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Evolutional analysis of human influenza A virus N2 neuraminidase genes based on the transition of the low-pH stability of sialidase activity[1]

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Abstract The 1957 and 1968 human pandemic influenza A virus strains as well as duck viruses possess sialidase activity under low-pH conditions, but human H3N2 strains isolated after 1968 do not possess such activity. We investigated the transition of avian (duck)-like low-pH stability of sialidase activities with the evolution of N2 neuraminidase (NA) genes in human influenza A virus strains. We found that the NA genes of H3N2 viruses isolated from 1971 to 1982 had evolved from the side branches of NA genes of H2N2 epidemic strains isolated in 1968 that were characterized by the low-pH-unstable sialidase activities, though the NA genes of the 1968 pandemic strains preserved the low-pH-stable sialidase. These findings suggest that the prototype of the H3N2 epidemic influenza strains isolated after 1968 probably acquired the NA gene from the H2N2 low-pH-unstable sialidase strain by second genetic reassortment in humans.

Key words: Influenza virus; Neuraminidase; Sialidase; Evolutional analysis

1. Introduction

New pandemic influenza A viruses seem to be generated by transmission of avian viruses to other animal species or by genetic reassortment between avian and other host viruses [1–3]. The H2 and H3 subtypes of hemagglutinin (HA) appeared in the 1957 and 1968 pandemic strains by genetic reassortment from the avian influenza viruses [4–6], while the N2 subtype of neuraminidase (NA) first appeared in the 1957 strain and was inherited in the 1968 pandemic strain from the prevalent H2N2 virus in humans [7,8]. With regard to the evolution of N2 NAs in humans, NA genes of H3N2 viruses isolated between 1968 and 1997 have been shown to share essentially a single lineage with short side branches [9,10]; however, little is known about the properties of the original strain from which the NA gene of the 1968 pandemic influenza virus was introduced [7,11–13].

Replication of human–avian reassortant influenza A viruses in ducks showed that reassortant viruses with the NA gene from the human strain A/Udorn/307/72 (H3N2) and all other genes from the duck virus failed to infect the duck intestine by oral inoculation [14]. In addition, a reassortant virus containing the NA gene from A/Singapore/1/57 (H2N2), but not a reassortant virus with the NA gene from later human viruses (H2N2), replicated in the intestinal tracts of ducks [15]. These observations suggest that the biological property of duck virus NA contributes to the host range restriction and that the property has been inherited in the 1957 human pandemic virus NAs. On the other hand, we found that sialidase activities in the 1957 and 1968 pandemic influenza A virus strains A/Singapore/1/57 (H2N2), A/Japan/305/57 (H2N2), A/Aichi/2/68 (H3N2) and A/Hong Kong/1/68 (H3N2), as well as in duck viruses were maintained under low-pH conditions, but that sialidase activities in human H3N2 strains isolated after 1968 disappeared under low-pH conditions [16].

In this study, we determined how the sialidase low-pH stability of the 1957 pandemic strains was introduced into late virus strains with the evolution of human influenza A viruses. We report here the evolution of human influenza A virus N2 NAs based on the transition of avian (duck)-like low-pH stability of sialidase activity and evolutional analysis of the nucleotide sequences of the NA genes in 22 influenza A virus strains isolated between 1957 and 1982.

2. Materials and methods

2.1. Viruses

The influenza A viruses tested were propagated in the allantoic sacs of 10-day-old embryonated eggs and purified by sucrose density gradient centrifugation as described previously [17].

2.2. Sialidase assay

Sialidase activity of each virus strain and each cell-expressed NA was determined by a fluorometric assay as described previously [16,18]. The assays were performed in duplicate.

2.3. NA gene sequence analysis

Each virion RNA was extracted with Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA). cDNAs of NA genes from influenza viruses were synthesized using AMV reverse transcriptase (RT) and amplified by polymerase chain reaction (PCR) with forward and reverse primer oligonucleotides as described previously [16] and *pfu*

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[1] Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB101671–AB101675, AB124653–AB124664.

