Japanese archipelago. Our data underscore the need for continued global monitoring of H5N1 HPAIVs and provide "early warning" signals for preparedness against the unprecedented situation in which the natural reservoirs maintain HPAIVs consistently, as is the case with nonpathogenic influenza A viruses.

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ORIGINAL ARTICLE

Improvement of the H5N1 influenza virus vaccine strain to decrease the pathogenicity in chicken embryos

Norikazu Isoda · Yoshihiro Sakoda · Masatoshi Okamatsu · Yoshimi Tsuda · Hiroshi Kida

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Abstract The avian influenza vaccine strain A/duck/Hokkaido/Vac-1/2004 (H5N1) (Vac-1) was found to be pathogenic in chicken embryos (CEs). In order to decrease the pathogenicity of Vac-1 in CEs, a series of reassortant viruses was generated between Vac-1 and A/Puerto Rico/8/1934 (H1N1) (PR8), and their pathogenicity and growth potential were compared in CEs. The results indicated that either the PB1 or PA protein was responsible for the pathogenicity of Vac-1 in CEs. The HA titers of the allantoic fluids of CEs inoculated with the recombinant H5N1 viruses, of which pathogenicity was lower than that of the recombinant Vac-1 prepared by reverse genetics in CEs, were equivalent to those of CEs inoculated with the recombinant Vac-1. One of the reassortant viruses, rg-PR8-PA/Vac-1 (H5N1), in which the PA gene was replaced with the corresponding gene of PR8, yielded allantoic fluids with the same HA titer as that of Vac-1, indicating that this reassortant should be a good candidate as an improved vaccine strain.

N. Isoda · Y. Sakoda · M. Okamatsu · Y. Tsuda · H. Kida (✉)
Laboratory of Microbiology,
Department of Disease Control,
Graduate School of Veterinary Medicine,
Hokkaido University, Kita-18 Nishi-9,
Kita-ku, Sapporo, Hokkaido 060-0818, Japan
e-mail: kida@vetmed.hokudai.ac.jp

Y. Tsuda · H. Kida
Research Center for Zoonosis Control,
Hokkaido University, Sapporo,
Hokkaido 060-0818, Japan

Introduction

Outbreaks of highly pathogenic avian influenza (HPAI) caused by H5N1 viruses have spread to 62 countries in Eurasia and Africa from Southeast Asia since 1996, and well over 400 million birds have died or been culled [27, 28]. This has greatly affected not only the poultry industry but also public health. H5N1 HPAI viruses infected 18 humans, and 6 died in Hong Kong in 1997 [2]. Since 2003, there have been 498 human cases of H5N1 virus infection, with 294 deaths in 15 countries as of May 6, 2010 [27]. "Stamping-out" is the basic measure for the control of HPAI. Vaccination may be an additional option when the disease spreads widely [18]. Inactivated H5 and H7 avian influenza virus vaccines have been prepared and evaluated by several research groups [9, 12, 20, 24, 25]. It is, therefore, important to assess the antigenicity, pathogenicity, and growth potential of the vaccine strains in chicken embryos (CEs).

CEs are currently used as the host in which influenza viruses can grow in sufficient amounts for vaccine production. It is well known that the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) are responsible for extensive replication of influenza viruses in embryonated chicken eggs [1, 4, 7, 14, 15]. Amino acid substitutions in the vicinity of the receptor-binding site of HA are responsible both for growth potential in CEs and antigenicity [1]. After several passages in embryonated chicken eggs, influenza A and B viruses with amino acid substitutions in the vicinity of the receptor-binding pocket on the HA molecule show high growth potential [11, 15, 16]. The NA contributes to enhancement of virus yield in embryonated chicken eggs [7]. Amino acid substitutions in the HA and/or NA, however, lead not only to extensive growth but also to antigenic variation of the virus [15]. Internal proteins, such as PB2 and NP, which are components of the ribonucleoprotein (RNP)

complex, may also contribute to the replication of influenza viruses in embryonated chicken eggs [16]. The pathogenicity of influenza viruses in CEs also affects the yield of the virus suspension, because embryo death makes it difficult to harvest infectious allantoic fluids due to postmortem change.

In a previous study, a non-pathogenic H5N1 reassortant influenza virus, A/duck/Hokkaido/Vac-1/2004 (H5N1) (Vac-1), was generated between A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1), both of which were isolated from migratory ducks in Asia [9]. Phylogenetic analysis of the H5 HA genes revealed that A/duck/Mongolia/54/2001 (H5N2) belonged to the Eurasian lineage [22]. In addition, antigenic analysis using a panel of monoclonal antibodies to the H5 HA proteins and antiserum to Vac-1 indicated that the HAs of HPAI viruses currently circulating in Asia were antigenically closely related to that of Vac-1 [22]. The inactivated vaccine prepared from Vac-1 was confirmed to be potent in a previous study [9]; however, the CEs in the eggs inoculated with Vac-1 died between 48 and 72 hours post-inoculation. Ideally, vaccine strains should be less pathogenic in chicken embryos for vaccine manufacture, although Vac-1 was defined as a nonpathogenic virus strain in 6-week-old chickens [9].

In the present study, it was revealed that the PB1 and PA proteins of Vac-1 influenza virus are responsible for pathogenicity in CEs. One of the reassortant viruses, rg-PR8-PA/Vac-1 (H5N1), in which PA gene was replaced with the corresponding gene of PR8, yielded allantoic fluids with the same HA titer as that of Vac-1, indicating that this reassortant should be a good candidate as a vaccine strain.

Materials and methods

Viruses

A/duck/Hokkaido/Vac-1/2004 (H5N1) was selected from reassortants between A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1), both of which were isolated from fecal samples from migratory ducks in Asia [9, 23]. The NA and NS gene segments of Vac-1 are derived from A/duck/Mongolia/47/2001 (H7N1), and the other 6 segments from A/duck/Mongolia/54/2001 (H5N2) [23]. Nucleotide and amino acid sequences of the eight genes of Vac-1 (H5N1) were submitted to the DNA Data Bank of Japan under accession numbers AB253760 (PB2), AB253761 (PB1), AB257726 (PA), AB263192 (HA), AB263193 (NP), AB263194 (NA), AB263195 (M), and AB263196 (NS) [9]. A/Puerto Rico/8/1934 (H1N1) was provided by St. Jude Children's Research Hospital, USA. PR8 and Vac-1 were propagated in 10-day-old embryonated chicken eggs at 35°C for 48 hours, and the allantoic fluids were then harvested.

