-7, -8) reassortant viruses, in addition to the 6:2 viruses (NIIDRG-1, -5) (Table 1). We performed 50% egg infective dose (EID₅₀) assays and found that infectivity increased

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Table 1. Differences in the amino acid sequences of HA and the infectivity titers of viruses

Virus ¹⁾	Passage history	Gene constellation	Amino acid at the indicated HA positions ²⁾					EID ₅₀ (log ₁₀ /EID ₅₀ ml) ³⁾	
			125	191	209	222	223	Exp. 1	Exp. 2
A/California/7/2009	—	—	N	L	K	D/G	Q/R	n.d.	n.d.
X-179A	E7/E1/E6	5:3	·	·	T	D	R	9.4	9.3
NIIDRG-1	LLC1E2	6:2	·	I	·	D	Q	8.3	8.4
NIIDRG-3	LLC1E2	5:3	·	I	·	D	Q	8.2	8.3
NIIDRG-5	LLC1E4	6:2	·	·	·	D	R	9.1	9.0
NIIDRG-6	LLC1E5	5:3	N/D	·	·	D	R	9.0	8.9
NIIDRG-7	LLC1E2	7:1	·	·	·	D	R	9.9	10.0
NIIDRG-8	LLC1E3	7:1	·	I	·	D	Q	9.3	9.1

¹⁾: Reassortant viruses were generated by co-transfecting 12 plasmid DNAs into qualified LLCMK2 cells using FuGENE HD Transfection Reagent (Roche) (LLC1) (8). Approximately 18 h post-transfections, the cells were washed, and the medium was replaced with Opti-Pro SFM medium (Invitrogen) containing 1 µg/ml of TrypZean (SIGMA). The supernatant was harvested at 48 h post-transfection and used to inoculate 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs to amplify the rescued viruses. For each reassortant virus, the genetic sequences of HA and/or NA and/or M derived from Cal7 were verified by sequencing the RT-PCR products amplified from viral RNA. All the manipulations were performed in accordance with the Good Manufacturing Practice of NIID.

²⁾: H1 numbering; all changes relative to A/California/7/2009.

³⁾: The EID₅₀ was determined as follows: each virus was serially diluted from 10⁻⁵ to 10⁻¹¹. Six 10 to 11 day-old embryonated chicken eggs were infected with 0.2 ml of virus for each dilution. An aliquot of the allantoic fluid was harvested from each of the eggs at 48 h post-infection. An HA assay was performed for each allantoic fluid, using 0.5% turkey erythrocytes. The EID₅₀ was calculated by using the formula of Reed and Muench. The results are from two separate experiments.

n.d., not done.

among the reassortant viruses (in the order 5:3, 6:2, and 7:1) possessing the identical amino acid residues at positions 222/223. In addition, the influence of 223R on the EID₅₀ was greater than that of 223Q, as determined by evaluating viruses with the same gene constellations (Table 1). The HA titers and EID₅₀ values were nearly parallel for each virus. Among the reassortant viruses, NIIDRG-7 exhibited the highest growth (HA titer, 1024) and EID₅₀ (Table 1). Residue 223 is located on the 220-loop of the receptor-binding site of HA. Avian H1HA has close contact between 223Q and the Gal-2 of α2-3 linked sialylated glycans. However, similar contacts between the A(H1N1)pdm09 HA of A/California/4/2009 and α2-3 linked sialylated glycans are absent (18). The presence of 223R instead of 223Q might overcome the unfavorable interactions between Cal7 HA and α2-3 linked sialylated glycans, which are prevalent in the chorioallantois of chicken eggs. In the case of 223Q viruses (NIIDRG-1, -3, and -8), all rescued viruses possessed an additional substitution, i.e., L191I. The L191I substitution might facilitate viral rescue and amplification in eggs, as was observed with other reassortant viruses derived from A/California/4/2009 (12). However, this substitution did not have a stronger influence on virus growth than that observed with 223R.

The total protein yield (TPY) of the purified NIIDRG-1, -3, and -5 to -8 and X-179A viruses was determined by using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, Ill., USA), according to the manufacturer instructions (Fig. 1). Among the viruses subjected to the assay, NIIDRG-7 exhibited the highest TPY (13.8 µg/ml allantoic fluid [AF]), whereas X-179A had a TPY of 10.4 µg/ml AF. The other viruses exhibited TPYs similar to or lower than that of X-179A (Fig. 1). A correlation between virus proliferation and TPY was observed primarily with the resultant viruses.

The HA yield of each virus was calculated using the TPY and the results of the SDS-PAGE analysis (Fig. 1). The HA content relative to the total protein content of

Fig. 1. Average yield (µg) of total protein, HA antigen per ml of allantoic fluids and the average HA content (%) of each virus concentrate. Ten-day-old embryonated chicken eggs were inoculated with NIIDRG-1, -3, and -5 to -8 viruses. Forty-eight hours post infection, the allantoic fluid was collected. The viruses were purified by ultracentrifugation using 20% sucrose at 35,000 rpm for 60 min at 4°C. The pellet was re-suspended in a small volume of buffer, which was used as the purified concentrate. The method for preparation of the virus concentrate differed from the method used by others (4,21); therefore, we re-assessed the total protein yield of X-179A and NIIDRG-7. We used viruses concentrated by methods similar to those used by others (4,21), that is, viruses were grown in 50 eggs and purified using 10–40% continuous sucrose gradient with centrifugation at 35,000 rpm for 35 min at 4°C. The total protein yield of X-179A and NIIDRG-7 was estimated at 4.5 mg/50 eggs and 6.25 mg/50 eggs, respectively. The HA content was calculated using the results from the SDS-PAGE analysis, which are shown in Figure 2. The SDS-PAGE results were captured and analyzed using a CS analyzer (ATTO), and the ratio of HA protein to total proteins was calculated. The total HA yields were calculated from the total protein yields and the relative HA contents. Each value is the average of values from 3 analyses.