Abbreviations: HA, hemagglutinin; NA, neuraminidase

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Table 1
Comparison of nucleotide sequences of the N2 NA genes in the 1967 and 1968 epidemic H2N2 strains, the 1968 pandemic H3N2 strains, and the H3N2 epidemic strains isolated after 1968

Strains	Low-pH stability	HA subtypes	Nucleotide at position[a]																					
			57	130	137	296	396	496	599	613	691	730	847	866	876	988	1033	1042	1050	1201	1226	1237	1327	1357	1381	1415
A/Georgia/1/67	yes	H2	T	T	C	C	C	A	A	T	A	C	G	G	C	C	G	C	G	A	C	C	A	T	A	T
A/Poland/6/67	yes	H2	T	T	C	C	C	A	A	T	A	C	G	G	C	C	G	C	G	A	C	C	A	T	A	T
A/Cordoba/522/67	yes	H2	T	T	C	C	C	A	A	T	A	C	G	G	C	C	G	C	G	A	C	C	A	T	A	T
A/Preg/1/68	yes	H2	T	T	C	C	C	A	A	T	A	C	G	G	C	C	G	C	G	A	C	C	A	T	A	T
A/Texas/68	no	H2	C	C	C	A	C	G	G	T	G	T	G	G	A	A	A	C	A	G	A	T	A	C	G	C
A/Berkeley/68	no	H2	C	C	C	A	C	G	G	T	G	T	G	G	A	A	A	C	A	G	A	T	A	C	G	C
A/Hong Kong/1/68	yes	H3	T	T	T	C	A	A	A	C	A	C	A	A	G	C	G	T	G	A	C	C	G	T	A	T
A/Aichi/2/68	yes	H3	T	T	T	C	A	A	A	C	A	C	A	A	G	C	G	T	G	A	C	C	G	T	A	T
A/Memphis/1/71	no	H3	T	C	T	A	C	G	G	T	G	T	G	G	G	C	G	C	A	G	A	T	A	T	G	C
A/Memphis/102/72	no	H3	T	C	T	A	C	G	G	T	G	T	G	G	G	C	G	C	A	G	A	T	A	T	G	C
A/Tokyo/6/73	no	H3	T	C	T	A	C	G	G	T	G	T	G	G	G	C	G	C	A	G	A	T	A	T	G	C
A/Kumamoto/55/76	no	H3	T	C	T	A	C	G	G	T	G	T	G	G	G	C	G	C	A	G	A	T	A	T	G	C
A/Yamanashi/2/77	no	H3	T	C	T	A	C	G	G	T	G	T	G	G	G	C	G	C	A	G	A	T	A	T	G	C
A/Texas/1/77	no	H3	T	C	T	A	C	G	G	T	G	T	G	G	G	C	G	C	A	G	A	T	C	T	G	C
A/Bangkok/1/79	no	H3	T	C	T	A	C	G	G	T	G	T	G	G	G	C	G	C	A	G	A	T	C	T	A	C
A/Victoria/2/82	no	H3	T	C	T	A	C	G	G	T	G	T	G	G	G	C	G	C	A	G	A	T	C	T	A	C

[a]Only the nucleotides that are different between A/Hong Kong/1/68 (H3N2) and A/Texas/68 (H2N2) are shown. Bold nucleotide sequences indicate the nucleotides that are conserved in A/Texas/68 (H2N2), A/Berkeley/68 (H2N2), and the H3N2 epidemic influenza strains isolated after 1968.

(Stratagene, La Jolla, CA, USA) or *Taq* (Takara Shuzo Co. Ltd., Tokyo, Japan) DNA polymerase. The PCR-derived double-stranded (ds)DNA was used as a template for automated sequencing on an Applied Biosystem 373A automated sequencer (Perkin-Elmer, Foster City, CA, USA). Seven internal forward primers and six reverse primers were used to sequence the coding region of the NA gene. The nucleotide sequences of NA genes were compared among viruses in the regions of amino acid residues 1–469.