Generation of recombinant viruses

A series of plasmids carrying the eight gene segments of PR8 was provided by Drs. E. Hoffmann and R. Webby, St. Jude Children's Research Hospital. The universal primer set for influenza A viruses was used for RT-PCR [6]. Each of the PCR products of the eight gene segments of Vac-1 was cloned into pCR 2.1 TOPO vector (Invitrogen). Eight segments of Vac-1 were cloned into a dual-promoter plasmid, pHW2000 [5]. The plasmids carrying either the PB1 or PA gene of PR8 or Vac-1 were used to construct chimeric gene segments. To exchange the PB1 genes between PR8 and Vac-1, the plasmids carrying the PB1 gene of either PR8 or Vac-1 were digested with *Mfe*I (Takara Bio; cleavage site, nucleotide position 719 in the PB1 gene) and *Bam*HI (Takara Bio; cleavage site, nucleotide position 1997 in PB1 gene), and both of their reading frames were divided into three fragments. The fragments were cloned, and the resulting 6 plasmids were designated as pHW-PB1/P719V (nucleotide position 1 to 719 of PR8, and the other region of Vac-1), pHW-PB1/V719P (nucleotide position 1 to 719 of Vac-1, and the other region of PR8), pHW-PB1/V719P1997V (nucleotide position 720 to 1997 of PR8, and the other regions of Vac-1), pHW-PB1/P719V1997P (nucleotide position 720 to 1997 of Vac-1, and the other regions of PR/8), pHW-PB1/V1997P (nucleotide position 1 to 1997 of Vac-1, and the other region of PR8), and pHW-PB1/P1997V (nucleotide position 1 to 1997 of PR8, and the other region of Vac-1). To exchange the PA gene between PR8 and Vac-1, plasmids carrying the PA gene of either PR8 or Vac-1 were digested with *Csp*45I (Toyobo; cleavage site, nucleotide position 706 in the PA gene) and *Sph*I (Takara Bio; cleavage site, nucleotide position 1240 in the PA gene), and both of their reading frames were divided into three fragments. The fragments were cloned, and the resulting 6 plasmids were designated as pHW-PA/P706V (nucleotide position 1 to 706 of PR8, the other region of Vac-1), pHW-PA/V706P (nucleotide position 1 to 706 of Vac-1, and the other region of PR8), pHW-PA/V706P1240V (nucleotide position 707 to 1240 of PR8, and the other regions of Vac-1), pHW-PA/P706V1240P (nucleotide position 707 to 1240 of Vac-1, and the other regions of PR8), pHW-PA/V1240P (nucleotide position 1 to 1240 of Vac-1, and the other region of PR8), and pHW-PA/P1240V (nucleotide position 1 to 1240 of PR8, and the other region of Vac-1).

Recombinant viruses were generated by reverse genetic methods according to Hoffman et al. [5]. Briefly, 293T cells and Madin-Darby canine kidney (MDCK) cells were cocultured in 35-mm dishes and transfected with 1 μg of each of the eight plasmids and 16 μl of TransIT-293T (Promega) in a total volume of 1 ml of OPTI-MEM (Gibco). After 30 hours, 1 ml of OPTI-MEM with 5 μg/ml

Table 1 MDTs of chicken embryos inoculated with wild-type and recombinant influenza viruses

Virus	Gene segment [a]								MDT ± SD (h) [b]
	PB2	PB1	PA	HA	NP	NA	M	NS	
wt-PR8	□	□	□	□	□	□	□	□	95.0 ± 9.8
wt-Vac-1	■	■	■	■	■	■	■	■	64.0 ± 11.9
rg-PR8	□	□	□	□	□	□	□	□	86.2 ± 13.2
rg-Vac-1	■	■	■	■	■	■	■	■	55.2 ± 5.9
rg-Vac-PB2-PB1-PA/PR8	■	■	■	□	□	□	□	□	*62.3 ± 8.6
rg-PR8-PB2-PB1-PA/Vac-1	□	□	□	■	■	■	■	■	**79.1 ± 12.9
rg-Vac-1-HA-NA/PR8	□	□	□	■	□	■	□	□	88.0 ± 10.1
rg-PR8-HA-NA/Vac-1	■	■	■	□	■	□	■	■	62.2 ± 14.3
rg-Vac-1-NP-M-NS/PR8	□	□	□	□	■	□	■	■	*68.4 ± 11.4
rg-PR8-NP-M-NS/Vac-1	■	■	■	■	□	■	□	□	60.4 ± 9.6
rg-Vac-1-PB2/PR8	■	□	□	□	□	□	□	□	82.2 ± 3.9
rg-PR8-PB2/Vac-1	□	■	■	■	■	■	■	■	**72.0 ± 15.5
rg-Vac-1-PB1/PR8	□	■	□	□	□	□	□	□	*53.3 ± 5.7
rg-PR8-PB1/Vac-1	■	□	■	■	■	■	■	■	**88.0 ± 18.7
rg-Vac-1-PA/PR8	□	□	■	□	□	□	□	□	*69.6 ± 9.3
rg-PR8-PA/Vac-1	■	■	□	■	■	■	■	■	**90.2 ± 18.1

[a] White; derived from A/Puerto Rico/8/1934 (H1N1), Black; derived from A/duck/Hokkaido/Vac-1/2004 (H5N1)

[b] Values significantly different from that of rg-PR8 at the 5% level are indicated by a single asterisk, and values significantly different from that of rg-Vac-1 at the 5% level are indicated by double asterisks

acetyltrypsin was added, and the cells were incubated at 35°C for 48 hours. One hundred μl of the supernatant was inoculated into 10-day-old embryonated chicken eggs, which were then incubated at 35°C for 48 hours.

Mean death time of CEs inoculated with recombinant influenza viruses

Wild-type or recombinant viruses generated by the reverse genetic method were inoculated into the allantoic cavities of ten 9-day-old embryonated chicken eggs at 100 times the 50% egg infectious dose (EID_{50}). All eggs were incubated at 35°C and observed for embryo death every eight hours until 120 hours post-inoculation. The mean death time (MDT) of the CEs was calculated as the mean hours until the death of all ten inoculated CEs. Differences in pathogenicity between rg-PR8 or rg-Vac-1 and the recombinant viruses were evaluated statistically using Student's t-test at the 5% level.

Comparison of the HA titer of recombinant viruses in embryonated chicken eggs

Each recombinant virus was inoculated into the allantoic cavities of six 9-day-old embryonated chicken eggs at a dosage of 100 EID_{50} and incubated at 35°C for 72 hours. Allantoic fluids of the eggs were collected every 6 or 12 hours starting at 24 hours post-inoculation. Dead CEs were not sampled further. An HA test was performed to assess the HA titer of each of the collected allantoic fluids. The HA titers of allantoic fluids of CEs at each time point were evaluated statistically using the Welch t-test at the 5% level [26].