NIIDRG viruses ranged between 23% (NIIDRG-3) and 30% (NIIDRG-7). NIIDRG-7 had the highest HA yield (4.1 µg/ml AF) whereas X-179A had an HA yield of 3.3

$\mu\text{g/ml}$ AF (Fig. 1). NIIDRG-6 and -8 also exhibited low HA yield ($2.5 \mu\text{g/ml}$ AF). The remaining viruses had yields much lower than that of NIIDRG-7.

The functional balance between HA and NA is known to affect the virus growth (19,20). The novel gene constellation of the A(H1N1)pdm09 virus may not result in the optimal balance between HA and NA activity. The difference in TPY between NIIDRG-5 and NIIDRG-7 is attributable to the imbalance in HA and NA activity. Cal7 HA is genetically close to HA in the triple-reassortant swine-like H1N1 virus, A/Wisconsin/10/98 (Wis98) (3). The similarity of HA1 is 93% between Cal7 and Wis98. To improve the compatibility of HA and NA, we developed another 6:2 reassortant virus, Cal7/Wis98 which harbored Cal7 HA and Wis98 NA with a PR8 backbone. The HA yield ($4.4 \mu\text{g/ml}$ AF) and the TPY ($15.2 \mu\text{g/ml}$ AF) were slightly higher than that of NIIDRG-7. This suggests that the low TPY and HA yield of NIIDRG-5 was a result of the poor compatibility of HA and NA of Cal7.

During the preparation of this manuscript, Harvey et al. reported a new CVV (6:2) with a chimeric sequence derived from PR8 and Cal7 HA (NIBRG-119). This new CVV exhibited improved growth as compared to the current vaccine virus (6:2) (NIBRG-121) (21). Based on their results, we created a new 7:1 reassortant virus by exchanging the 3'- and 5'-non-coding region domains, signal peptide, transmembrane (TM) domain, and cytoplasmic tail of Cal7 HA with those of PR8 (NIIDRG-7.1) (Fig. 2A). NIIDRG-7.1 exhibited an improved HA content ($40.1 \pm 1.23\%$) (Fig. 2B) and HA yield ($5.2 \mu\text{g/ml}$ AF). However, it still had a TPY ($13.1 \mu\text{g/ml}$ AF) similar to that of NIIDRG-7. Our result proved the hypothesis proposed by Harvey et al.; they stated that the TM domain of PR8HA plays a role in the efficient incorporation of HA into viral particles, which is caused by the increased association of HA with glycosphingolipid-enriched, detergent-resistant plasma membrane domains. In the case of NIBRG-119, the TPY was higher, but not the HA yield. Although both viruses showed similar HA yields, the mechanisms responsible for improvement might be different.

We performed hemagglutination inhibition (HAI) assays using ferret antisera against the reassortant viruses in order to determine their antigenic characteristics

(Table 2). All NIIDRG viruses were antigenically similar to wild-type A(H1N1)pdm09, Cal7, and A/Narita/1/2009, and the vaccine virus X-179A. However, the NIIDRG viruses were distinct from A/Brisbane/59/2007, a past seasonal H1N1 virus (Table 2).

To improve the protein/antigen yields of A(H1N1)pdm09 CVVs, genetic alterations have been introduced into the packaging signal and the oligosaccharide moiety of Cal7 HA (21,22). In this study, we showed that the composition of HA and NA must be controlled to achieve a high protein yield. Although NA immunogenicity of the original pandemic virus is significant, if the antigen yield is extremely low, as observed for the A(H1N1)pdm09 CVV, we can also con-

Fig. 2. (A) Schematic diagram of the HA chimera. Domains derived from Cal7 and PR8 are shown in white and black, respectively. NCR, non-coding region; SP, signal peptide; TM/CT, transmembrane and cytoplasmic tail domains of the HA. (B) SDS-PAGE analysis of NIIDRG-7 and NIIDRG-7.1. The purified viruses ($6 \mu\text{g}$) were treated with (+) or without (-) 10 units of PNGase F according to the manufacturer's instructions (New England Biolabs). Samples were incubated in a total volume of $20 \mu\text{l}$ at 37°C overnight, and the reducing agent was added before the samples were loaded on 10% SDS-PAGE gels. The relative ratio of HA to NP is shown as the average of the values.

Table 2. Antigenic analysis of viruses

Virus	Titer ¹⁾ of ferret antiserum against indicated virus:			
	A/Brisbane/59/2007	A/California/7/2009	A/Narita/1/2009	NYMC X-179A
A/Brisbane/59/2007	640	< 10	< 10	< 10
A/California/7/2009	< 10	2560	10240	2560
A/Narita/1/2009	< 10	2560	10240	5120
NYMC X-179A	< 10	5120	10240	5120
NIIDRG-1	< 10	2560	5120	1280
NIIDRG-3	< 10	1280	5120	1280
NIIDRG-5	< 10	1280	5120	1280
NIIDRG-6	< 10	1280	5120	1280
NIIDRG-7	< 10	2560	5120	2560
NIIDRG-8	< 10	1280	5120	1280

¹⁾: HAI assay was performed with 0.5% turkey erythrocytes. HAI titers are presented as the reciprocal value of the highest serum dilution which inhibited hemagglutination ability of the viruses. The homologous titers are boldfaced.

sider the development of a CVV with a different gene constellation without genetic alterations.

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Conflict of interest None to declare.

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