2.4. Evolutional analyses

Nucleotide distance matrices were estimated by the three-parameter method based on the number of total substitutions. Evolutional trees were constructed by the neighbor-joining method [19]. All of the calculations were done using BIORESEARCH/SINCA ver. 3.0 (Fujitsu, Tokyo, Japan). The nucleotide sequences (amino acid positions 1–469) of A/Poland/6/67 (H2N2), A/Georgia/1/67 (H2N2), A/Cordoba/522/67 (H2N2), A/Preg/1/68 (H2N2), and A/Bangkok/1/79 (H3N2) were obtained from a published sequence analysis [13].

2.5. Cloning of NA genes

Viral RNAs from A/Adachi/2/57 (H2N2), A/Japan/305/57 (H2N2), A/Singapore/1/57 (H2N2), A/Ann Arbor/6/60 (H2N2), A/Murakami/4/64 (H2N2), A/Georgia/1/67 (H2N2), A/Preg/1/68 (H2N2), A/Berkeley/68 (H2N2), A/Texas/68 (H2N2), A/Aichi/2/68 (H3N2), A/Hong Kong/1/68 (H3N2), A/Memphis/1/71 (H3N2), A/Duck/Hong Kong/273/8/78 (H2N2), and A/Duck/Hong Kong/47/5/76 (H7N2) were extracted with Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) and amplified using an RT-PCR kit ver. 2.0 (Takara, Shuzo Co. Ltd., Tokyo, Japan) with the primer 5′-AGCAAAAGCAGG-3′. Full-length cDNAs of the NA genes were amplified by PCR with a set of primers containing *Eco*RI and *Xho*I restriction enzyme sites and *pfu* DNA polymerase (Stratagene, La Jolla, CA, USA). PCR products were digested with *Eco*RI and *Xho*I restriction enzymes (Takara, Tokyo, Japan) for 20 h at 37°C and then purified by 1% agarose gel electrophoresis. Each fragment was subcloned between the *Eco*RI and *Xho*I restriction enzyme sites of the plasmid expression vector pCAGGS/MCS [20] to generate pCAGGS-Ada57NA (Ada57NA), pCAGGS-Jap57NA (Jap57NA), pCAGGS-Sin57NA (Sin57NA), pCAGGS-Ann60NA (Ann60NA), pCAGGS-Mur64NA (Mur64NA), pCAGGS-Geo67NA (Geo67NA), pCAGGS-Per68NA (Per68NA), pCAGGS-Ber68NA (Ber68NA), pCAGGS-Tex68NA (Tex68NA), pCAGGS-Aic68NA (Aic68NA), pCAGGS-HK68NA (HK68NA), pCAGGS-Mem71NA (Mem71NA), pCAGGS-DKHK78NA (DKHK78NA), and pCAGGS-DKHK76NA (DKHK76NA) respectively using a DNA ligation kit ver. 2.0 (Takara Shuzo Co. Ltd., Tokyo, Japan).

3. Results

3.1. Low-pH stability of sialidase activities of human H2N2 viruses

To investigate the change in pH stability of sialidase activities with evolution of human H2N2 viruses, we first examined the relative sialidase activities of nine H2N2 epidemic strains isolated between 1957 and 1968 by treatment with two different pH conditions for 30 min as previously described [16]. The results together with results for sialidase activities of H3N2 epidemic strains previously tested [16] are shown in Table 1. Five strains isolated in 1960 (A/Ann Arbor/6/60), 1967 (A/Poland/6/67, A/Georgia/1/67, and A/Cordoba/522/67), and 1968 (A/Perg/1/68) retained their sialidase activities at pH 4.0 as did the pandemic strain A/Adachi/2/57 (H2N2), while the sialidase activities of four strains isolated in 1964 (A/Murakami/4/64 and A/Taiwan/1/64) and 1968 (A/Texas/68 and A/Berkeley/68) completely disappeared under the same pH condition. The difference between the enzymatic activities of the three H2N2 strains isolated in the 1968 epidemic was confirmed by the pH profiles of the sialidases. The stability of A/Preg/1/68 (H2N2) sialidase was clearly different to the stabilities of A/Texas/68 (H2N2) and A/Berkeley/68 (H2N2)