Results

MDTs of CEs inoculated with reassortant influenza viruses

The MDT of CEs inoculated with rg-PR8 and rg-Vac-1 generated by reverse genetic methods was 86.2 and 55.2 hours, respectively (Table 1). Although the MDTs of these recombinant viruses were not equal to those of the respective wild-type viruses, significant differences were not found between the MDTs of wild-type and recombinant viruses. The MDT of either the wild-type or reassortant of Vac-1 was approximately 30 hours shorter than that of PR8. The MDT of CEs inoculated with rg-Vac-1-PB2-PB1-PA/PR8 (H1N1), which had PB2, PB1, and PA gene

Table 2 MDTs of chicken embryos inoculated with influenza viruses with a chimeric gene segment

Virus	Gene segment [a, b]				MDT ± SD (h) [c]
	PB2	PB1	PA	Other genes	
rg-PR8	White	White	White	White	86.2 ± 13.2
rg-Vac-1	Black	Black	Black	Black	55.2 ± 11.9
		1 – 240 – 665 – 758			
rg-PB1/P719V	Black	White / Black / Black	Black	Black	69.0 ± 20.0
rg-PB1/V719P	White	Black / White / White	White	White	77.6 ± 23.0
rg-PB1/V719P1997V	Black	Black / White / Black	Black	Black	※※82.4 ± 17.4
rg-PB1/P719V1997P	White	White / Black / White	White	White	※68.0 ± 16.2
rg-PB1/V1997P	Black	Black / Black / White	Black	Black	58.6 ± 17.3
rg-PB1/P1997V	White	White / White / Black	White	White	81.4 ± 21.3
			1 – 235 – 413 – 716		
rg-PA/P706V	Black	Black	White / Black / Black	Black	※※86.0 ± 20.0
rg-PA/V706P	White	White	Black / White / White	White	※53.0 ± 10.4
rg-PA/V706P1240V	Black	Black	Black / White / Black	Black	※※82.4 ± 21.0
rg-PA/P706V1240P	White	White	White / Black / White	White	85.0 ± 23.9
rg-PA/V1240P	Black	Black	Black / Black / White	Black	69.6 ± 20.3
rg-PA/P1240V	White	White	White / White / Black	White	※67.2 ± 14.2

[a] White; derived from A/Puerto Rico/8/1934 (H1N1), Black; derived from A/duck/Hokkaido/Vac-1/2004 (H5N1)

[b] Chimeric parts are described in Materials and methods

[c] Values significantly different from that of rg-PR8 at the 5% level are indicated by single asterisks, and values significantly different from that of rg-Vac-1 at the 5% level are indicated by double asterisks

segments from Vac-1 and the others from PR8, was 62.3 hours. The MDT of CEs inoculated with rg-PR8-PB2-PB1-PA/Vac-1 (H5N1), which had PB2, PB1, and PA gene segments from PR8 and the others from Vac-1, was 79.1 hours. The pathogenicity of PR8 in CEs increased upon recombination of these three gene segments from Vac-1, and conversely, that of Vac-1 decreased upon recombination of these gene segments from PR8. The MDTs of CEs inoculated with rg-Vac-1-HA-NA/PR8 (H5N1) and rg-PR8-HA-NA/Vac-1 (H1N1) were not significantly different from those inoculated with rg-PR8 and rg-Vac-1, respectively. It is thus postulated that the virus glycoproteins of Vac-1 are not responsible for pathogenicity in CEs. The MDT of CEs inoculated with rg-Vac-1-NP-M-NS/PR8 (H1N1), which had NP, M, and NS gene segments from Vac-1 and the others from PR8, was 68.4 hours. Although an aggravation of the pathogenicity of recombinants in CEs upon the introduction of the gene segments from Vac-1 was observed, no significant difference was found in the pathogenicity in CEs between rg-Vac-1 and rg-PR8-NP-M-NS/Vac-1. These results indicate that, although it is possible that some of these three genes should be responsible for pathogenicity, it is unlikely that they are responsible for the pathogenicity of PR8 or Vac-1 in CEs.

The present results of the MDT test indicate that the viral polymerase proteins of Vac-1 are responsible for pathogenicity in CEs. To identify the virus protein(s) responsible for the pathogenicity in CEs, 6 clones of single gene reassortant viruses were prepared, and the MDT of each recombinant virus was determined. The MDTs of CEs inoculated with recombinant viruses possessing 7 gene segments from Vac-1 and one from PR8 were significantly different from those of CEs inoculated with rg-Vac-1. In addition, for three recombinant PR8 viruses possessing 7 gene segments from PR8 and one from Vac-1, the MDTs of CEs inoculated with each of the recombinant viruses except rg-Vac-1-PB2/PR8 (H1N1) were significantly different from those of CEs inoculated with rg-PR8. Recombinant PR8 viruses with either the PB1 or PA gene of Vac-1 were more pathogenic in CEs than rg-PR8, and recombinant Vac-1 viruses with either of the genes of PR8 were less pathogenic in CEs than rg-Vac-1. These results indicate that the PB1 and PA proteins of Vac-1 are responsible for the pathogenicity in CEs.

Identification of amino acid region(s) responsible for pathogenicity in CEs

Chimeric genes of the PB1 and PA gene segments of either PR8 or Vac-1 were constructed to identify the amino acid residue(s) of the encoded proteins responsible for the pathogenicity in CEs on the basis of the MDTs (Table 2).

A mutant PR8 virus with the pHW-PB1/P719V1997P gene was more pathogenic in CEs than rg-PR8. On the other hand, a mutant Vac-1 with the pHW-PB1/V719P1997V gene showed lower pathogenicity in CEs than rg-Vac-1. Sequence analysis showed that the nucleotide region from 720 to 1997 in the PB1 gene encodes amino acids 240 to 665 of the PB1 protein, indicating that this region of the PB1 protein is responsible for the pathogenicity of Vac-1 in CEs. The MDT of CEs inoculated with rg-PA/P706V (H5N1), which had a chimeric PA gene from nucleotide position 706 to 1240, was 86.0 hours. The MDT of CEs inoculated with rg-PA/V706P (H1N1), which was also chimeric at the same residues, was 53.0 hours. Sequence analysis showed that the nucleotide region from 1 to 707 in the PA gene encoding amino acids 1 to 235 in the PA protein is responsible for the pathogenicity of Vac-1 in CEs.

Comparison of HA titers of recombinant viruses in embryonated chicken eggs

The HA titers of recombinant viruses in embryonated chicken eggs were compared. Each of the recombinant H5N1 viruses that showed low pathogenicity in CEs was inoculated into 9-day-old embryonated chicken eggs. The infectious allantoic fluids were collected every 6 or 12 hours starting at 24 hours post-inoculation. Maximum HA titers of the allantoic fluids of embryonated chicken eggs inoculated with rg-Vac-1 and those inoculated with the other recombinant viruses were between 128 and 512 (Fig. 1). HA titers of rg-PR8-PB1/Vac-1 (H5N1) at 48 and 72 hours post-inoculation were significantly lower than those of rg-Vac-1. On the other hand, HA titers of rg-PR8-PA/Vac-1 (H5N1) were not lower than those of rg-Vac-1 at any of the time points. These results indicate that, for the four H5N1 recombinant viruses generated from Vac-1, which are less pathogenic in CEs than Vac-1, the HA titer of rg-PR8-PA/Vac-1 (H5N1) in CEs stayed at a high level similar to that of Vac-1 for 72 hours after inoculation.

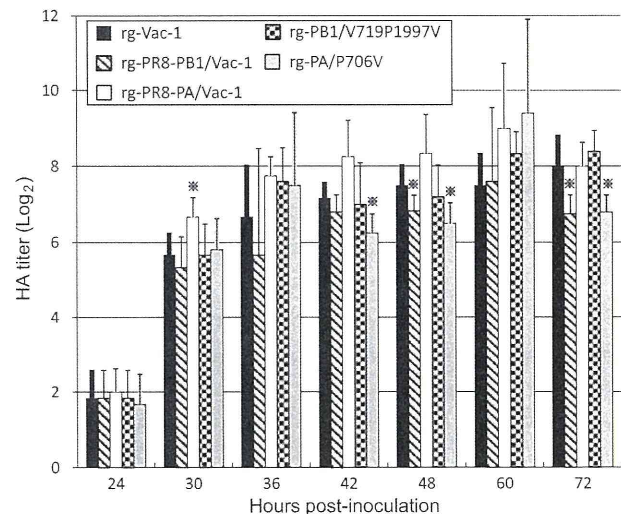

Fig. 1 Virus growth in chicken embryos. Each of six recombinant virus strains generated by reverse genetics was inoculated into six 9-day-old embryonated chicken eggs and incubated at 35°C for 72 hours. The allantoic fluids of the eggs inoculated with each virus were collected every 6 or 12 hours, starting at 24 hours post-inoculation, and samples were titrated by HA test. The mean (bar) and standard deviation (line) of the HA titer at each time point are shown. The differences in HA titer at each time point were evaluated statistically using the Welch t-test. An asterisk indicates a significant difference between the HA titers of the allantoic fluids of embryonated chicken eggs inoculated with rg-Vac-1 and those of each recombinant virus at the 5% level

Discussion

An influenza virus strain, A/duck/Hokkaido/Vac-1/2004 (H5N1), was generated by genetic reassortment between non-pathogenic H5N2 and H7N1 isolates from migratory ducks in order to prepare an inactivated avian influenza vaccine [9, 21, 23]. In the previous study, it was shown that the NA and NS gene segments of Vac-1 are derived from A/duck/Mongolia/47/2001 (H7N1), and the other segments from A/duck/Mongolia/54/2001 (H5N2) [23]. Although the pathogenicity of Vac-1 in CEs was not high (MDT = 64.0 hours), it should ideally be less pathogenic in CEs. In the present study, the contribution of the PB1 and PA proteins of the vaccine strains to pathogenicity in CEs was demonstrated. HA titers of the allantoic fluids of CEs inoculated with four of five recombinant viruses that were less pathogenic than Vac-1 were equal to that of rg-Vac-1 in CEs. The other recombinant virus, rg-PR8-PA/Vac-1 (H5N1), grew more efficiently than rg-Vac-1. Therefore, the present data provide information concerning how to establish a good vaccine strain with high growth potential and low pathogenicity in CEs.

Between PR8 and Vac-1, 10 amino acid differences were found from positions 240 to 666 in the PB1 protein, and seven amino acid differences were found from positions 1 to 235 in the PA protein (Table 3). In the present study, recombinant viruses with 10 and 7 amino acid substitutions in the PB1 and PA proteins, respectively, did not show significant differences in the MDT of CEs (data not shown). It has been shown that the virus polymerase proteins of avian influenza viruses are responsible for pathogenesis in different host animals [8, 13]. In the case of acquisition of pathogenicity in chickens by serial intracerebral passages, amino acid substitutions were identified not only in the HA but also in the internal proteins, including the PB1 and PA proteins [13]. The PB1 and PA proteins of H5N1 HPAI viruses were responsible for

Table 3 Amino acid differences in PB1 and PA proteins between PR8 and Vac-1

Virus	Amino acid at each position in the virus proteins																
	PB1 (from 240 to 665)										PA (from 1 to 235)						
	325	375	383	398	473	563	577	640	645	654	20	28	55	57	65	100	213
A/Puerto Rico/8/1934 (H1N1)	M	S	D	E	L	I	I	M	M	N	T	L	N	Q	L	A	K
A/duck/Hokkaido/Vac-1/2004 (H5N1)	I	N	E	D	V	R	L	V	V	S	A	P	D	R	S	V	R

lethality in mallard ducks, although the mechanism by which these two proteins play roles in pathogenicity has not been clarified [8]. It has been reported that strong interferon beta antagonism due to the accumulation of the virus NS1 proteins in the cytoplasm of infected cells is associated with high pathogenicity of the virus in avian hosts, including embryonated chicken eggs [10]. In the present study, it was considered that the virus pathogenicity in CEs may be associated with viral polymerase activity. However, we were not able to provide conclusive evidence relating to viral polymerase activity using a minigene luciferase assay and pathogenicity in CEs (data not shown). The association between the viral polymerase and pathogenicity in CEs needs to be clarified in a further study.

The crystal structure and function of the amino terminus of the PA protein have been analyzed [3, 17, 19, 29]. It has been shown that amino acid residues 1 to 209 in the PA protein contain the site of endonuclease activity [3]. The PB2 protein binds the 5' cap of host pre-messenger RNA that has been cleaved by the virus endonuclease, and the viral polymerase synthesizes viral messenger RNA. It has also been reported that amino acid residues 163 to 178 of the PA protein are directly or indirectly involved in complementary RNA promoter binding, suggesting a novel function for the PA protein in modulating promoter binding [17]. The sites responsible for these activities are located in the region of amino acids 1 to 235 in the PA protein.

In order to account for the defect in the avian influenza vaccine strain, the virus proteins responsible for pathogenicity in CEs were identified. Rg-PR8-PA/Vac-1 (H5N1), which shows lower pathogenicity in CEs than rg-Vac-1 and gives a high HA titer in CEs that is similar to that of Vac-1, was obtained as a new vaccine strain by using reverse genetics. The present data indicate that replacement of PB1 and PA genes of vaccine strains with those of the PR8 strain decreases their pathogenicity in CEs. This procedure could be applied for the establishment of influenza vaccine strains.

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Predicting the Antigenic Structure of the Pandemic (H1N1) 2009 Influenza Virus Hemagglutinin

Manabu Igarashi[1], Kimihito Ito[1], Reiko Yoshida[1], Daisuke Tomabechi[1], Hiroshi Kida[1,2,3], Ayato Takada[1]*

1 Department of Global Epidemiology, Hokkaido University Research Center for Zoonosis Control, Sapporo, Japan, 2 Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan, 3 OIE Reference Laboratory for Highly Pathogenic Avian Influenza, Sapporo, Japan

Abstract

The pandemic influenza virus (2009 H1N1) was recently introduced into the human population. The hemagglutinin (HA) gene of 2009 H1N1 is derived from "classical swine H1N1" virus, which likely shares a common ancestor with the human H1N1 virus that caused the pandemic in 1918, whose descendant viruses are still circulating in the human population with highly altered antigenicity of HA. However, information on the structural basis to compare the HA antigenicity among 2009 H1N1, the 1918 pandemic, and seasonal human H1N1 viruses has been lacking. By homology modeling of the HA structure, here we show that HAs of 2009 H1N1 and the 1918 pandemic virus share a significant number of amino acid residues in known antigenic sites, suggesting the existence of common epitopes for neutralizing antibodies cross-reactive to both HAs. It was noted that the early human H1N1 viruses isolated in the 1930s–1940s still harbored some of the original epitopes that are also found in 2009 H1N1. Interestingly, while 2009 H1N1 HA lacks the multiple *N*-glycosylations that have been found to be associated with an antigenic change of the human H1N1 virus during the early epidemic of this virus, 2009 H1N1 HA still retains unique three-codon motifs, some of which became *N*-glycosylation sites via a single nucleotide mutation in the human H1N1 virus. We thus hypothesize that the 2009 H1N1 HA antigenic sites involving the conserved amino acids will soon be targeted by antibody-mediated selection pressure in humans. Indeed, amino acid substitutions predicted here are occurring in the recent 2009 H1N1 variants. The present study suggests that antibodies elicited by natural infection with the 1918 pandemic or its early descendant viruses play a role in specific immunity against 2009 H1N1, and provides an insight into future likely antigenic changes in the evolutionary process of 2009 H1N1 in the human population.