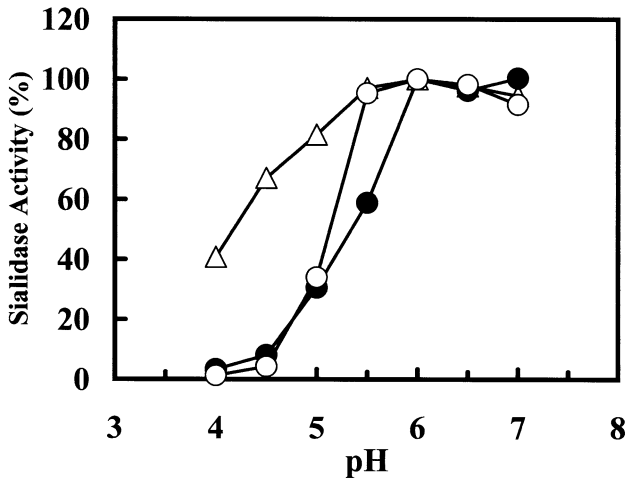

Fig. 1. The pH profiles of A/Preg/1/68 (H2N2), A/Texas/68 (H2N2), and A/Berkeley/68 (H2N2) sialidases. Sialidase activities of each virus under various pH conditions were measured as described in Section 2. Sialidase activity is expressed as a percentage relative to the activity of each virus at optimal pH. The results are shown as means for duplicate experiments. A/Preg/1/68 (open triangles), A/Texas/68 (open circles), A/Berkeley/68 (closed circles).

under low-pH conditions (Fig. 1). The data showed that the low-pH stability of sialidase activity in the 1957 pandemic viruses was preserved in some of the H2N2 epidemic strains isolated up until 1968 and the 1968 H3N2 pandemic strains, but the stability disappeared in the H3N2 virus strains isolated after 1968.

3.2. Sialidase activities of cell-expressed NAs under low-pH conditions

To confirm that the NA genes of H2N2 strains tested are responsible for the stability of the sialidase activities under low-pH conditions, 293T cells were transfected with each expression plasmid vector containing the NA gene from A/Adachi/2/57 (H2N2), A/Japan/305/57 (H2N2), A/Singapore/1/57 (H2N2), A/Ann Arbor/6/60 (H2N2), A/Murakami/4/64 (H2N2), A/Georgia/1/67 (H2N2), A/Preg/1/68 (H2N2), A/Berkeley/68 (H2N2), A/Texas/68 (H2N2), A/Aichi/2/68 (H3N2), A/Hong Kong/1/68 (H3N2), A/Memphis/1/71 (H3N2), A/Duck/Hong Kong/273/8/78 (H2N2), and A/Duck/Hong Kong/47/5/76 (H7N2). The low-pH stability of sialidase activities of cell-expressed NAs was estimated as the relative ratio of fluorescence of released 4-MU at pH 4.0 normalized to that at pH 5.0. The N2 NAs in the 1957 and 1968 human pandemic virus strains as well as those in duck viruses retained their sialidase activities at pH 4.0, but the sialidase activities of the NAs in the 1964 and 1968 epidemic H2N2 viruses except A/Perg/1/68 (H2N2) practically disappeared under the same pH condition (Fig. 2). In all cases, pH stability of the sialidase activities of the cell-expressed NAs tested coincided with that of the viruses.

3.3. Evolution of N2 NA gene

To examine the change in the low-pH stability of sialidase activities of the pandemic strains with the evolution of human influenza virus N2 NA genes, we determined the NA nucleotide sequences (the regions of amino acid residues 1–469) of the H2N2 viruses that are not available in the GenBank database except the epidemic strains previously reported [13]. Evolutional analysis of the N2 NA genes of human influenza A viruses in addition to N2 NA genes of H3N2 viruses previously tested [16] showed that the N2 NA genes have evolved essentially as a single lineage from 1957 to 1982, but the NA genes of 1968 H3 pandemic strains (A/Aichi/2/68 and A/Hong Kong/1/68) were located on a branch distinctly different from the branches of the NA genes represented by the low-pH-stable sialidase strains (A/Poland/6/67, A/Georgia/1/67, A/Cordoba/522/67, and A/Perg/1/68) and the low-pH-unstable sialidase strains (A/Texas/68 and A/Berkeley/68) isolated in 1967 and 1968 (Fig. 3). The evolutional tree also indicated that the NA genes of H3N2 viruses isolated from 1971 to 1982 have evolved from the side branch of NA genes represented by the 1968 H2N2 strains in which low-pH-unstable sialidase activity was preserved. To determine whether the prototype of the H3N2 epidemic influenza strains isolated after 1968 was generated by second genetic reassortment between the 1968 pandemic virus and the previously circulating H2N2 virus in addition to A/Texas/68 and A/Berkeley/68, nucleotide sequence homology of the NA genes between A/Memphis/1/71 (H3N2) and A/Hong Kong/1/68 (H3N2) was compared with the homology between A/Memphis/1/71 (H3N2) and A/Texas/68 (H2N2). Although the nucleotide sequences (the regions of amino acid residues 1–469) of the NA genes of A/Hong Kong/1/68 (H3N2) and A/Texas/68 (H2N2) are very similar to that of the 1971 epidemic H3N2 strain (e.g. 96.81 and 97.66% identical with A/Hong Kong/1/68 and A/Texas/68 NAs, respectively), as shown in Table 1, 18 of 24 nucleotide substitutions observed between A/Hong Kong/1/68 (H3N2) and A/Texas/68 (H2N2) were conserved in A/Texas/68 (H2N2), A/Berkeley/68 (H2N2), and the H3N2 epidemic influenza strains isolated after 1968. The data indicate that the prototype of the H3N2 epidemic influenza strains isolated after 1968 probably acquired the NA gene from the H2N2 low-pH-unstable sialidase strain by second genetic reassortment in humans.

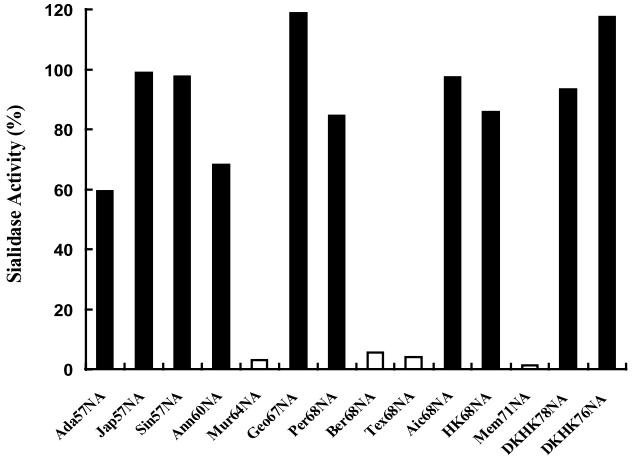

Fig. 2. Sialidase activity of cell-expressed NA. The pCAGGS/MCS expression plasmid construct for each NA gene was transfected in 293T cells and the sialidase activity of each cell-expressed NA under different pH conditions was measured as described previously [18]. The sialidase activity at pH 4.0 is expressed as a percentage relative to the activity of each cell at pH 5.0. The open columns and closed columns represent the NAs from the low-pH-unstable sialidase strains and the NAs from the low-pH-stable sialidase strains, respectively.