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* E-mail: atakada@czc.hokudai.ac.jp

Introduction

In April 2009, pandemic (H1N1) 2009 influenza virus (2009 H1N1) was first found in patients with febrile respiratory illness in the United States and Mexico, and has spread rapidly across the world by human-to-human transmission. On the 11th of June 2009, the World Health Organization declared a global pandemic of 2009 H1N1 infection. H1N1 influenza virus caused a pandemic in 1918 (1918 H1N1) [1], and its descendant virus with highly altered antigenicity of the viral surface protein, hemagglutinin (HA) has been causing "seasonal flu" in humans.

The 2009 H1N1 resulted from genetic reassortment between the recently circulating swine H1 viruses in North America and the avian-like swine viruses in Europe [2]. Phylogenetic analysis showed that the HA gene of 2009 H1N1 was derived from the so-called "classical swine H1N1" virus, which likely shares a common ancestor with the recent human H1N1 virus [2]. Accordingly, it has been reported that the early strains of the classical swine H1N1 virus, which was first identified in North America in 1930, were antigenically similar to the prototype strain of 1918 H1N1, A/South Carolina/1/1918 (SC1918), detected from a few victims of the pandemic in 1918 [3,4]. Since antigenic changes occur more slowly in swine than in the human population [5], HA of the classical swine H1N1 virus was antigenically highly conserved until the late 1990s [4,6], raising the possibility that the recently emerged 2009 H1N1 may still retain an antigenic structure similar to that of SC1918 and the early isolates of its descendants.

In this study, we generated three-dimensional (3D) structures of the HA molecules of 1918 H1N1, its descendent, recent seasonal H1N1 viruses, and 2009 H1N1, and compared their antigenic structures to look for evidence for the existence of shared epitopes for neutralizing antibodies. Since the 2009 H1N1 HA antigenic sites will be targeted by antibody-mediated selection pressure in humans in the near future, we further discuss possible directions of antigenic changes in the evolutionary process of this pandemic virus.

Results and Discussion

It is known that the H1 HA molecules have four distinct antigenic sites: Sa, Sb, Ca, and Cb [7,8,9,10] (Figure 1). As a result, these sites consist of the most variable amino acids in the HA molecule of the seasonal human H1N1 viruses that have been subjected to antibody-mediated immune pressure since its

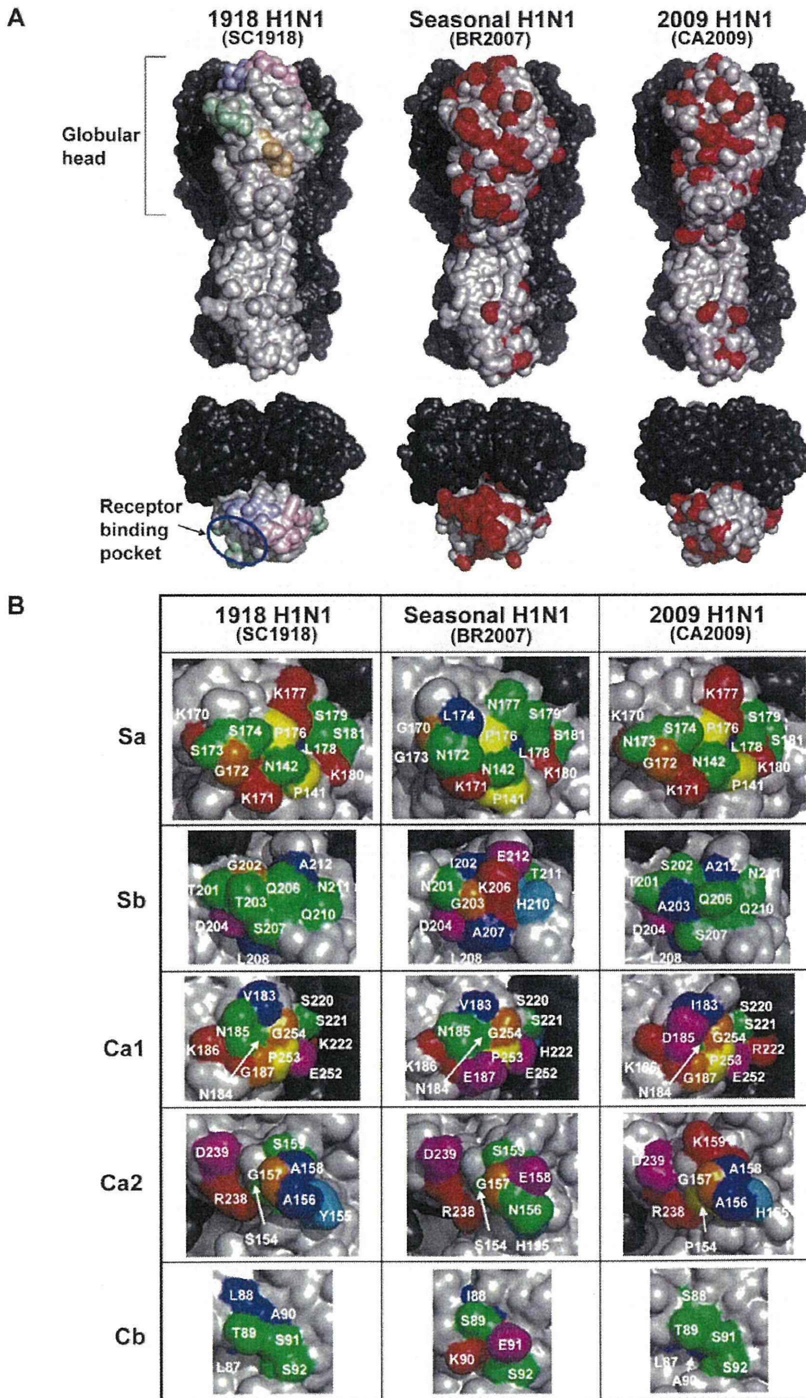

Figure 1. Comparison of the structures of antigenic sites on the HA molecules among 1918 H1N1 (SC1918), recent seasonal H1N1 (BR2007), and 2009 H1N1 (CA2009). Three-dimensional models of the H1 HA molecules of SC1918, BR2007, and CA2009 were constructed based on the HA crystal structures of A/South Carolina/1/18, A/Puerto Rico/8/34, and A/swine/Iowa/30, respectively (PDB codes: 1RUZ, 1RU7, and 1RUY, respectively). Models with solvent-accessible surface representation were generated by a molecular modeling method as described in the Methods section. Molecular surface of the HA trimers viewed on its side (upper) and top (lower) are shown (A). One monomer (center) is colored gray and the others are colored dark gray. The antigenic sites, Sa (light pink), Sb (light blue), Ca (pale green), and Cb (light orange) are indicated on the model of SC1918 HA. The spatial locations of amino acid residues that are distinct from those of SC1918 HA are shown in red on the models of BR2007 and CA2009 HAs. Each amino acid residue is mapped on the close-up views of each antigenic site of SC1918, BR2007, and CA2009 HAs (B). The Ca site is divided into subregions, Ca1 and Ca2. Amino acids are colored by the default ClustalX color scheme [29]: Trp, Leu, Val, Ile, Met, Phe, and Ala (blue); Lys and Arg (red); Thr, Ser, Asn, and Gln (green); Cys (pink); Asp and Glu (magenta); Gly (orange); His and Tyr (cyan); Pro (yellow).
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Table 1. Amino acid similarity in the HA antigenic sites among recent seasonal H1N1 (BR2007), 2009 H1N1 (CA2009), and 1918 H1N1 (SC1918).

Antigenic sites	No. amino acids involved	No. of amino acids identical to SC1918	
		BR2007	CA2009
Sa	13	8	12
Sb	12	4	10
Ca	19	13	13
Cb	6	2	5

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emergence in 1918 [3]. To investigate the structures of these antigenic sites of 2009 H1N1, 3D structures of the HA molecules of SC1918, the recent seasonal human H1N1 virus A/Brisbane/59/2007 (BR2007), and 2009 H1N1 A/California/04/2009 (CA2009) [2] were constructed by a homology modeling approach, and compared by mapping all the amino acid residues that were distinct from those of SC1918 HA (Figure 1 and Table S1). We found that most of these antigenic sites of BR2007 HA predominantly contained altered amino acid residues if compared with SC1918. By contrast, amino acid residues at these positions were relatively conserved in CA2009 HA. Notably, the Sa and Sb sites that contain many amino acids involved in neutralizing epitopes near the receptor binding pockets [8,10] remain almost intact in CA2009 HA (Table 1), suggesting that antibodies raised by natural infection with SC1918 or its antigenically related descendant viruses play a role in specific immunity against CA2009.

We then constructed 3D structures of the representative strains of seasonal H1 viruses that had been isolated since 1934, and tracked the amino acid substitutions on their HA molecules (Figure 2 and Figure S1). We confirmed that amino acid substitutions associated with the antigenic changes gradually accumulated on the globular head region of HA and were distributed over four distinct antigenic sites. However, it was noted that the early isolates represented by the A/Puerto Rico/8/1934 and A/Bellamy/1942 strains, but not the strains isolated after the

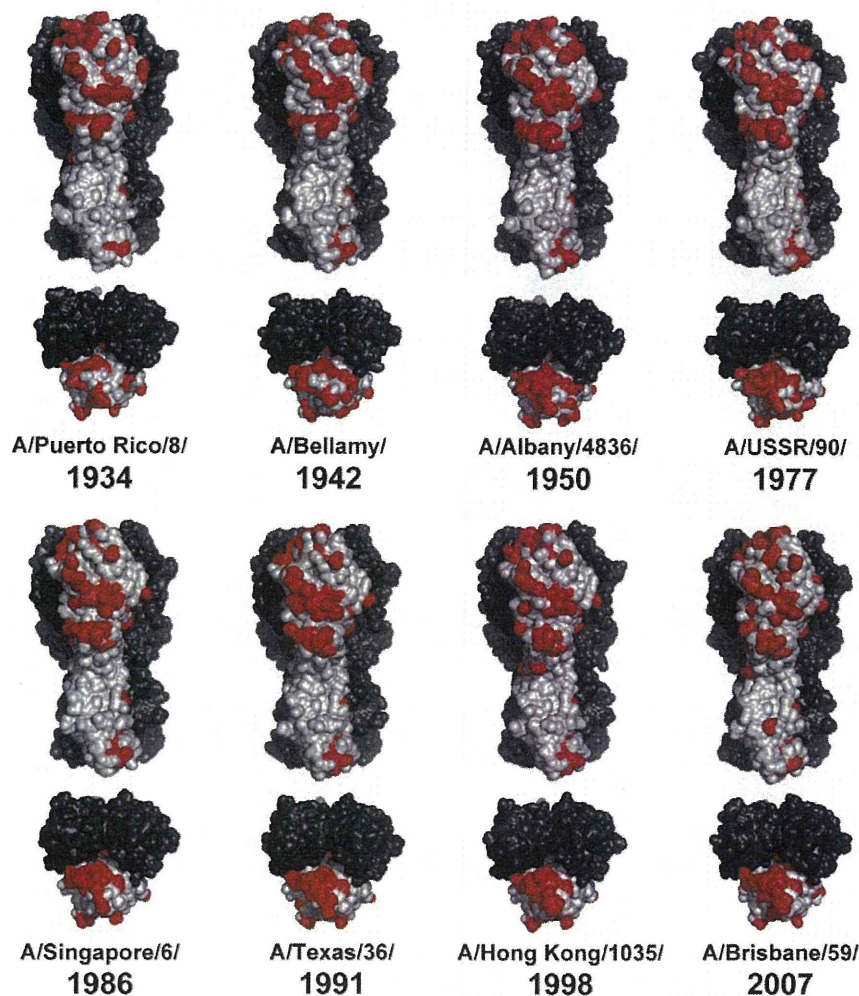

Figure 2. Amino acid substitutions associated with antigenic changes of seasonal human H1N1 virus HAs. All models were generated and shown by a molecular modeling method as described in the Methods section and the legend of Figure 1.
doi:10.1371/journal.pone.0008553.g002

1950s, still harbored unchanged amino acids forming potential neutralizing epitopes in the Sa and Sb sites (Figure 2). It seems likely that most of the amino acids on these antigenic sites were eventually substituted in the late 1940s (Figure S1).

It is well-documented that antigenic changes of HA occasionally result in the acquisition of carbohydrate side chains on the HA molecule [8,11]. Since the carbohydrate side chains in the vicinity of antigenic sites mask the neutralizing epitopes on the HA surface, amino acid substitutions associated with acquisition of carbohydrate chains are believed to efficiently generate antigenic variants. Accordingly, recent seasonal H1N1 viruses have acquired 4–5 *N*-glycosylation sites (Asn-Xaa-Ser/Thr, where Xaa is any amino acid except Pro) in the globular head region of HA [12,13], whereas SC1918 HA had only one site, at Asn 104 (Figure 3).

Interestingly, CA2009 also has a single potential *N*-glycosylation site at the same position in the globular head region of HA (Figure 3), despite the fact that the classical swine H1N1 virus emerged in the early 1900s and was circulating in the pig population until recently. This prompted us to estimate the potential of 2009 H1N1 to acquire additional *N*-glycosylation sites on its HA, which may be related to its future evolutionary process in the human population. We previously defined a three-codon motif that becomes an *N*-glycosylation site with a single-nucleotide mutation as "*Cand1*", and suggested that the presence of the *Cand1* sites in the HA sequence is one of the key factors for human influenza A viruses to rapidly acquire *N*-glycosylation sites during the early epidmic in the human population [13]. We compared the number of the *Cand1* sites in the HA globular head region between SC1918 and CA2009 (Figure 3 and Table S1). We found that CA2009 HA possessed three *Cand1* sites on the antigenic sites Sa and Ca, all of which were also present at the same position in SC1918 HA (positions of the first Asn residue, 177, 179, and 184). Of these, the *Cand1* sites with positions at 177 and 179 had actually become potential *N*-glycosylation sites in human H1N1 viruses, although these two sites did not exist concurrently [12]. It is noted that these two *Cand1* sites are still present on the surface of CA2009 HA, suggesting the likelihood of additional *N*-glycosylation at these sites during future antigenic changes of 2009 H1N1 HA.