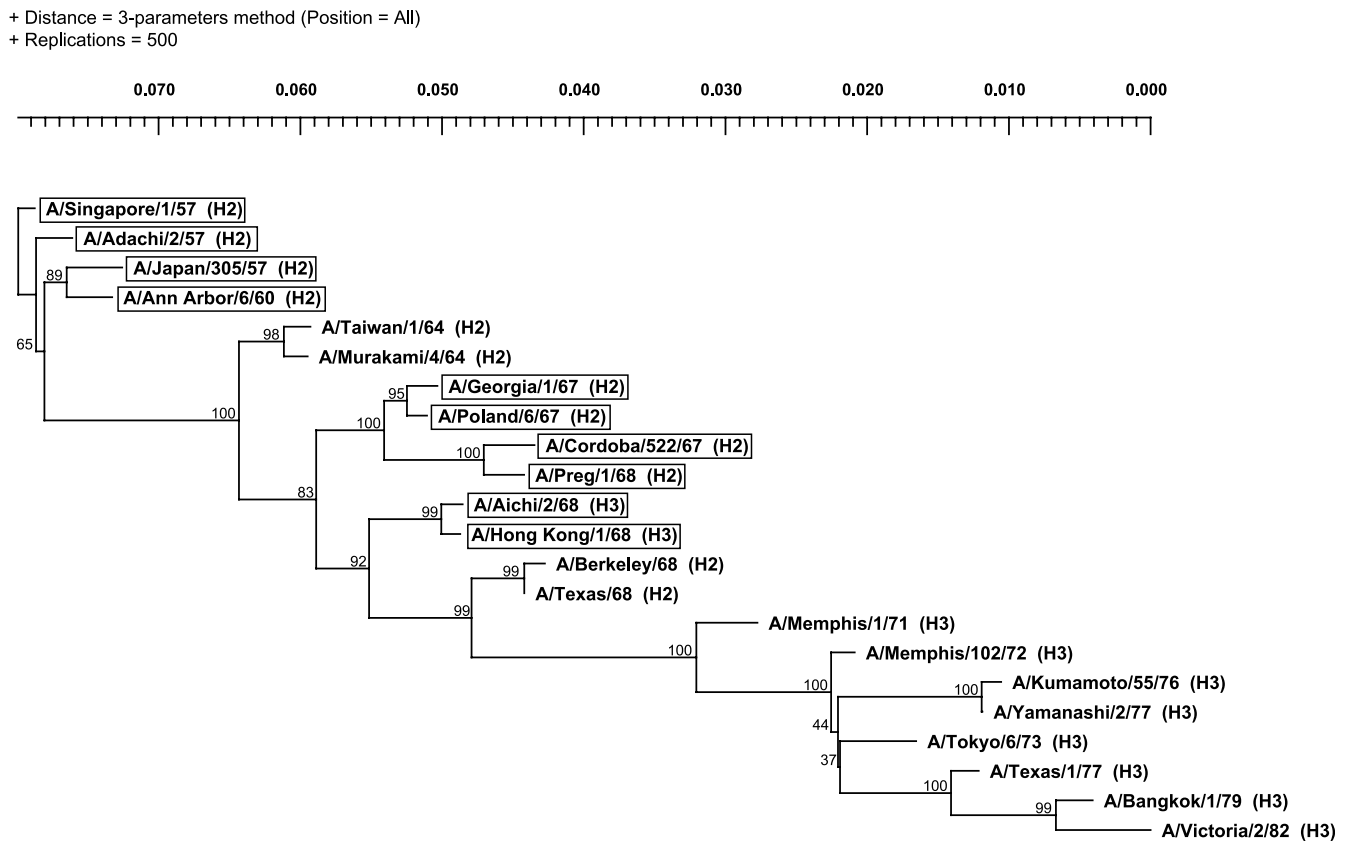

Fig. 3. Evolutional tree of N2 NA genes of human H2N2 and H3N2 influenza A viruses isolated between 1957 and 1982. The nucleotide sequences (amino acid positions 1–469) of the influenza virus N2 NAs tested except for A/Poland/6/67 (H2N2), A/Georgia/1/67 (H2N2), A/Cordoba/522/67 (H2N2), A/Preg/1/68 (H2N2), and A/Bangkok/1/79 (H3N2) were determined as described in Section 2. Nucleotide distance matrices were estimated by the three-parameter method based on the number of total substitutions. Evolutional trees were constructed by the neighbor-joining method. Framed viruses indicate the strains that possess low-pH-stable sialidase activity.

4. Discussion

All of the 15 HA and nine NA subtypes of influenza A viruses have been isolated from wild aquatic birds, which seem to play an important role as the reservoir for the viruses transmitted to other animals [3]. The N2 gene of NA first appeared in human influenza viruses in the 1957 outbreak of H2N2 subtype and was inherited in the 1968 H3N2 pandemic virus [7,8]; however, there is little information on the variation in N2 NA genes of H2N2 viruses or on the generation of the 1968 pandemic virus [11–13].