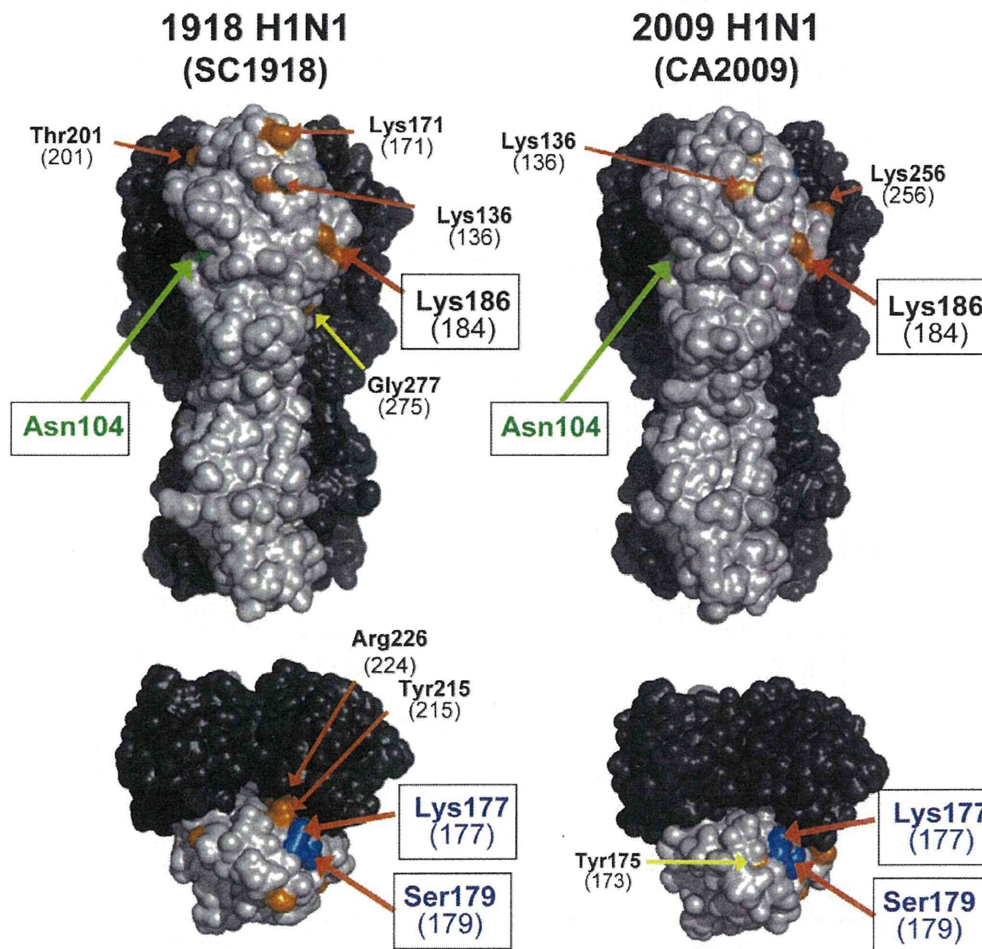

Figure 3. Comparison of the *N*-glycosylation potential of HA between SC1918 and CA2009. Residues shown in green represent Asn at the actually existing *N*-glycosylation sites. Residues shown in orange or blue represent the amino acids in *Cand1* sites that require a nucleotide substitution to produce *N*-glycosylation sites. Residues shown in blue represent the amino acids that were actually substituted, resulting in the acquisition of *N*-glycosylation sites during the antigenic evolution of human H1N1 viruses. Numbers in parentheses show the positions of Asn residues that may be linked to carbohydrate chains, if respective *Cand1* sites mutate to have *N*-glycosylation sites. All models were generated as described in the Methods section and the legend of Figure 1.
doi:10.1371/journal.pone.0008553.g003

In this paper, we employed 3D structures constructed by a homology modeling method to map amino acid residues on the antigenic sites of HA. When compared to the presentation of simple primary sequences, the 3D presentation has following advantages: (a) There are several amino acid residues that are buried beneath the surface of the HA molecule, even if they are included in the antigenic sites described by the primary amino acid sequences. Since such amino acid residues do not directly contribute to the interaction with antibodies, the surface structures of antigenic sites that are accessible for antibodies can be compared more precisely in the presentation by 3D models than by the primary amino acid sequence. (b) An epitope likely consists of multiple amino acid residues belonging to different antigenic regions presented by the primary amino acid sequence. Such conformational epitopes can be illustrated only by the 3D presentation. (c) One of the purposes of this study is to provide a structural basis to confirm antigenic similarity between the 1918 H1N1 and the pandemic 2009 H1N1 viruses. For this purpose, we employed a homology modeling method rather than simply mapping on the existing crystal structure (e.g. 1918 H1N1 HA), since this method is generally used to generate a 3D structure of a protein molecule if there is no available crystal structure of the target protein [14]. Thus, we believe that this method produces more likely HA structure models of the viruses whose HA crystal structure are not available (e.g. CA2009). In fact, our homology modeling approach suggests that several amino acid residues were occasionally buried beneath or exposed to the surface of HA molecule, depending on the substitutions found in the viruses examined (Figure 1B and Figure S1). The homology modeling approach might enable us to analyze such dynamics of antigenic changes at molecular levels.

Our analysis indicated that 2009 H1N1 had undergone less significant antigenic changes of HA in the pig population than human H1N1 virus since their emergence in the early 1900s. The Centers for Disease Control and Prevention reported that vaccination with recent (2005–2009) human H1N1 viruses was unlikely to provide protection against 2009 H1N1 [15]; however, cross-reactive antibodies were detected in 33% of people aged 60 and over. Another report showed that appreciable neutralizing antibodies against CA2009 were present in the sera collected from individuals born before 1918 [16]. Our 3D models provide a protein-structural basis supporting these observations, and further suggest that infection with the 1918 H1N1 or early human H1N1 viruses (viruses present before the 1940s), but not with antigenically divergent human H1N1 viruses circulating after the 1950s, elicited cross-neutralizing antibodies to 2009 H1N1.

This virus will soon be subjected to complex immunological selection pressure by the antibody response that will be induced in the human population by vaccination and/or natural infection with homologous viruses, and pre-existing immunity cross-reactive to the early descendants of 1918 H1N1. In the present study, we showed that the antigenic structure of 2009 H1N1 HA might still be similar, at least in part, to that of the 1918 H1N1 HA. We speculate that the 2009 H1N1 HA antigenic sites involving the conserved amino acids will soon be targeted by neutralizing antibodies in humans. Thus, it is of interest to monitor whether these antigenic sites of 2009 H1N1 will undergo similar patterns of amino acid substitutions to those seen in seasonal H1N1 viruses during its epidemic period (Figure 4). Interestingly, we found that some of the recent variants of the 2009 H1N1 virus (as of November 3, 2009) have indeed undergone substitutions identical to those predicted in Figure 4. Although the present study still needs to be supported by experimental data, our approach may provide new perspectives on collective immunity against 2009 H1N1 and an insight into future antigenic changes of this new human pandemic influenza virus.

Methods

Sequence Data of HA Genes

Nucleotide sequences for HA genes of SC1918 (AF117241), BR2007 (CY030230), CA2009 (FJ966082), A/Puerto Rico/8/1934/Mount Sinai (AF389118), A/Bellamy/1942 (CY009276), A/Albany/4836/1950 (CY021701), A/USSR/90/1977 (DQ508897), A/Singapore/6/1986 (CY020477), A/Texas/36/1991 (AY289927), and A/Hong Kong/1035/1998 (AF386777) [2,3,17,18,19,20] were obtained from Influenza Virus Resource at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html).

Molecular Modeling

MODELLER 9v6 [21] was used for homology modeling of HA structures. After one hundred models of the HA trimer were generated, the model was chosen by a combination of the MODELLER objective function value and the discrete optimized protein energy (DOPE) statistical potential score [22]. After addition of hydrogen atoms, the model was refined by energy minimization (EM) with the minimization protocols in the Discovery Studio 2.1 software package (Accelrys, San Diego, CA) using a CHARMm force field. Steepest descent followed by conjugate gradient minimizations was carried out until the root mean square (rms) gradient was less than or equal to 0.01 kcal/mol/Å. The generalized Born implicit solvent model [23,24] was used to model the effects of solvation. The HA model was finally evaluated by using PROCHECK [25], WHATCHECK [26], and VERIFY-3D [27]. All figures are shown as a solvent-accessible surface representation prepared by PyMOL (DeLano Scientific LLC) [28]. All HA structures constructed by a homology modeling method are available in Supplementary Files S1, S2, S3, S4, S5, S6, S7, and S8.

Sequence Data Analyses for *N*-Glycosylation Sites

Custom-made programs were developed with the Ruby language and used for investigating the numbers of potential *N*-glycosylation sites and candidate codons (*CandI*) in HA sequences. The programs are available upon request.

Supporting Information

Table S1
Found at: doi:10.1371/journal.pone.0008553.s001 (0.04 MB PDF)

Figure S1 Amino acid substitutions of seasonal human H1N1 virus HAs shown in close-up views of each antigenic site. The strains used in this analysis are corresponding to those shown in Figure 2. Amino acids are colored according to the scheme in the legend of Figure 1B.
Found at: doi:10.1371/journal.pone.0008553.s002 (1.02 MB PDF)

File S1 PDB file of the homology model of H1 HA (A/California/04/2009) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s003 (0.20 MB ZIP)

File S2 PDB file of the homology model of H1 HA (A/Bellamy/1942) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s004 (0.20 MB ZIP)

File S3 PDB file of the homology model of H1 HA (A/Albany/4836/1950) after energy minimizations.

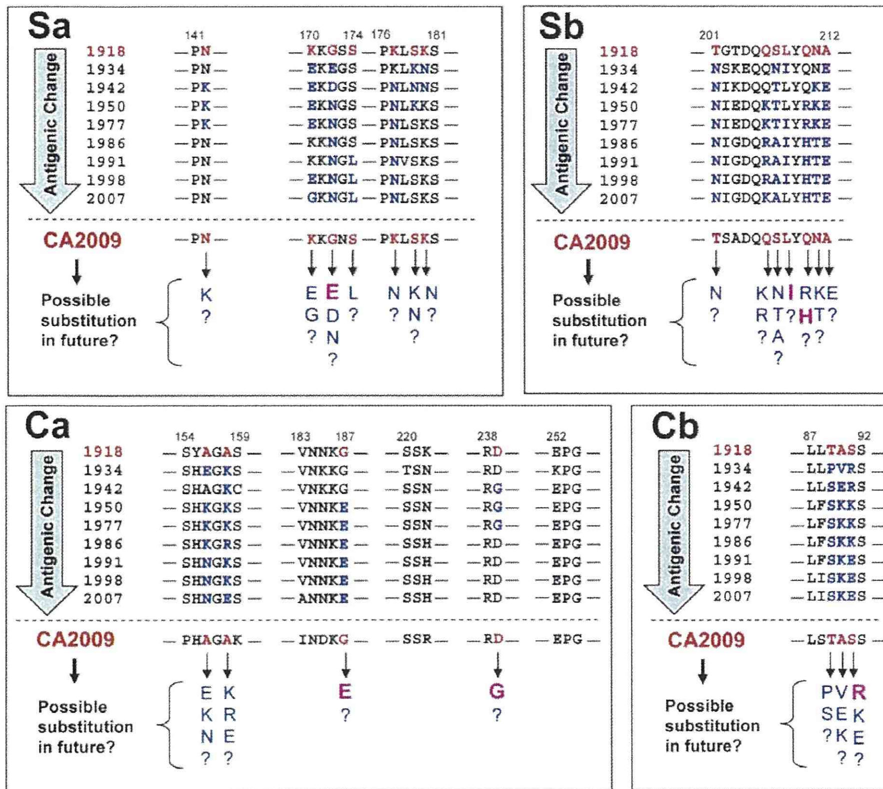

Figure 4. Prediction of the future amino acid substitutions on the antigenic sites of 2009 H1N1 HA. Amino acid sequences of HA antigenic sites of human H1N1 viruses are shown. Sequence data are corresponding to those of virus strains shown in Figures 1 and 2. Amino acid residues shared between 1918 H1N1 (SC1918) and 2009 H1N1 (CA2009) are shown in red, and those that have been substituted since 1934 are shown in blue. Amino acid residues indicated by arrows represent the predicted substitutions which might be associated with antigenic changes of 2009 H1N1 in the near future. The amino acid substitutions which have already been found in the recent variants of the 2009 H1N1 virus (as of November 3, 2009) are shown in bold pink letters.
doi:10.1371/journal.pone.0008553.g004

Found at: doi:10.1371/journal.pone.0008553.s005 (0.20 MB ZIP)

File S4 PDB file of the homology model of H1 HA (A/USSR/90/1977) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s006 (0.20 MB ZIP)

File S5 PDB file of the homology model of H1 HA (A/Singapore/6/1986) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s007 (0.20 MB ZIP)

File S6 PDB file of the homology model of H1 HA (A/Texas/36/1991) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s008 (0.20 MB ZIP)

File S7 PDB file of the homology model of H1 HA (A/Hong Kong/1035/1998) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s009 (0.20 MB ZIP)

File S8 PDB file of the homology model of H1 HA (A/Brisbane/59/2007) after energy minimizations.
Found at: doi:10.1371/journal.pone.0008553.s010 (0.20 MB ZIP)

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Author Contributions

Conceived and designed the experiments: MI KI HK AT. Analyzed the data: MI RY DT. Wrote the paper: MI AT.

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