In the current study, we investigated the change in low-pH stability of sialidase activity with the evolution of N2 NA genes in human viruses. Our data suggest that the low-pH stability of the 1957 pandemic virus NA was maintained in some of the H2N2 epidemic virus strains isolated up until 1968 and inherited in the 1968 pandemic strains. We do not know why the 1968 pandemic virus strains possess the avian (duck)-like NA gene. The H3 HA of the 1968 pandemic strain that had been introduced from avian species may have required the avian (duck)-like NA gene to support productive virus replication.

Serological studies [21] and peptide maps [22] have shown that the NAs of H2N2 influenza virus strains isolated between 1967 and 1968 were closely related to the 1968 H3N2 pandemic strains. Previous characterization of the N2 NA genes in H2N2 viruses isolated in 1967 and 1968 also suggested that the NA gene of A/Poland/6/67 (H2N2) was genetically closest to that of the 1968 H3N2 pandemic strains [13]. Our evolutional analysis indicated that the NA genes of the H2N2 strains isolated between 1967 and 1968 were split into two branch clusters, which were composed of the groups of NA genes represented by low-pH-stable and low-pH-unstable sialidase strains. The 1968 H3N2 pandemic strains were genetically derived from the group of NA genes represented by low-pH-stable sialidase strains, including A/Poland/6/67 (H2N2). On the other hand, the evolutional tree revealed that the NA genes of H3N2 strains isolated after 1968 evolved from the side branches of NA genes of the 1968 H2N2 epidemic strains, which had low-pH-unstable properties, not from the side branches of the 1968 H3N2 pandemic strain NA genes. Our findings indicate that the prototype of the H3N2 epidemic influenza strains isolated after 1968 probably generated from the H2N2 low-pH-unstable sialidase strain by second genetic reassortment in humans.

Kobasa et al. have shown that early human H2N2 viruses retained avian-like substrate specificity in the NA gene [23] and that the NA of A/Singapore/1/57 (H2N2), but not the NAs from a series of human H2N2 viruses such as A/England/12/62 and A/Korea/426/68 isolated between 1960 and 1968 [15], also possessed a high level of sialidase activity that was correlated with efficient viral growth in the intestines of orally inoculated ducks. They suggested that the reduction in the high level of sialidase activities of later H2N2 strains arose from adaptation of the NA for maintenance of a balance [24–28] between the sialidase activity and the reduced

receptor binding to growth in humans. On the other hand, the properties of the avian (duck)-like low-pH stability of the 1957 pandemic NAs had been maintained in some of the later H2N2 viruses that are not thought to possess high levels of specific sialidase activity as well as A/England/12/62 and A/Korea/426/68. Using chimeric NAs in which portions of the low-pH-stable N2 NAs were replaced with the corresponding regions of the low-pH-unstable N2 NAs, we found that the amino acid regions responsible for the low-pH instability of sialidase activity in Tex68NA and Mem71NA were the same but that the region in Mur64NA was different. We also found that consensus amino acid regions responsible for low-pH stability did not exist in the 1957 and 1968 pandemic NAs but that substitutions of both amino acid residues Arg to Lys at position 344 (corresponding to nucleotide G to A substitution at position 1050) and Phe to Leu at position 466 (corresponding to nucleotide T to C substitution at position 1415) in HK68 NA remarkably reduced the degree of low-pH stability of sialidase activity and that a single amino acid change, Leu to Phe at position 466 was sufficient to confer low-pH stability of sialidase activity of HK68 NA to Tex68 NA. Both of the amino acid residues are located near known calcium ion-binding sites [18]. We therefore generated mutant influenza A viruses that included N2 NA genes, in which pH stability of the sialidase activities was changed, by using a plasmid-driven reverse genetics system. The NA mutant viruses showed a significant difference in viral infection (unpublished data). The reason for the loss of the low-pH stability of sialidase activity in the early stage of the emergence of the 1968 H3N2 pandemic influenza virus is not known. The property of the avian (duck)-like low-pH stability in the NA genes of the H3N2 virus may be unfavorable to adaptation of the virus to humans. Further studies are needed to verify this hypothesis. Our findings should contribute to elucidation of the epidemiology of influenza A virus on the transmission of avian (duck) viruses to humans.

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